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ORIGINAL ARTICLE

Histomorphometric analysis of the soleus muscle in different phases of ischemic stroke in an animal model

Análise histomorfométrica do músculo sóleo em diferentes fases do acidente vascular cerebral isquêmico em modelo animal

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KEYWORDS
Stroke
Skeletal Muscle
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ABSTRACT

Objective: To analyze the muscle mass and cross-sectional area of the muscular fiber of the hemiparetic soleus in an animal model, in order to identify the muscular adaptations that occur in ischemic stroke.
Method: Twelve *Rattus norvegicus* were divided into 2 groups: stroke group (n=6) and control group (n=6). Each group was subdivided into two subgroups, with evaluations at 7 days (CG7 and SG7) and 21 days (CG21 and SG21) after the accident. Their soleus muscles were removed for muscle mass analysis and cross-sectional area of the muscular fibers (CSAMF) measurement. The adopted statistical significance was 5%.
Result: Significant differences in the muscle mass were observed between CG7 (0.120 ± 0.005 g) vs. SG7 (0.100 ± 0.004 g; p=0.035), and between CG21 (0.130 ± 0.010 g) vs. SG21 (0.078 ± 0.006; p=0.012). Significant differences in the relative muscle mass were observed between CG7 (0.044 ± 0.002 g) vs SG7 (0.039 ± 0.003; p=0.025), and CG21 (0.044 ± 0.003) vs. SG21 (0.028 ± 0.002; p=0.011). The CSAMF showed significant differences between CG7 (2,322 µm² [2312-2453]) vs. SG7 (2,056 µm² [2,022-2,135]; p=0.012), and CG21 (2,667 µm² [2,692-2,845]) vs. SG21 (2,050 µm² [2,034-2,161]; p=0.006).
Conclusion: In this study in animal models of ischemic stroke, there was a significant loss of muscle mass, and this loss was accentuated in the longer term of the injury, highlighting the importance of future research on types of muscle fibers and applicability in human patients.

RESUMO

Objetivo: Analisar a massa muscular e a área de secção transversal da fibra muscular do sóleo hemiparético em um modelo animal, a fim de identificar as adaptações musculares que ocorrem no acidente vascular cerebral isquêmico.
Método: Doze *Rattus norvegicus* foram divididos em 2 grupos: grupo AVC (n=6) e grupo controle (n=6). Cada grupo foi subdividido em dois subgrupos, com avaliações aos 7 dias (CG7 e SG7) e 21 dias (CG21 e SG21) após o acidente. O músculo sóleo foi

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PALAVRAS-CHAVE
AVC
Músculo Esquelético
Histologia

removido para análise de massa muscular e medição da área de secção transversal das fibras musculares (CSAMF). O nível de significância estatística adotado foi de 5%. **Resultado:** Diferenças significativas na massa muscular foram observadas entre CG7 (0,120 ± 0,005 g) vs. SG7 (0,100 ± 0,004 g; p=0,035), e entre CG21 (0,130 ± 0,010 g) vs. SG21 (0,078 ± 0,006; p=0,012). Diferenças significativas na massa muscular relativa foram observadas entre CG7 (0,044 ± 0,002 g) vs. SG7 (0,039 ± 0,003; p=0,025), e CG21 (0,044 ± 0,003) vs. SG21 (0,028 ± 0,002; p=0,011). A CSAMF mostrou diferenças significativas entre CG7 (2.322 µm² [2.312-2.453]) vs. SG7 (2.056 µm² [2.022-2.135]; p=0,012), e CG21 (2.667 µm² [2.692-2.845]) vs. SG21 (2.050 µm² [2.034-2.161]; p=0,006).
Conclusão: Neste estudo em modelos animais de acidente vascular cerebral isquêmico, houve uma perda significativa de massa muscular, e essa perda foi acentuada no longo prazo da lesão, destacando a importância de futuras pesquisas sobre tipos de fibras musculares e aplicabilidade em pacientes humanos.

INTRODUCTION

Stroke remains a significant challenge to global health, affecting the central nervous system and presenting an estimated prevalence in the general population that ranges from 0.5% to 0.7%[1]. In Brazil, stroke maintains its sad position as the leading cause of death, contributing to approximately 68,000 deaths annually[2]. In addition to high mortality, stroke imposes specific morbidity, with sequelae rates ranging from 24% to 54%, significantly impacting the quality of life of affected individuals[3].

In addition to clinical challenges, stroke represents a substantial burden on public healthcare systems, generating economic and social implications expressed through hospital expenses, rehabilitation services and premature retirements[4].

In the recovery trajectory after a stroke, there is a sequence of phases including the flaccid phase (acute), the recovery phase (subacute), and the spastic phase (chronic)[5]. Each phase is characterized by specific characteristics, and its duration is influenced by the severity of the injury, age, level of physical resilience, patient motivation and therapeutic approach. In animal models, researchers consider the first week as the acute phase of stroke and the third week as the chronic phase to conduct research[6].

A stroke located in the internal capsule can cause paralysis or weakness on the side opposite the injury, as this structure contains descending motor fibers that pass through the corticospinal tract and control voluntary movements on the contralateral side of the body, including the lower limbs[7].

Studies reveal notable changes in muscle morphology and mitochondrial enzyme activity in the paretic muscles of post-stroke patients, associated with physical disability[8]. The paretic side generally has a smaller proportion of slow fibers, a greater proportion of fast and fatiguable fibers, as well as smaller muscle fiber size and reduced oxidative enzyme activity[9].

The assessment of muscle mass, which refers to the total amount of muscle tissue, and relative muscle mass, which considers the proportion of muscle mass in relation to total body weight, plays a fundamental role in this context[10]. Muscle mass is essential for motor function and the ability to recover post-stroke[11]. Therefore, a comprehensive understanding of the effects of stroke on the musculoskeletal system is crucial, providing useful information for more effective rehabilitation strategies[12].

In this context, the present study aims to investigate the muscular architecture of the paretic soleus muscle in an animal model of ischemic stroke. The primary objective is to identify and characterize muscular adaptations occurring during the first and third weeks after the ischemic event, offering insight into the acute changes that influence motor function and post-stroke recovery.

METHODS

This was a laboratory-based study conducted using an animal model[13]. The procedures were carried out at the Laboratory of Neuroanatomy and Neurophysiology, State University of the Midwest (UNICENTRO), Paraná, Brazil. All experiments were conducted in accordance with the Ethics Committee on Animal Use (CEUA) of the same university, under protocol approval number 020/2018.

Sample

The sample consisted of 12 *Rattus norvegicus*, Wistar lineage, male, aged two months, obtained from the animal facility at the State University of Londrina (UEL). The animals were housed in a dedicated room under a 12-hour light/dark cycle (lights on from 7 am to 7 pm) with a room temperature maintained at 23 ± 1 °C using a Sprint 7000 BTU air conditioner. The animals were randomly distributed before starting the study through the "Graphpad by Dotmatics" website, and divided into 4 groups, with 3 animals per cage. The cages used in the experiment were constructed from unbreakable autoclavable acrylic, measuring 41x34x16 cm, equipped with a wire mesh lid and a 700 mL polypropylene water bottle with a stainless-steel sipper tube. Animals had ad libitum access to food and water but were subjected to an 8-hour fast and 4-hour water restriction prior to surgery[14].

Stroke group (SG)

The experimental group consisted of six animals divided into two subgroups: the 7-day experimental group (SG7) with euthanasia on the 8th day and the 21-day experimental group (SG21) with euthanasia on the 22nd day. In both groups, stereotactic surgery was performed involving electrode implantation followed by electrolytic lesioning of the left internal capsule to induce ischemic stroke. After

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euthanasia, the right soleus muscle was dissected, weighed using a precision balance, and stored in 10% formaldehyde for 24 h for subsequent histomorphometric analysis.

Control group (CG)

The control group consisted of six animals divided into two subgroups: the 7-day control group (CG7) with euthanasia on the 8th and the 21-day control group (CG21) with euthanasia on the 22nd day. Animals in this group did not undergo surgical procedures but were handled similarly during the experiment. After euthanasia, the right soleus muscle was dissected, weighed using a precision balance, and stored in 10% formaldehyde for 24 hours for subsequent histomorphometric analysis.

Surgical procedure

Animals from the SG (n = 6) were intraperitoneally anesthetized with a solution of 80 mg/kg of ketamine (Ketamine, Syntec, 10 mL vial) and 15 mg/kg of xylazine (Dopaser, Hertap, 10 mL vial) and secured in a stereotactic apparatus (David Kopf, USA). Their heads were immobilized using the external auditory meatus and upper incisors. After removing part of the overlying tissue, the skull was drilled with a dental drill to implant the electrode into the internal capsule. The implantation site was determined based on stereotactic coordinates[15]. With coordinates of AP= 1.72 mm, ML= -3.4 mm, and DV= 4.4 mm relative to bregma as the reference, aligning the lambda and bregma sutures in the same horizontal plane. Once implanted, the electrodes were fixed to the calvaria using self-polymerizing acrylic resin (VIPIFLASH Autopolymerizable®). Animals were allowed to rest for five days, after which they were re-anesthetized and returned to the stereotactic apparatus, where they received a 20 mA current for 45 seconds from a DC power supply MPS-3005. The electrodes were made with number 34 enameled wire approximately 10 mm long[15].

Postoperative care

After the procedure, analgesics were administered to control pain. The animals received tramadol at a dose of 2 mg/kg, diluted in 0.2 mL of water, via oral gavage every 12 hours. The animals' behavior was assessed, including their ability to move affected limbs, such as their hind legs, and to perform basic activities, such as walking, running and maintaining balance. The ability to perform specific tasks, such as reaching for food or moving around the cage, was also observed. There was no sample loss in the present study[16].

Euthanasia

The animals were anesthetized with 80 mg/kg of ketamine and 15 mg/kg of xylazine. After confirming the anesthetic state, they received 175 mg/kg of Thiopental intraperitoneally[17].

Dissection and weighing procedure

After euthanasia, the right soleus muscle was carefully dissected. First, the skin of the leg was removed to expose the underlying muscles. Then, using sterile surgical instruments, the soleus muscle was isolated from adjacent tissues, such as fascia and tendons. The proximal and distal muscle insertions were carefully released, allowing the complete removal of the muscle without damaging its structures. The dissected muscle was then weighed using a precision analytical balance (Metter/Toledo) with a minimum capacity of 10 mg and a maximum of 210 g to obtain its exact mass. During the weighing process, the muscle was periodically dripped with saline solution (NaCl 0.9%) to prevent tissue drying. The absolute mass (expressed in grams) and its relationship with the body mass of the respective animal (relative mass, expressed as a percentage) were recorded. Finally, the muscle was fixed in 10% formaldehyde for 24 h for subsequent histomorphometric analysis[18].

Histomorphometric analysis

The soleus muscle was selected due to its role as a typically gravity-antagonistic muscle often affected by spasticity, resulting in the typical motor deficit seen after stroke in humans. Histological sections of the soleus muscle stained with hematoxylin and eosin (HE) were evaluated. To assess the cross-sectional area of muscle fibers (CSAMF), ten photos of each muscle were captured, with one histological section chosen from each. The choice of the section was based on the absence of artifacts, i.e., a higher quantity of muscle fibers, a lower quantity of reagent deposits, and the presence of undistorted, torn, or poorly focused fibers. Subsequently, the selected section was focused under a 10x and 40x objective on a light microscope (Olympus CX21) and photographed using a digital camera (Sony CCD IRIS Camera). Later, the image was transferred to a computer where 100 fibers in the central region of the histological section were randomly selected, without knowledge of the experimental group, to ensure a blinded analysis. CSAMF measurements were conducted using the Image J software (version 1.45q). To measure the diameters of the muscle fibers of the soleus muscle of rats, ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used. Initially, the scale was calibrated using a micrometric blade, which had marks spaced at intervals of 100 µm. The image of the micrometric slide was imported into ImageJ, and a line was drawn between two 100 µm marks. Using ImageJ's "Set Scale" function, we enter the known distance as 100 µm and the measurement unit as "µm". This calibration was then applied globally to all subsequent images. Using the line tool, a line was drawn across the widest point of each muscle fiber, with the "Measure" function the diameter was recorded in micrometers. Multiple fibers were measured for each sample, and recorded for statistical analysis. The scale was previously calibrated using a micrometric blade[19].

Statistical analysis

The Shapiro-Wilk test was used to assess normality, and Levene's test was used to assess data homogeneity. Therefore, a one-way ANOVA test followed by Tukey's post-hoc analysis was employed to compare the groups. All tests were carried out using the SPSS software (version

22.0®). The graphs were produced on Microsoft Excel. The significance level was set at 5%.

RESULTS

In Figure 1 it is possible to observe that there was no statistically significant difference between the initial body mass between the groups (p=0.119). There was also no statistically significant difference between CG7 and SG7 in final body mass (p=0.112). The presence of a stroke did not have a significant impact on body mass compared to the control group over a 7 day period in this sample, as shown in Figure 2.

However, upon extending the analysis to 21 days, a significant change was observed. CG21 showed a significantly higher body mass compared to both CG7 and SG7, (p=0.027). When comparing CG21 with SG21, the difference in body mass was also statistically significant (p=0.014), indicating that the stroke negatively influenced body mass gain over the 21-day period. These results are shown in Figure 2.

When analyzing muscle mass after 7 days, a significant difference was observed, with SG7 displaying lower muscle mass compared to CG7 (p=0.035). Extending the observation to 21 days accentuated the disparity. SG21 recorded the lowest muscle mass among all groups, with a significant difference compared to CG7 (p=0.012), SG7 (p=0.018), and SG21 (p=0.024). These findings are illustrated in Figure 3.

Analyzing relative muscle mass after 7 days revealed a significant difference, where SG7 exhibited lower relative muscle mass compared to CG7 (p=0.025). Extending the observation to 21 days accentuated the disparity, SG21 recorded the lowest relative muscle mass among all groups, with a significant difference compared to CG7 (p=0.009), SG7 (p=0.014), and CG21 (p=0.011). These results can be observed in Figure 4.

In CG7, a larger mean transversal area was observed in relation to SG7, showing a significant difference between them (p=0.012). Extending the observation to 21 days, CG21 displayed a notable increase in cross-sectional area compared to all groups, particularly in its comparison with the respective SG21, showing a statistically significant difference (p=0.006). These findings are detailed in Table 1.

In Figure 5, histological sections from the control and stroke groups at different periods (7 and 21 days) are presented. These figures were used to calculate the CSAMF, the results of which are presented in Table 1.

DISCUSSION

The results of this study indicate that stroke caused changes in both body mass and muscle mass of animals

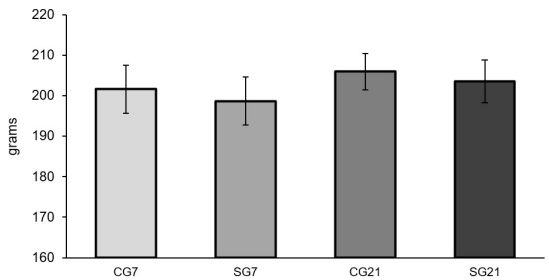

Figure 1 – Comparison of initial body mass between control groups and groups with stroke over different periods. Values are presented as mean ± standard deviation. CG7: Control Group 7 days (201.6 ± 5.9 g); SG7: Stroke Group 7 days (198.7 ± 5.9 g); CG21: Control Group 21 days (205.9 ± 4.5 g); SG21: Stroke Group 21 days (203.5 ± 5.3 g).

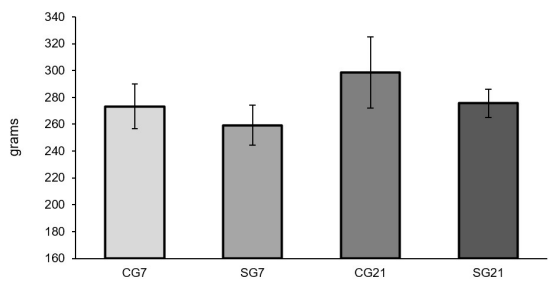

Figure 2 – Comparison of final body mass between control groups and groups with stroke over different periods. Values are presented as mean ± standard deviation. CG7: Control Group 7 days (273.2 ± 16.6 g); SG7: Stroke Group 7 days (259.1 ± 14.9 g); CG21: Control Group 21 days (298.6 ± 26.4 g); SG21: Stroke Group 21 days (275.6 ± 10.5 g).

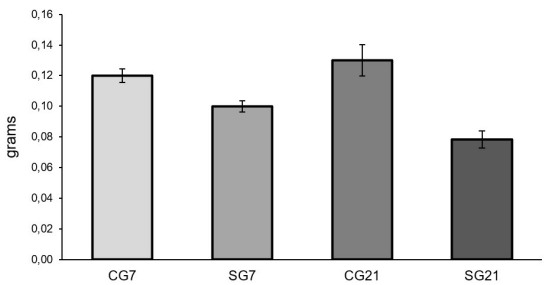

Figure 3 – Comparison of muscle mass between control groups and groups with stroke over different periods. Values are presented as mean ± standard deviation. CG7: Control Group 7 days (0.120 ± 0.005); SG7: Stroke Group 7 days (0.100 ± 0.004); CG21: Control Group 21 days (0.130 ± 0.010); SG21: Stroke Group 21 days (0.078 ± 0.006).

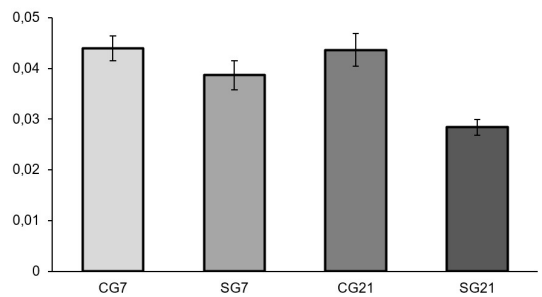

Figure 4 – Relative muscle mass of control groups and groups with stroke over different periods. Values are presented as mean ± standard deviation. CG7: Control Group 7 days (0.044 ± 0.002); SG7: Stroke Group 7 days (0.039 ± 0.003); CG21: Control Group 21 days (0.044 ± 0.003); SG21: Stroke Group 21 days (0.028 ± 0.002).

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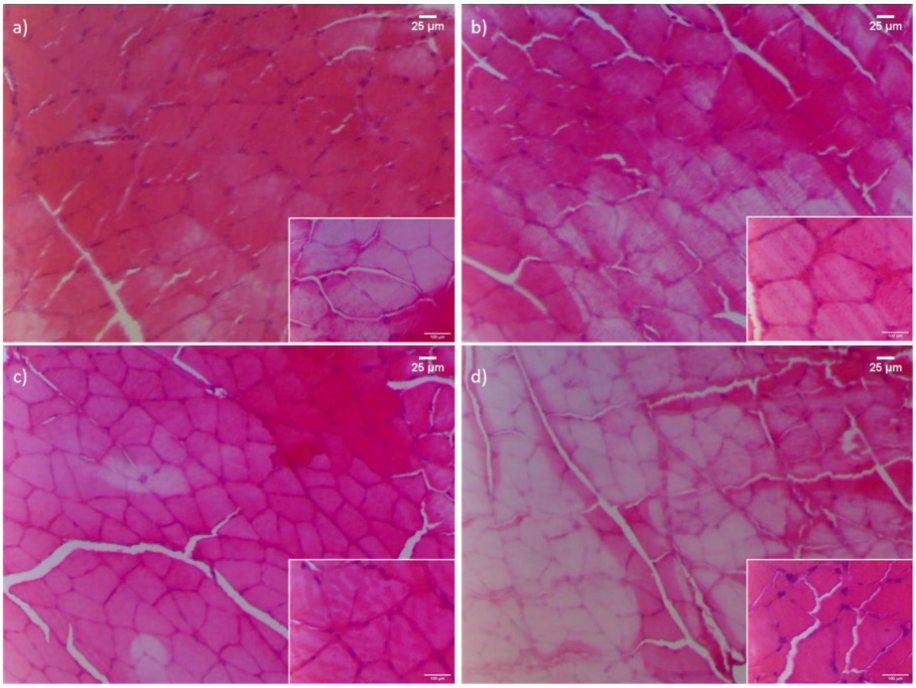

Figure 5 – Soleus muscle morphology in rats with and without stroke at 7 and 21 days, using hematoxylin and eosin staining. (a) CG7: Control Group 7 days; (b) SG7: Stroke Group 7 days; (c) CG21: Control Group 21 days; (d) SG21: Stroke Group 21 days. Scale bar 25µm, 10X; scale bar 100 µm, 40X.

Table 1 – Comparison between the groups of the cross-sectional area of muscle fibers (CSAMF) in different periods.

Groups	CG7 (n=3)	SG7 (n=3)	CG21 (n=3)	SG21 (n=3)
CSAMF (µm²)	2,322 (2,312-2,453)	2,056 (2,022-2,135)	2,667 (2,692-2,845)	2,050 (2,034-2,161)

Values are presented as median and 95% confidence interval. CG7: Control Group 7 days; SG7: Stroke Group 7 days; CG21: Control Group 21 days; SG21: Stroke Group 21 days.

after 7 and 21 days of the ischemic event, compared to their respective control groups. The CG21 and SG21 groups showed higher body weight compared to the CG7 and SG7 groups, and this increase was justified by the normal growth of the animals[20].

However, when comparing the stroke groups with their respective controls, a loss of body mass can be observed, both in the short term (7 days) and the long term (21 days). Unintentional weight loss after a stroke is a common occurrence, emphasizing the need for constant monitoring of the nutritional status of these patients[21]. Monitoring body weight becomes especially crucial in cases of severe stroke, feeding difficulties, low pre-albumin levels, and compromised glycemic metabolism[22].

To assess the implications of stroke on muscle tissue, we conducted an analysis of absolute muscle mass and the cross-sectional area of muscle fibers. Our results indicate a significant reduction in both parameters in the stroke groups, especially in the long term (SG21), when compared to control groups. This highlights that despite being free in the cage, reduced mobility of the affected hemi-body resulted in changes in muscle composition after the ischemic event[23].

Stroke triggers muscular abnormalities, originating from a combination of denervation, disuse, remodeling, and spasticity, contributing to a complex pattern of changes and atrophy[24]. Adaptive structural changes in muscle tissue begin approximately 4 hours after a stroke, possibly related to the interruption of synaptic transmission from motor neurons and a reduction in the number of motor units. Additionally, inactivity and immobilization after a stroke are crucial factors for the loss of muscle mass[25].

It is crucial to implement muscle rehabilitation interventions in the early stages of stroke to minimize potential damage to muscle function, promoting a more effective patient recovery[26]. Inactivity resulting from prolonged bed rest can have adverse effects, slowing the recovery process and contributing to increased mortality and morbidity among stroke patients[27]. Early mobilization of these patients should be encouraged to minimize the impact of disuse on muscle mass, especially considering the observation of a reduction in both absolute and relative muscle mass[28].

This study on post-stroke muscle changes highlights the complexity of these adaptations over time, revealing short- and long-term effects. The inclusion of a control group

may help distinguish between stroke-induced changes and those related to natural growth. However, some important limitations of this study include the lack of analysis of muscle fiber types in paralyzed muscle and the lack of assessment of activity levels and functional outcomes in animals. Furthermore, the study was performed in a small animal model sample, requiring caution when extrapolating results to human patients due to possible differences in muscular responses to stroke.

CONCLUSION

This study highlights that in animal models subjected to ischemic stroke, not only was there a loss of body mass, but a significant decrease in muscle mass and a relative reduction in this mass were also observed. Furthermore, a decrease in cross-sectional area was observed, assessed through soleus muscle biopsies in hemiparetic animal models. These findings highlight the need for future investigations focusing on specific types of muscle fibers and the applicability of the results to human patients.

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