

## Effects of physical activity on skeletal muscle histomorphometry of murine cachexia tumor model

### Efeitos da atividade física na histomorfometria muscular esquelética de modelo tumoral murino de caquexia

Tayrine Resende de Oliveira<sup>1</sup> João Vitor Nunes Lopes<sup>2</sup> Iaggo Raphael David<sup>3</sup> Frederico Sander Mansur Machado<sup>4</sup> Mariana Rocha Alves<sup>5</sup> Alfredo Mauricio Batista de Paula<sup>6</sup> Renato Sobral Monteiro Júnior<sup>7</sup> Vinicius Dias Rodrigues<sup>8</sup> <sup>1</sup>Universidade Estadual de Montes Claros (Montes Claros). Minas Gerais, Brazil. tayrineoliveirauni@gmail.com<sup>2</sup>Corresponding author. Faculdades Integradas do Norte de Minas (Montes Claros). Minas Gerais, Brazil. joaolopes@outlook.com.br<sup>3</sup>Universidade Paulista (Vitória da Conquista). Bahia, Brazil. iaggoraphaell@gmail.com<sup>4,6-8</sup>Universidade Estadual de Montes Claros (Montes Claros). Minas Gerais, Brazil. machado.frederico@outlook.com, ambpatologi@gmail.com, monteirojuniorms@gmail.com, viniciuslabex@hotmail.com<sup>5</sup>Universidade Federal Fluminense (Montes Claros). Minas Gerais, Brazil. marianarochaalves13@gmail.com

**RESUMO | INTRODUÇÃO:** A caquexia associada ao câncer (CAC) é uma síndrome paraneoplásica que ocorre com indivíduos com câncer que é caracterizada pela diminuição gradual de tecidos muscular esquelética e de tecido adiposo, promovendo um quadro de consumação física do indivíduo. A ocorrência de CAC determina de forma significativa a uma pior qualidade de vida e de sobrevivência de indivíduos com câncer. A CAC não pode ser revertida pelo amparo nutricional convencional. Tratamentos não-farmacológicos empregados para a CAC tem reportado a realização de atividade física para possibilitar adaptações neurais e de hipertrofia muscular em indivíduos com a síndrome. **OBJETIVO:** Verificar os efeitos da atividade física em ambiente enriquecido na histomorfometria da musculatura esquelética de camundongos C57BL/6 submetidos ao modelo tumoral murino de caquexia associada ao câncer (CAC). **MÉTODOS:** Foram utilizados 38 camundongos C57BL/6 fêmeas, distribuídos aleatoriamente nos grupos de estudo. Todos os animais foram submetidos ao modelo tumoral murino singênico de Melanoma Cutâneo (MTMSMC) com a inoculação subcutânea de células B16F10. Os grupos de estudo são animais do experimento diagnóstico CRC (n = 12), animais sedentários (n = 11) e animais submetidos à prática de atividade física em ambiente enriquecido (n = 15). O seguimento do estudo ocorreu por um período de dez dias. Após esse momento, todos os animais foram sacrificados e amostras de tecido muscular esquelético do gastrocnêmio foram submetidas às análises histomorfométricas. **RESULTADOS:** Os resultados da análise inferencial do peso absoluto e relativo muscular esquelético não diferiram entre os grupos do estudo. Todas as comparações das variáveis apresentaram tamanho do efeito pequeno. A análise histomorfométrica muscular revelou que a área da fibra muscular não diferiu entre os grupos do estudo. Contudo, essa área muscular, apresentou tamanho do efeito pequeno. **CONCLUSÃO:** Os resultados apresentados mostraram que a realização de atividade física no ambiente enriquecido não influenciou na área da fibra do músculo do gastrocnêmio de camundongos C57BL/6 submetidos ao MTMSMC, associada à CAC.

**PALAVRAS-CHAVE:** Roedores. Câncer. Sarcopenia. Atividade locomotora.

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**ABSTRACT | INTRODUCTION:** Cancer-associated cachexia (CAC) is a paraneoplastic syndrome that occurs in individuals with cancer, which is characterized by the gradual decrease in skeletal muscle and fat tissue, promoting an individual's physical consumption. The occurrence of CAC significantly determines a worse quality of life and survival for individuals with cancer. CAC cannot be reversed by conventional nutritional support. Non-pharmacological treatments used for CAC have reported physical activity to enable neural adaptations and muscle hypertrophy in individuals with the syndrome. **OBJECTIVE:** To verify the effects of physical activity in an enriched environment on the histomorphometry of skeletal musculature of C57BL/6 mice submitted to the murine tumor model of cancer-associated cachexia (CAC). **METHODS:** 38 female C57BL/6 mice were used, randomly distributed in groups of study. All animals were subjected to the cutaneous murine tumor model of Cutaneous Melanoma (MTMSMC) with subcutaneous inoculation of B16F10 cells. The study groups are CRC diagnostic experiment animals (n = 12), sedentary animals (n = 11) and animals submitted to physical activity in an enriched environment (n = 15). The study was followed up for a period of ten days. After that moment, all animals were sacrificed and samples of skeletal muscle tissue from the gastrocnemius were submitted to histomorphometric analyzes. **RESULTS:** The results of inferential analysis of absolute and relative skeletal muscle weight did not differ between the study groups. All comparisons of the variables showed a small effect size. Muscle histomorphometric analysis revealed that the muscle fiber area did not differ between the study groups. However, this muscular area had a small effect size. **CONCLUSION:** The results presented showed that physical activity in the enriched environment did not influence the fiber area of the gastrocnemius muscle of C57BL/6 mice submitted to MTMSMC, associated with CAC.

**KEYWORDS:** Rodents. Cancer. Sarcopenia. Locomotive activity.

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A type of cancer with a high incidence in the world is cutaneous melanoma<sup>1-4</sup>, which according to Zacharias et al.<sup>5</sup>, promotes the accelerated development of metastasis. Barnhill<sup>6</sup> stated that a small pigmentation on normal skin exposed to the sun, may be a sign of a small melanoma tumor. A negative prognosis is the development of a multifactorial syndrome that is characterized by the loss of skeletal and adipose muscle tissue, called cachexia<sup>7</sup>. When the scientific literature is verified, the non-pharmacological possibilities for the treatment of cancer-associated cachexia do not have sufficient information on the subject.

Perez-Leighton, et al.<sup>8</sup> combined spontaneous physical activity with the enriched environment, a methodology designed for rodents, allowing involuntary movement without severe control of the intensity of activity. There are several examples of an enriched environment, including the use of objects as a form of toys, to enable the exploration of the place, it is important to note that this junction improves neurogenesis and neurotrophic factors<sup>9</sup>.

In the study by Rodrigues, et al.<sup>10</sup> reports a significant improvement in muscle strength and volume of the right rear limb, using the animal physical activity model, a fact that points to a methodological possibility of physical activity with experimentation using an animal model.

Despite this methodological possibility of physical activity, there are no studies in the literature that show effects of this activity on the cachectic picture associated with cancer. Thus, the aim of this study was to verify the effects of physical activity in an enriched environment on the histomorphometry of the gastrocnemius muscle of C57BL / 6 mice with cachexia associated with the syngeneic tumor model of cutaneous melanoma.

### Ethical aspects

The present study was analytical, prospective and with a quantitative approach. Tumor induction of B16-F10 cells of the cutaneous melanoma strain occurred in all animals in this study. The work was submitted for analysis by an ethics committee on animal experimentation and welfare and had a favorable opinion for execution. It is important to note that all experiments took place after systematic preparation by the research team for total control over the actions that involve an experimental study. The work was submitted to analysis by an ethics committee on animal experimentation and welfare (CEEBEA / Unimontes) and had a favorable opinion for execution (131/2017).

### Study groups

38 female C57BL / 6 mice were used, which were randomly assigned to three groups with two distinct phases of the study. In the first phase of the study, 12 animals were used to characterize the cachectic condition. Subsequently, the second phase of the study started with the sedentary group (CRC) (n = 11), did not perform any type of intervention (remained in the housing box), the experimental group (CRC-ATF) (n = 15), performed activity physics in an enriched environment. The CRC group had a smaller number of animals, as 4 rodents died during the experiment. The number of animals was based on the study by Rodrigues, et al.<sup>10</sup>.

### Syngene murine tumor model

The animals were submitted to the cutaneous melanoma murine tumor model (MTMSMC). Briefly, the animals were inoculated in the subcutaneous with 5x10<sup>5</sup> cells of murine cells of cutaneous melanoma B16-F10, resuspended in 50µL, in the dorsal subcutaneous region, close to the base of the neck (flank). The inoculation of this quantity of viable cells in the subcutaneous region has the capacity to complete a mitotic cycle in 24 hours and to develop the tumor within 3-4 days<sup>11,12</sup>.

## Enriched environment for physical activity

In order to familiarize the animals to the intervention with the enriched environment, prior to tumor induction, two sessions of 30 minutes of physical activity (PA) took place in this context. It is important to note that the structural variables (speed, time in motion, among others) during the experiment were not controlled.

Physical activity was organized in an enriched environment 60 cm long, 30 cm wide and 45 cm high. This environment contained seesaw, wheels, balls and tunnels (10). The intervention consisted of 7 exposure sessions for 30 minutes, with intervals of 48 hours, totaling 14 days of experimentation. The intervention with physical activity in the enriched environment occurred after the diagnosis of cachexia.

## Diagnosis of cancer-associated cachexia

The diagnosis of cachexia was established as soon as the mice submitted to MTMSC showed a loss of at least 5% of the body weight, disregarding the weight of the tumor, during the progression of the tumor<sup>13</sup>. A linear regression equation was used to define the relationship between tumor volume and tumor weight. The measurements were performed 12 days after the inoculation of tumor cells in a sample of 12 animals, in order to calculate the tumor weight throughout the experiment. Each day, a mouse was sacrificed and the tumor volume (mm<sup>3</sup>) and mass (g) were measured to obtain the characterization and progression of the tumors<sup>14</sup>. At the end of the experiment, data were obtained from different tumor stages and the relationship between mass and volume was defined in the linear equation ( $R^2 = 0.9892$ ). Once the tumor weight measurement was defined, these values were subtracted from the body weight measurement of each animal per day. Thus, after performing the cachexia diagnostic experiment, the tenth day was established for the diagnosis of cachexia after tumor induction for all animals, thus, the experimental group started the intervention, at each session they had a 48-hour interval to the next session. After the seventh session, the surviving animals were euthanized<sup>11,12</sup>.

## Biological samples

After 26 days from the beginning of the experiment, all 26 animals in the control and experimental groups were anesthetized with ketamine / xylazine (75 mg / kg and 5 mg / kg, respectively) and euthanized by cervical dislocation<sup>13,14</sup> to perform tissue collection skeletal muscle cells of the gastrocnemius. The collected tissues were weighed using an analytical precision digital scale (A. Cientifica EEQ9003E)<sup>10,13</sup>.

Biological samples collected (gastrocnemius muscle) were weighed individually, sectioned and placed part in 4% paraformaldehyde fixing solution (w: v), 4h fixation time, at room temperature and part in RNA holder and tissue tek for freezing at -80° C. The samples fixed in 4% buffered formalin solution were blocked in paraffin and subjected to microscopic cuts to perform the morphometric studies.

## Muscle histomorphometric analysis

The right posterior gastrocnemius muscles were blocked in paraffin and microsected at 7µm and marked with Hematoxylin-Eosin (HE). Sections of the skeletal muscles were made at 90° and 180° for the longitudinal axis of the muscle fibers. Three different locations (histological slides) of skeletal muscle tissue randomly indicated in the visualization were photographed. Sections of the tissue were photographed using an Olympus BX50 microscope (Olympus Corp., JAP). The area of the transversal muscle fiber was measured using the Image J software (imagej.nih.gov). All data were expressed as the mean ± SEM.

## Statistical analysis

The collected data were analyzed statistically using SPSS (Statistical Package for the Social Sciences) software version 20.0. The confidence level adopted in all analyzes was set at 95% ( $p < 0.05$ ). The Shapiro-Wilk test was performed to verify normality, then the independent Student T test was selected for inferential analysis of the dependent variables. The effect size was also calculated and classified according to Cohen<sup>15</sup>.

## Results

The results in table 01 show the inferential analysis of the absolute and relative weight of the gastrocnemius muscle, with no significant difference ( $p < 0.05$ ) between the groups. In addition, all comparisons of the variables showed a small effect size according to Cohen<sup>15</sup>. The means of the groups were compared using the independent Student's t test.

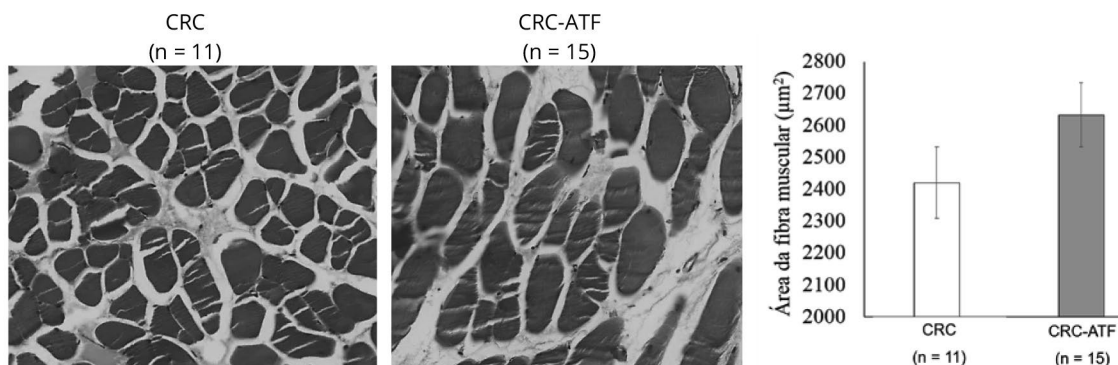
**Table 1.** Inferential analysis of the absolute and relative weight of the gastrocnemius muscle of the right rear limb of C57BL / 6 mice from the murine syngeneous tumor model of cutaneous melanoma and with cachexia

Variables	CRC (n = 11)	CRC-ATF (n = 15)	P value	TE
Body weight (g)	20,22 ± 1,72	19,82 ± 2,70	0,666	P (0,01)
Weight of m. gastrocnemius (g)	0,13 ± 0,05	0,13 ± 0,04	0,529	P (0,02)
Weight of m. gastrocnemius (%)	0,68 ± 0,19	0,66 ± 0,13	0,565	P (0,01)

\*TE= Effect size \*\* P = Little

The results in figure 01 show the histomorphometric analysis of the gastrocnemius muscle fiber area, in the inferential analysis there was no significant difference ( $p < 0.05$ ) between the groups, however, the size of the small effect was verified according to Cohen<sup>15</sup>. The inferential analysis using the independent Student's T test does not show any significant difference between the groups, in addition, the size of the small effect was verified (0.21).

**Figure 1.** Hismorphometric analysis of the fiber area of the gastrocnemius muscle of the right rear limb of C57BL / 6 mice from the murine syngeneous tumor model of cutaneous melanoma and with the occurrence of cachexia submitted to physical activity in an enriched environment and controls



## Discussion

The findings of this study show that physical activity in an enriched environment in C57BL / 6 mice with cachexia associated with the murine syngeneic tumor model of cutaneous melanoma has no contribution to the morphological and histomorphometric variables analyzed.

In the study by Rodrigues, et al. (2019)<sup>10</sup> it was demonstrated that the physical activity model in an enriched environment using healthy C57BL / 6 mice showed significant improvements in muscle strength and volume in the right rear limb, such research presents a methodological possibility of physical activity with C57BL mice / 6.

Muscle atrophy in cancer-associated cachexia occurs due to the inflammatory condition<sup>16</sup>, caused by the induction and increased production of proinflammatory cytokines (TNF- $\alpha$  and IL-6)<sup>16,17</sup>, this promotes an increase in oxidative stress<sup>18</sup> and an increase of protein catabolism as a function of the ubiquitin proteasome system<sup>19</sup>.

The outcome of our study did not result in significant, non-significant implications, possibly due to the small number of sessions performed after the cachectic condition, it is important to note that trainable characteristics, when not properly controlled, can negatively impact morphological adaptations<sup>20</sup>.

The possible results with more sessions of physical activity in an enriched environment could show the possibility of protection of the muscular structure, probably due to the physical activity promoting the increase of antioxidant enzymes (Glutathione peroxidase, Mn-SOD mitochondrial and Superoxide), favoring antagonistically in the picture of muscular atrophy<sup>21-23</sup>.

Thinking about the preservation of muscle tissue, the work of Ballarò, et al. (2019)<sup>24</sup> presents the investigation due to oxidative stress in the presence and absence of moderate aerobic training in albino cachectic mice Balb / c with colorectal carcinoma, thus, the moderate exercise used in the experimental intervention was able to mitigate the decrease in muscle mass and to avoid loss of muscle strength, such a situation was associated with reduced levels of reactive oxygen species.

The project to which this study belongs are the first steps to understand the effects of physical activity in an enriched environment in C57BL / 6 mice submitted to MTMSMC associated with CAC. However, this study presented limitations (lack of a control group with the practice of physical activity in an enriched environment) that may have influenced the results, and the potential that the theme may still contribute to the understanding of the subject.

## Conclusions

The results of this study demonstrate that physical activity in the enriched environment did not influence bodyweight (g), weight of the musician. gastrocnemius (g), weight of the music. gastrocnemius (%) and area of the gastrocnemius muscle fiber of C57BL / 6 mice submitted to MTMSMC, associated with CAC.

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## Author contributions

Oliveira TR participated in data collection and animal management. Lopes JVN participated in the writing of the article. Melo WCMA, Alves MR participated in the statistical design. David IR participated in animal management. Machado FSM participated in the laboratory analysis. De Paula AMB participated in the statistical design, data collection. Monteiro Júnior RS participated in the statistical design, animal management. Rodrigues VD coordinated the research and writing of the article.

## Competing interests

No financial, legal or political competing interests with third parties (government, commercial, private foundation, etc.) were disclosed for any aspect of the submitted work (including but not limited to grants, data monitoring board, study design, manuscript preparation, statistical analysis, etc.).



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