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L-leucine dietary supplementation modulates muscle protein degradation and increases pro-inflammatory cytokines in tumour-bearing rats

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ABSTRACT

Cancer cachexia is characterised by involuntary weight loss associated with systemic inflammation and metabolic changes. Studies aimed at maintaining lean body mass in cachectic tumour-bearing hosts have made important contributions reducing the number of deaths and improving the quality of life. In recent years, leucine has demonstrated effective action in maintaining lean body mass by decreasing muscle protein degradation. Currently, there is a growing need to understand how leucine stimulates protein synthesis and acts protectively in a cachectic organism. Thus, this study aimed to assess the effects of a leucine-rich diet on protein degradation signalling in muscle over the course of tumour growth. Animals were distributed into four experimental groups, which did or did not receive 2×10^6 viable Walker-tumour cells. Some were fed a leucine-rich diet, and the groups were subsequently sacrificed at three different time points of tumour evolution (7th, 14th, and 21st days). Protein degradation signals, as indicated by ubiquitin-proteasome subunits (11S, 19S, and 20S) and pro- and anti-inflammatory cytokines, were analysed in all experimental groups. In tumour-bearing animals without nutritional supplementation (W7, W14, and W21 groups), we observed that the tumour growth promoted a concurrent decrease in muscle protein, a sharp increase in pro-inflammatory cytokines (TNFα, IL-6, and IFNγ), and a progressive increase in proteasome subunits (19S and 20S). Thus, the leucine-supplemented tumour-bearing groups showed improvements in muscle mass and protein content, and in this specific situation, the leucine-rich diet led to an increase on the day in cytokine profile and proteasome subunits mainly on the 14th day, which subsequently had a modulating effect on tumour growth on the 21st day. These results indicate that the presence of leucine in the diet may modulate important aspects of the proteasomal pathway in cancer cachexia and may prevent muscle wasting due to the decrease in the cachexia index.

1. Introduction

Cachexia is one of the most important effects of some types of cancer. Among other symptoms, involuntary weight loss and malnutrition are the most common cachectic characteristics observed in cancer patients. This syndrome leads to intense host tissue wasting and subsequent intense body weight loss, especially due to the loss of adipose tissue and muscle mass [1]. Walker 256 rat carcinoma cells originated from a tumour that produced effects resembling those of various cachectic cancers; therefore, these cells are extensively used to establish experimental models of cachexia in rats [2].

Many studies have indicated that the most important goal during cancer progression is the maintenance of lean body mass to improve the prognosis, reduce death, and maintain cancer patients' quality of life. In recent years, leucine has demonstrated efficacy in maintaining lean body mass by stimulating muscle protein synthesis. Among the branched-chain amino acids (BCAA), leucine assumes an important role in the regulation of protein metabolism. *In vivo* and *in vitro* studies have corroborated the fact that a diet supplemented with leucine stimulates protein synthesis, especially in skeletal muscle [3–6]. Similarly, other studies have indicated that leucine supplementation may even reverse or minimise protein catabolism [7,8].

Only a few studies are related to alterations in the tumour-bearing organism throughout tumour evolution. Usually, the literature presents studies that have analysed only one-time point for tumour evolution (for example, rats in the pre-agonic stage). A relevant issue in the present work is the point at which nutritional supplementation begins to prevent/lessen the onset of tumour effects. Therefore, studying the modulatory effects caused by leucine supplementation over time course experiments was beneficial for helping to define the point at which nutritional supplementation began to exert its benefits on lessening tumour effects or improving host responses.

The modulation of pro- and anti-inflammatory interleukins is an important characteristic of the immune system during the pathological

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state [9]. Interleukin levels are changed in tumour-bearing organisms in order to prevent/lessen tumour growth [10–12]. Nutritional supplementation has a role in fostering cytokine action in the tumour-bearing host, thus indirectly modulating tumour effects [13].

Currently, our understanding regarding the mechanism by which leucine imparts a protective effect in skeletal muscle must be expanded in order to better comprehend the reversion of protein catabolism. Therefore, the main aim of this study was to assess leucine effects in relation to the time course of tumour progression by assessing various proteins critical to the proteolytic process in gastrocnemius muscle tissue in Walker 256 tumour-bearing rats.

2. Materials and methods

2.1. Diets

The semi-purified isocaloric diets included normal protein (C), 18% protein (AIN-93G) [14] and leucine (L), or 18% protein plus 3% L-leucine. The diets were supplemented with approximately 70% carbohydrates (sucrose, dextrin, and starch), 7% fat (soybean oil), and 5% fibre (purified micro-cellulose) in addition to vitamin and mineral mixes, cysteine, and choline. The control diet contained 1.6% L-leucine, whereas the leucine-rich diet contained 4.6% L-leucine as based on previous experimental studies [8,15].

2.2. Animals

Female Wistar rats (n = 72 animals; 90 days old; weighing 180–200 g) were obtained from the animal facilities centre at UNICAMP (CEMIB/State University of Campinas, Brazil), and the rats received food and water ad libitum under a 12-h light-dark cycle and constant temperature (22 ± 2 °C) and humidity (50–60%). The choose to use female rats was based on our previous research [7,16–19] which showed that during tumour development the female reproductive cycle stopped at the diestrus stage and did not affect the results acquired in this kind of experimental tumour model. The animals were distributed into 12 groups according to the inoculation of Walker 256 tumour cells (1×10^6 viable tumour cells counted by trypan blue exclusion), the tumour growth time points (7, 14, and 21 days after inoculation) and the provision of a leucine-rich diet. Each group contained six animals, which were treated according to Table 1.

The rats remained in collective cages throughout the experimental period, and groups of animals were sacrificed 7, 14, or 21 days after tumour cell inoculation to evaluate and establish trends in the protein degradation-associated cell signalling profiles. The gastrocnemius muscles were then dissected; portions were weighed, quickly frozen in liquid nitrogen, and then stored in a bio-freezer for further biochemical and molecular analyses. The general United Kingdom Coordinating Committee on Cancer Research guidelines for animal welfare were followed, and the institutional committee approved the protocols based on ethical standards in animal research (CEEA/IB/UNICAMP, protocol number #2418-1) [20].

2.3. Western blot assay

The gastrocnemius muscles were homogenised with homogenisation buffer (20 mM Tris, 1 mM dithiothreitol (DTT), 2 mM adenosine triphosphate (ATP) and 5 mM $MgCl_2$), centrifuged, and divided into aliquots for the analysis of total protein [21]. We also verified the expression of various key proteins of the ubiquitin-proteasome system (proteasome subunits 20Sα, 19S, and 11S; Affinity, USA; diluted 1:1500) in the muscle. In total, 2.5 μg of muscle homogenate protein was resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (12%) and transferred to a 0.45-μm pore size nitrocellulose membrane. The expression of ubiquitin-proteasome system proteins was assessed with antibodies against the following subunits: 20Sα (Affinity USA, 1:1000 dilution), 19S-dependent proteasome unit (ATP, Affinity USA, 1:1500 dilution) and 11S subunit (Affinity USA, 1:1000 dilution). The presence of these subunits was determined using the following secondary antibodies: anti-rabbit, anti-mouse and anti-goat, respectively. The expression of these proteins was standardised using GAPDH as a loading control. Enhanced chemiluminescence (Amersham GE Healthcare, USA) was used to visualise the bands. The densitometry analysis of the protein bands was performed using Image Capture (Amersham GE Healthcare) and Gel Pro II software.

2.4. Serum cytokine analysis

Several cytokines (IL-4, IL-6, IL-10, TNF, and IFNγ) were analysed using multiplex kits (Multiplex Kit, Millipore, USA) with coupling beads and cytokine-specific capture antibodies. Plasma samples previously prepared with antibody conjugated-beads against the cytokines were aliquoted into a multiwell plate. The plate was stirred overnight at 4 °C and washed twice with sheath fluid buffer supplied by the manufacturer (Millipore, USA) to remove material not bound to the beads. Following the addition of the detection antibodies, the plate was stirred for 2 h at room temperature, 24 °C. Phycoerythrin-conjugated streptavidin was then added, and the plate was further incubated for 30 min. Fluorescence was measured using a Luminex®200™ system (Luminex Corporation, TX, USA). The analyses performed with xPonent® 3.1 Software (Luminex Corporation, TX, USA) provided by the Luminex®200™ system.

2.5. Statistical analysis

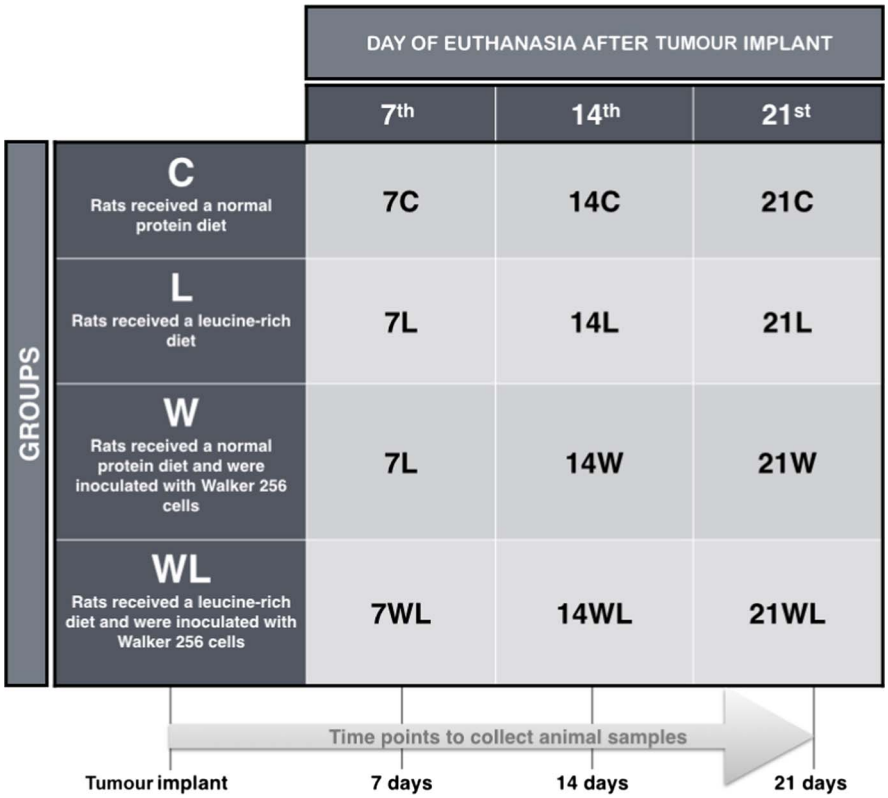
The results are expressed as the mean ± standard error of the mean, using the Graph Pad Prism 6.0 software (Graph-Pad Software, Inc.). For comparisons among multiple groups (such as C, W, L, and LW), data were evaluated with analysis of variance (two-way ANOVA) to determine the effects of diet and/or tumour growth on all parameters, followed by post hoc comparison using Bonferroni's test. P values < 0.05 were considered significant [22].

3. Results

3.1. Body weight and tumour development

The body weight of the animals increased in the leucine-treated groups. This increase was approximately 2.5% in the 7L group and became significant on the 14th and 21st days at approximately 6.5% in the 14L group and 7% in the 21L group when compared with the respective 7C, 14C, and 21C groups (Fig. 1A and C). These data showed that diet accounted for 9.3% of the total variance and influenced the whole body weight evolution ($P = 0.0118$). Among the tumour-bearing groups, the W group had a significant decrease in body weight evolution; despite having reductions in body weight, the leucine group (7WL, 14WL, and 21 WL) showed an increase in body weight compared to W groups (Fig. 1A). The body weight gain (Fig. 1C) showed that tumour effects accounted for 66% of total variance and reduced the body weight in tumour-bearing groups compared to C and L groups ($P < 0.0001$). The interaction effect, however, showed that diet accounted for 26% of the total variance and modulated tumour effects preferentially over body weight in WL group ($P = 0.0432$). Despite the nutritional differences, no differences in tumour development were noted between the W and WL groups; the final tumour weight was 37.28 g ± 3.51 g in the 21 W group and 40.46 g ± 3.31 g in the 21 WL group (Fig. 1B). This type of tumour represents an experimental model of cachexia, and a 10% tumour-to-body weight ratio indicates that the host is suffering from the effects of cachexia (Fig. 1B). To assess cachexia, we used the cachexia index [19,23], which corresponds to the percentage of the initial body weight minus the carcass mass plus tumour weight added to the body mass gain of the control group

Table 1
Experimental procedures.



divided by the initial body mass plus body mass gain of the control group [2,19]. The final cachexia index was 16.98% ± 1.45 in the 21W group and 13.72% ± 1.00 in the 21WL group (P = 0.0474) and showed less carcass spoliation in the leucine-treated group (Fig. 1D). The cachexia indices are in accordance with experimental assays described by Borges and colleagues [23]. In a recent publication, we have shown that leucine-rich diets could improve whole body protein mass leading to a reduction in the cachexia index [19].

3.2. Serum cytokine profiles

Serum cytokine profiles are presented in Fig. 2. The pro-inflammatory cytokines TNFα, IL-6, and IFNγ were increased in the serum of all tumour-bearing rats on the 14th and 21st days of the experiment. These enhanced levels were higher in the WL group on the 14th day than in the W group (Fig. 2A–C. These data show that diet accounted for 9.2–21.9% of the total variance, modulating the effect of the Walker tumour over these pro-inflammatory cytokines, indicating that the interaction is very significant. The anti-inflammatory cytokines IL-4 and IL-10 also exhibited similar profiles in both the 14 W and 14 WL groups, indicating that the altered values only occurred after 14 days of tumour growth (Fig. 2D and E). After analysing these data, it was observed that the increase in IL-4 and IL10 levels was found only in 14 WL and accounted for 21% of total variance. This result indicated that interaction of diet and tumour effects was very significant (P = 0.0019). Using this technique to analyse the TNFα-to-IL-10 ratio (Fig. 2F), we observed an increase in the ratio in the W group compared with the C and L groups but not the WL group (Fig. 2F). The analyses showed that tumour effects accounted for 42.9% of total variance; this effect was considered very significant (P = 0.0002).

3.3. Muscle protein content

The final muscle weight showed that the tumour-bearing groups lost muscle mass when compared with the control rats, especially the W group which had a reduction on 21st day; the tumour effects accounted for 54.4% of variance, and this muscle wasting effect was significant (Fig. 3A). The total muscle protein concentrations in the C group were assessed after sacrificing on the 7th, 14th, and 21st days, and no significant differences were observed. We chose to use the C group as a baseline group to simplify further analyses. The L group exhibited a similar muscle protein content profile that did not differ from that of the C group (Fig. 3B). When compared with the C group, the total muscle protein content was significantly reduced in the 14W and 21 W groups; these data showed that the tumour effect accounted for 14.1% of total variance and was significant (P = 0.0448) (Fig. 3B). On the other hand, when compared with the C and L groups, the WL group exhibited no differences in muscle protein content after 7 and 14 days but exhibited a significant decrease in muscle protein on the 21st day when compared with only the control group (P = 0.0481) (Fig. 3B).

3.4. Muscle proteasome subunits

The ubiquitin-proteasome pathway is the most important pathway for protein degradation in skeletal muscle. Evaluating key proteins allows for investigation of the tumour effects on muscle tissue wasting in addition to the modulatory effects of a leucine-rich diet; therefore, we evaluated the proteasome subunits 11S, 19S, and 20S (Fig. 3C–E, respectively). Fig. 3C presents the results regarding 11S subunit expression. When compared with the C group, the 7 W group showed that 11S subunit expression increased by 167.8%, while approximately

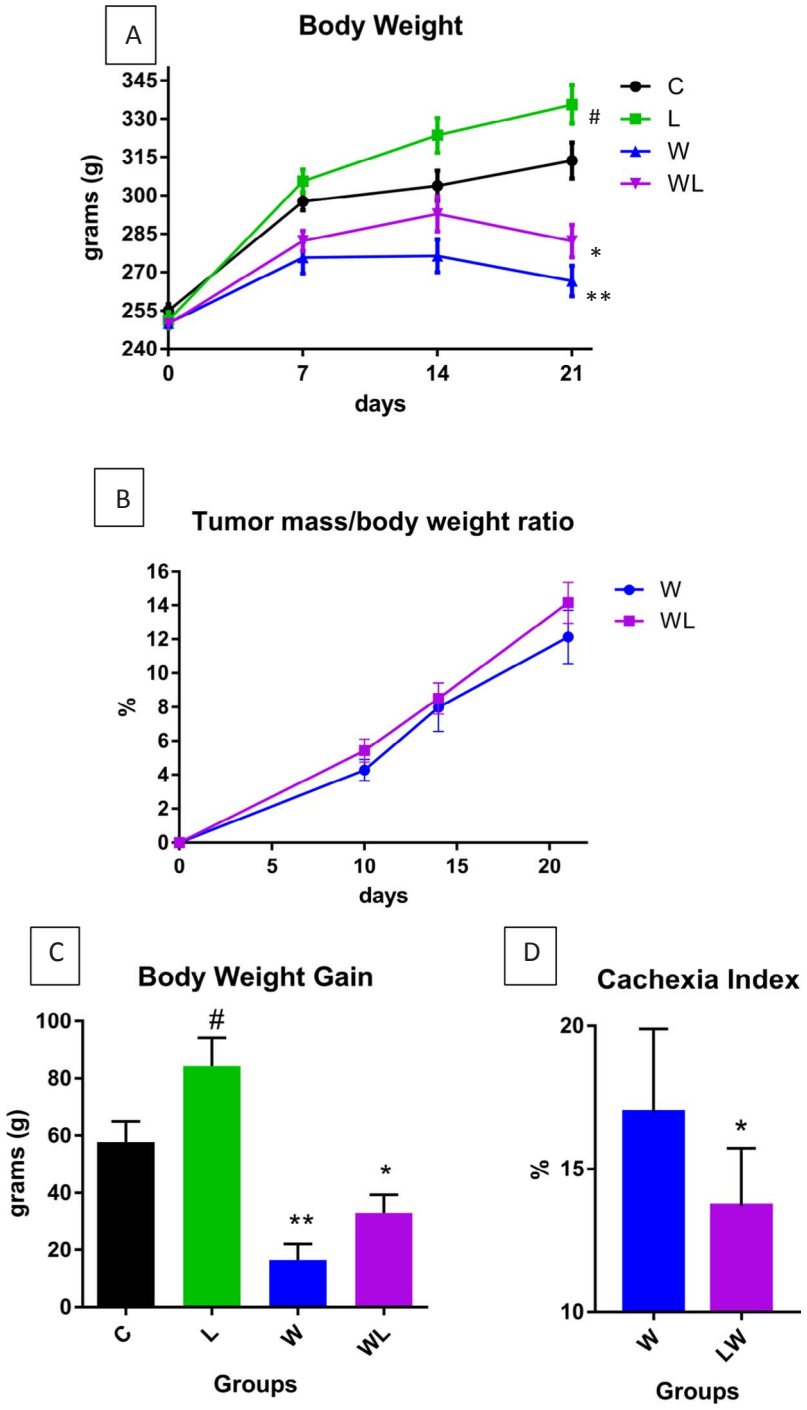


Fig. 1. Body weight (g; A), tumour-body weight ratio (%; B), final body weight gain (g; C) and cachexia index (%; D) at various time points in tumour-bearing rats ± to leucine nutritional supplementation compared with non-tumour-bearing animals. Data are plotted as the mean ± SD. Legend: C: Rats fed a normal protein diet; L: Rats fed a leucine-rich diet; W: Rats fed a normal protein diet and inoculated with Walker 256 tumour cells; WL: Rats fed a leucine-rich diet and inoculated with Walker 256 tumour cells. Samples were collected on the 7th, 14th, and 21st days after the beginning of the experiment. Cachexia index = [(initial body mass − carcass mass + tumour weight + body mass gain of control)/(initial body mass + body mass gain of control)] × 100% [19,21]. # Significant difference as a result of dietary effects, two-way ANOVA with Bonferroni testing, n = 6 per group; ** significant differences as a result of tumour effects, two-way ANOVA with Bonferroni testing, n = 6 per group; * significant differences as a result of leucine modulating the tumour effect, two-way ANOVA with Bonferroni testing, n = 6 per group. $P \leq 0.05$.

126.8% and 165.6% increases were observed in the 14 W and 21 W groups. The increases in the 11S subunit expression were lower in the 7 WL and 21 WL groups when compared with the C group (7 WL = 123.5% and 21 WL = 130.4%), and these values were 26% and 21% lower than those in the corresponding W groups (7 W and 21 W, respectively; Fig. 3C). These data showed that tumour effects accounted for 79.1% of the total variance and were extremely significant ($P < 0.0001$). When compared with the C group, in the 7L, 14L, and 21L groups, 11S expression was increased by approximately 84%, 47%, and 57%, respectively; these data showed that diet had an influence on modulating the 11S expression under leucine supplementation ($P = 0.0004$).

Compared with the C group, 19S subunit expression was increased in the W group on the 7th day (7 W = 189.2%), stabilised on 14th day (14 W = 187.3%), and then increased again on the 21st day (21 W = 243.9%) (Fig. 3D); the tumour effect accounted for 81.7% of

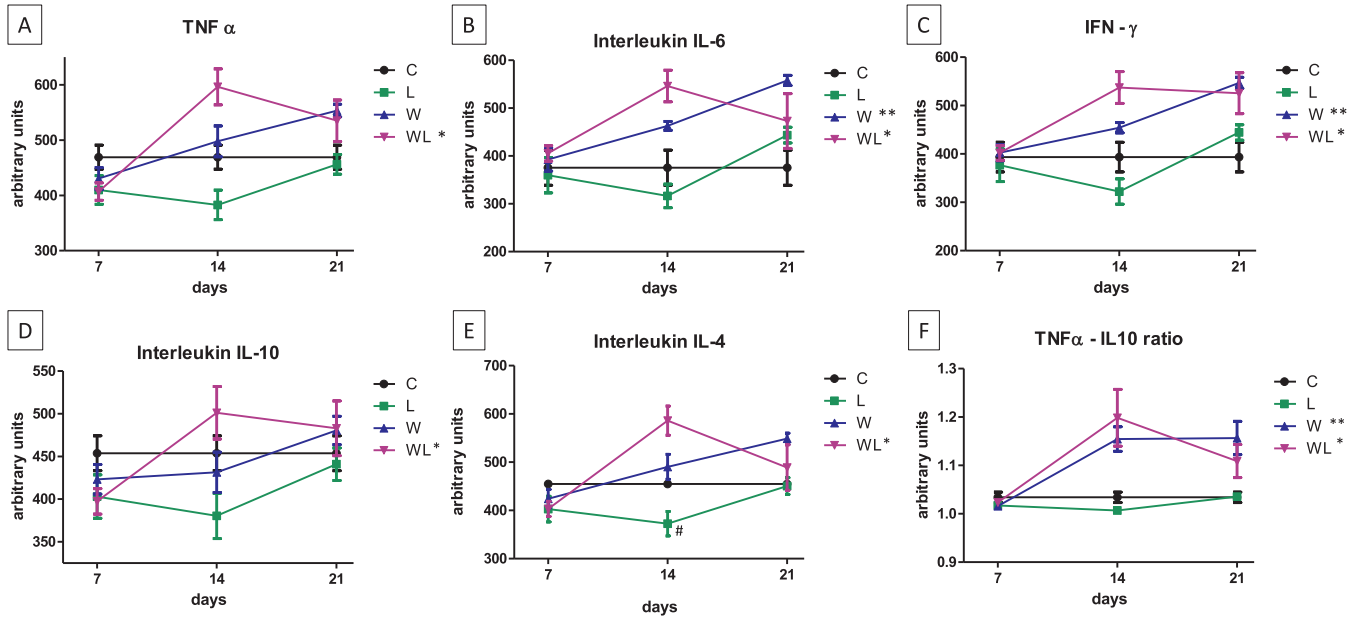


Fig. 2. Serum levels of the pro-inflammatory cytokines TNFα, IL-6 and IFNγ and the anti-inflammatory cytokines IL-4 and IL-10 at various time points in tumour-bearing rats subjected or not subjected to leucine nutritional supplementation compared with non-tumour-bearing animals. The TNFα and IL-10 ratio profiles in Wistar rats with tumours and leucine nutritional supplementation. Data are plotted as the mean ± SD. Legend: C: Rats fed a normal protein diet; L: Rats fed a leucine-rich diet; W: Rats fed a normal protein diet and inoculated with Walker 256 tumour cells; WL: Rats fed a leucine-rich diet and inoculated with Walker 256 tumour cells. Samples were collected on the 7th, 14th, and 21st days after the beginning of the experiment. **Significant differences as a result of tumour effects, two-way ANOVA with Bonferroni testing, n = 6 per group; *Significant differences as a result of leucine modulation of tumour effects, two-way ANOVA with Bonferroni testing, n = 6 per group, $P \leq 0.05$.

variance, being considered extremely significant ($P < 0.0001$). We also observed increased 19S expression levels during the experiment in the WL group, but the increases were not as high as those found in the W group on the 7th and 21st days (7WL = 135.7%; 14WL = 190.3%; and 21WL = 157.2%) (Fig. 3D); in this case, the interaction was considered very significant in modulating the Walker tumour effects ($P < 0.0001$). The L group exhibited an increase in the 19S subunit expression only on the 7th day when compared with the C group (7L = 178.7%) (Fig. 3D).

Proteasome subunit 20S exhibited increased expression in both the W and WL groups on the 7th and 21st days when compared with the respective control groups, and these increases were even higher on the 14th day (Fig. 3E); these data showed that the tumour accounted for 74.6% of total variance and led to an increase of proteasome subunit expression ($P < 0.0001$). In addition, when compared with the C group, we observed an approximately 29–50% increase in 20S expression in the L group; although the ANOVA two-way analysis showed that the diet accounted for 0.12% of the total variance, diet had no effects overall ($P = 0.7115$).

4. Discussion

Previous studies [7,8,17,19] have demonstrated that the loss of skeletal muscle induced by cancer-cachexia could be minimised by leucine supplementation. To our knowledge, the BCAA, leucine, is widely recognised as an attenuator of catabolic stimuli, and for the first time, this work highlights information on the evolution of Walker 256 tumour effects by reporting time course profiles as modulated by the leucine-rich diet scheme. In fact, our results demonstrated increases in various pro- and anti-cachectic cytokines and enhancement in various key proteins involved in the ubiquitin-proteasome pathway in gastrocnemius muscle tissue; these effects are strongly related to the decrease in muscle protein content that starts on the 14th day of tumour development. Importantly, we verified that a leucine-supplemented diet modulated the expression of these proteins, especially during the late phase (the 21st day of the experiment) of tumour-derived harmful effects, thereby preserving muscle protein and minimising the cachexia index (Figs. 3B and 1D, respectively).

The Walker 256 tumour has been extensively studied and is considered an experimental model of cachexia [2,24]. Thus, in our previous studies, we considered a cachectic state to be induced by the Walker 256 tumour when the tumour-to-body weight ratio was greater than 10% [7,25]. Here, we verified on the 21st experimental day that the tumour accounted for > 10% of the body weight, and the cachexia index was almost 17%. This information, in conjunction with body weight gain, clearly indicated that tumour growth induced a harmful effect in tumour-bearing hosts, especially in the W group. Despite the presence of a tumour, less intense body weight wasting was observed in the WL group than in the W group, as confirmed by a lower cachexia index of approximately 14%. As indicated in our previous reports and other studies, despite not increasing survival time and not reducing the tumour evolution, the leucine supplementation did attenuate protein degradation and improved protein synthesis, and these actions were also related to cachexia amelioration [7,25–28]. In fact, we hypothesised that unchanged survival time and tumour mass did not mean a jeopardise process but that leucine-rich diet would improve the whole body protein mass and total adipose body mass, as previously seen [7,29,30], which are important keys to minimise the cachectic state and to improve the host responses.

Lean body mass loss is considered a key component of the cancer process, and it is primarily established by tumour-induced host responses, which are controlled, at least in part, by a variety of pro-inflammatory cytokines and anti-inflammatory cytokines [29–31]. Cytokines such as TNFα, IL-6, and IFNγ in addition to other factors produced by tumours or host tissues have been suggested to play a critical role in tissue wasting in cancer cachexia, especially that of the skeletal muscle mass [15]. Therefore, the pro-inflammatory cytokine profiles reported in this work were also associated with an increase in tumour mass, especially when this mass was approximately 8% of the body weight (on the 14th day of the experiment). At this time, the pro-cachectic cytokine profiles clearly indicated a crescent-shaped evolution in the W group that achieved a maximum value on the 21st day. Other studies also demonstrated that increased pro-inflammatory cytokine levels were associated with increased tumour mass [32,33].

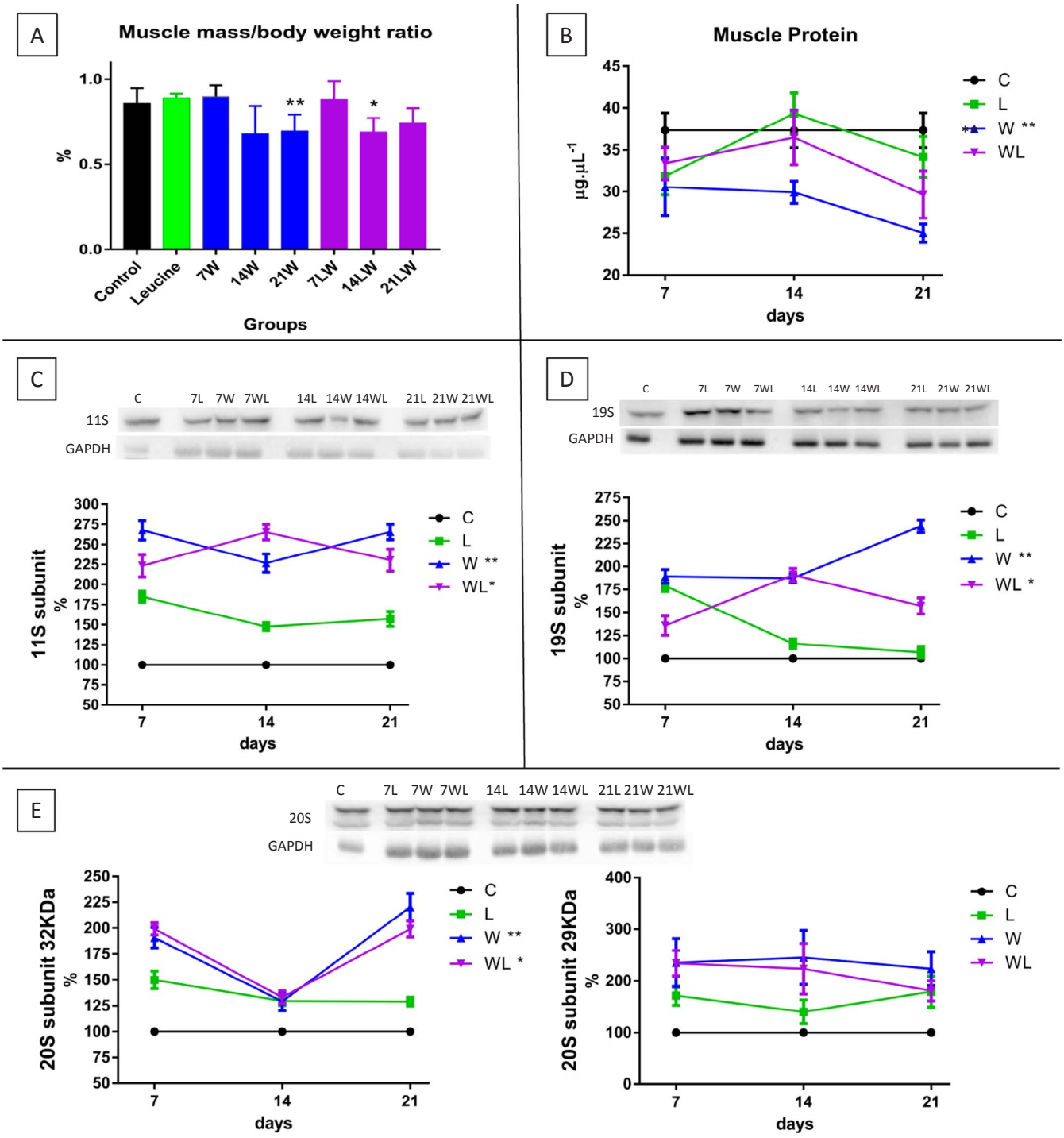


Fig. 3. Muscle mass-to-body weight ratio (%; A), total muscle protein concentrations (µg/µL; B) and proteasome subunits: 11S (% of arbitrary unity; C), 19S (% of arbitrary unity; D), and 20Sα (subunits 29 kDa and 32 kDa; (% of arbitrary unity; E)) in the gastrocnemius muscle tissue of Wistar rats with or without tumour and leucine nutritional supplementation. Data are plotted as the mean ± SD. Legend: C: Rats fed a normal protein diet; L: Rats fed a leucine-rich diet; W: Rats fed a normal protein diet and inoculated with Walker 256 tumour cells; WL: Rats fed a leucine-rich diet and inoculated with Walker 256 tumour cells. Samples were collected on the 7th, 14th and 21st days after the beginning of the experiment. **Significant differences as a result of tumour effects, two-way ANOVA with Bonferroni testing, n = 6 per group; *Significant difference as a result of leucine modulation of tumour effects, two-way ANOVA with Bonferroni testing, n = 6 per group. $P \leq 0.05$.

Here we showed that a different profile was observed in the tumour-bearing group fed a leucine-rich diet (the WL group) because the maximum value was achieved at an earlier time point (on the 14th day) than that in the W group; in parallel, this parameter was associated with an earlier increase in anti-inflammatory cytokines, including IL-4 and IL-10. As these anti-cachectic cytokines were also present in animals with a tumour subjected to a leucine-rich diet (those in the WL group), these cytokines could have contributed to an earlier induction of the anti-inflammatory process and protected the body against tumour-induced negative effects, thereby minimising the cachectic state. In fact, we noticed that the pro-inflammatory cytokine expression-to-muscle protein ratio (data not shown) showed that the W group had even higher ratio than WL group, suggesting an intense muscle wasting process in W, which was minimised in WL especially on 21st day. The relation between anti-inflammatory (IL-4 and IL-10) to muscle protein showed that the W group had lower values compared to WL, suggesting

that leucine supplementation led to improvement and antecipated the anti-inflammatory responses and amelioration of muscle wasting.

This fact could be noticed as protein degradation via ubiquitin-proteasome in the WL group showed some differences when compared with the W group. During the process of protein degradation in cachexia, myofibrillar proteins are primarily conjugated to ubiquitin, which serves as a signal for degradation by the large proteolytic 26S proteasome complex, which requires ATP to perform its functions [34]. Previous reports have provided evidence of increased 20S subunit expression in tumour-bearing animals, including animals bearing Walker 256 tumours [7,8,25,35]. Here, we analysed the proteasome subunits at various time points during tumour development in such tumour-bearing animals with muscle protein decrease. Our results demonstrated that this proteolytic system, increasing on the 7th day after tumour cell inoculation, could be an acute response to tumour-related effects. This process proceeded until the 21st day for both tumour-bearing groups and the process correlated with decreased muscle weight, especially in the W group. Since, the 26S proteasome is a complex with protease activity that degrades polyubiquitinated proteins via an ATP-dependent process [36], the increase in the expression of 20Sα subunits (29 and 32 kDa bands) likely indicated that the proteolytic process was activated in gastrocnemius muscle tissue in both tumour-bearing groups, and this activation was slightly more intense in the W group. Despite the tumour-induced wasting effect, the nutritional supplementation in the WL group led to a slightly decreasing trend ($P = 0.084$) in 20S subunit expression, suggesting a modulation of this proteolytic via. One regulatory complex of the 20S proteasome, which is an ATP-dependent 19S subunit or PA700 [37,38], had a progressive increase in the W group, and this increase was counteracted by modulated leucine nutritional support. This finding is consistent with the fact that leucine ameliorated the muscle protein content by causing a reduction in protein wasting. The 11S complex [39] is another regulatory complex that also appears to play an important role in 20S proteasome activation [40], which increased in tumour-bearing rats, indicating an effect of tumour evolution-induced muscle wasting. In contrast to this effect, the WL group exhibited an 11S subunit profile that was opposite to that observed in the W group, suggesting that leucine supplementation in tumour-bearing animals most likely modulated the process of protein degradation on the 21st day, given that the nutritional scheme led to reduced 11S subunit expression. Of the W and WL groups, the highest expression of proteasome subunits was observed in the W group, providing evidence that a leucine-rich diet aided in reducing protein breakdown in cachectic organisms. Therefore, the time course profiles of the proteasome proteolytic pathway revealed a trend of increasing expression in the tumour-bearing groups, confirming the well-known process of cachexia, as supported by previous publications [8,35]. Despite those facts, the administration of a leucine-rich diet likely prevented/minimised the onset of tumour effects, attenuating the tumour-induced protein degradation as evidence of maintenance of muscle protein content. Thus, the process of cachexia can be minimised upon administration of a leucine-rich diet. Further studies are currently underway to determine the precise role of leucine nutritional supplementation on protein metabolism in the skeletal muscle of tumour-bearing animals.

5. Conclusion

Results indicate that a leucine-rich diet improved body weight and muscle protein most likely via modulating pro-inflammatory cytokines and increasing anti-inflammatory cytokines. In addition, muscle protein maintenance was associated with leucine's modulatory effects on proteasome subunit expression and amelioration of the cachexia index. Overall, despite the presence of a tumour, leucine nutritional supplementation exhibited several protective advantages against harmful tumour-induced effects and, as showed here, a relevant impact of our results is the onset of leucine action (14th day) in which began to lessen the tumour effects and improving the host responses.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Authors' contributions

BC performed *in vivo* experiments and most of the biochemical/molecular assays and contributed to the discussion of the results in addition to the manuscript preparation.

AGO contributed to the experimental assays and molecular assays, discussion of the results, and manuscript preparation.

MCCGM was responsible for the experimental design, the interpretation and discussion of the results, and the final manuscript revision.

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