

Leucine-rich diet minimises liver glycogen mobilisation and modulates liver gluconeogenesis enzyme expression in tumour-bearing cachectic rats

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Abstract

Aims

Cachexia is defined as a complex metabolic syndrome that is associated with tissue damage. Some studies have shown that the liver metabolic alterations contribute to overall host tissue wasting. Knowing that leucine acts as cell signalling, we evaluated hepatic metabolism in Walker 256 tumour-bearing rats and investigated the modulatory effects of a leucine-rich diet.

Methods and Results

Wistar rats were distributed into 4 groups: control (**C**) and tumour-bearing (**W**) groups, fed a control diet, and leucine (**L**) and leucine tumour-bearing (**LW**) groups, which fed a leucine-rich diet. After tumour evolution (21 days), liver samples were collected, and assessed the glycogen content via histological periodic acid-Schiff (PAS) staining and performed the molecular and biochemical analysis. A higher liver-to-body weight rate was observed in W and LW groups, whereas a lower muscle-to-body weight ratio was observed only in W group. Hepatic glycogen content was lower only in W group, which had a greater number of hepatocyte nuclei; these parameters were unchanged in LW rats. Moreover, phosphoenolpyruvate carboxykinase (PEPCK), glycogen synthase, and lactate dehydrogenase (LDHA) gene expressions were higher in liver tissue from W group than in LW group. However, liver alkaline phosphatase and γ GT activities, and also liver AMP-activated protein kinase (AMPK) expression were higher in both tumour-bearing groups.

Conclusions

Our results suggest that a leucine-rich diet has a protective effect on the loss of skeletal muscle and also minimises the liver failure induced by Walker 256 tumours. Despite the lack of protection against liver damage, the leucine-rich diet modulated liver energy stores, likely decreasing the futile Cori cycle and reducing energy expenditures.

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Introduction

Cachexia is a multifactorial syndrome that significantly affects patient quality of life and is responsible for nearly to 20-30% of all cancer-related deaths [1, 2]. Cancer cachexia induces metabolic alterations that include primarily disorders of protein turnover, leading to significant skeletal muscle wasting, with or without fat loss, and subsequent involuntary body weight loss [3]. Cachexia leads to chronic inflammation, which elevates circulating levels of C-reactive protein (CRP) and pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α) [4], which may play an important role in the mechanisms that underlie muscle wasting [5].

Moreover, a study performed by Narsale and colleagues [4] showed that chronic exposure to IL-6 induces hyperplasia in the hepatic tissue. This liver hypertrophy may contribute to cachexia progression in a cachectic mouse model by elevating resting energy expenditures [4]. The liver tissue is partly responsible for the systemic metabolic rate by regulating pathways involved in the utilisation, transport, storage and breakdown of glucose and fat. These important liver functions could be jeopardised in the presence of cancer, leading to the abnormal expression of liver enzymes, which leads to a poor prognosis in most cancers [6].

The majority of neoplastic cells preferentially use glucose as an energy source, resulting in increased glucose metabolism in cancer cells [7]. In cancer patients who are losing weight, hepatic gluconeogenesis is

enhanced [8], probably to provide the tumour cells with de novo synthesised glucose, which is produced by using amino acids from skeletal muscle breakdown, glycerol from lipolysis and lactate from tumour aerobic glycolysis (Warburg effect) [9]. The gluconeogenesis process depends on the adenosine triphosphate (ATP) supply, which leads to energy depletion under low serum glucose conditions, contributing to protein breakdown, followed by tissue wasting and weight loss in cancer patients [10]. If we consider skeletal muscle tissue wasting to be the main source of amino acids for liver gluconeogenesis, it is plausible to assume that energy depletion contributes to skeletal muscle loss, which in turn causes the cachectic state [11, 12].

Several studies are currently investigating strategies to inhibit or minimise skeletal muscle wasting and consequently improve quality of life for cancer patients [13-15]. One of these strategies is nutritional supplementation with leucine, which is a branched-chain amino acid (BCAA) that plays an important role in skeletal muscle metabolism [16]. Leucine efficiently improves skeletal muscle protein synthesis in patients [17, 18] and in animals, as shown previously [19-22]. Leucine alone and in a complete BCAA mixture stimulates protein synthesis and decreases protein proteolysis [23-25]. Leucine regulates protein synthesis in the skeletal muscle following food intake, stimulating the mTOR pathway and inhibiting the ubiquitin-proteasome pathway [23, 26]. Moreover, a recent study performed by Takegoshi and colleagues [25] revealed that oral BCAA supplementation in patients with liver cirrhosis potentially suppresses the incidence of hepatocellular carcinoma, inducing anti-fibrotic factors and preventing apoptosis.

Therefore, this study aimed to determine if a leucine-rich diet could modulate the liver damage caused by tumour growth, improving hepatic enzymes and restoring liver glycogen stores, even in the cachectic state.

Material and Methods

Animals and diet

Female Wistar rats (approximately 90±10 days old, obtained from the Animal Facilities at the State University of Campinas, UNICAMP, Brazil) that weighed approximately 265±10 g were housed in collective cages under controlled environmental conditions (light and dark 12/12 hr; temperature 22±2°C; and humidity 50-60%). The animals were monitored daily, weighed 3 times/week and given food and water *ad libitum*. Semi-purified diets were constructed in accordance with the American Institute of Nutrition (AIN-93; [27]), and the leucine-supplemented diet was enriched with 3% L-Leucine, as described in our previous works [19, 28-30]. Both diets (control (C) and leucine (L)) contained similar

amounts of nitrogen (approximately 2.84 g N₂/100 g diet), for a protein content of approximately 18%.

Walker 256 tumour inoculation

The Walker 256 tumour is widely used as an experimental model of cancer cachexia syndrome [19, 30-32]. Walker 256 carcinosarcoma cells (2.5×10^6 viable cells) were injected subcutaneously into the right flank of the experimental rats on the first day of the experiment. The general guidelines of the UKCCCR (United Kingdom Co-ordinating Committee on Cancer Research, 1998) [33] regarding animal welfare were followed, and the experimental protocol was approved by the Institutional Committee for Ethics in Animal Research (CEEA/IB/UNICAMP, protocol # 2677-1).

Experimental protocol

The animals were randomly distributed into four experimental groups according to tumour implantation status and leucine nutritional supplementation. Two groups were fed a control diet (18% protein): C, control group (n=9) and W, Walker 256 tumour-bearing group (n=9). The other two other groups were fed a leucine-rich diet (18% protein + 3% leucine): L, leucine control group (n=8) and LW, leucine Walker 256 tumour-bearing group (n=9). All rats were monitored and weighed 3 times/week. At the end of the nutritional supplementation period (i.e., 21 days after tumour evolution), the animals were euthanised without an overnight fast, and blood, gastrocnemius muscle and liver samples were collected. The collected tissues were resected and weighed, and some liver fragments were immediately frozen directly in liquid nitrogen and stored at -80°C for biochemical analyses, western blotting and real-time PCR. Additional fragments of liver tissue were immediately fixed in 4% paraformaldehyde for 24 hours before being processed for histology with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining. The blood samples were centrifuged at 10,000 × g at 4°C for 10 min, and serum was stored at -20°C for biochemical analyses.

Biochemical analyses

Serum was analysed to determine the total protein, glucose and bilirubin contents, as well as some enzyme activities, such as alkaline phosphatase (ALP), gamma-glutamyltransferase (γGT), glutamate pyruvate transaminase (GPT), and glutamate oxaloacetate transaminase (GOT). ALP, γGT, GPT, GOT and cholesterol were also measured in the liver tissue. The serum total protein content was determined using the Biuret reactive reagent, and the absorbance was read at 540 nm. The glucose levels were measured using the glucose-oxidase reactive reagent, and the absorbance was read at 540

nm. Serum ALP activity was measured using a P-NPP substrate solution (60 mM nitrophenyl phosphate (P-NPP), 10 mM MgCl₂·6H₂O, pH 10.3). The enzymatic reaction proceeded for 30 min at 37°C, and the absorbance was read at 405 nm. γGT levels were measured using an enzymatic assay with a γGT substrate (γ-carboxyl-4-nitroanilide), and the absorbance was read at 405 nm within 10 minutes. The GPT and GOT levels in the serum and liver tissue were measured using an enzymatic assay kit (Bioclin, Sao Paulo, Brazil), with a GPT and GOT substrate (L-Alanine + α-ketoglutarate; L-Aspartate + α-ketoglutarate), with the enzymatic catalytic reactions performed according to manufacturer instructions. The absorbance was read at 365 nm using a microplate reader (Hidex Chameleon™ V plate reader, Switzerland). Cholesterol levels were measured using an enzymatic assay with a substrate (4-aminophenazone, phenol, cholesterol oxidase, cholesterol esterase and peroxidase). The reaction was performed according to the manufacturer's instructions (Laborclin), and the absorbance was read at 500 nm (Hidex Chameleon™ V plate reader, Switzerland).

Histology

The fixed liver tissues were embedded in paraffin, cut into 5 μm sections and stained with H&E and PAS. H&E-stained slides were analysed to determine the hepatocyte area, the nuclear hepatocyte area and the number of hepatocyte nuclei. These analyses were achieved by counting 5 fields (500 μm² each) on one slide from each animal, with at least six rats per group. The images were captured using a Leica DMLS microscope at a magnification of 200X. PAS staining was analysed to determine the liver glycogen content. The Image-Pro Premier 9.1.4 software was used to measure the glycogen content, using the tool Line Profile, which represents the pixel intensity value as a line drawn on the image. Thus, higher intensity pixels indicate higher glycogen content.

Western blotting

Liver tissue samples were homogenised in protein extraction buffer (20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 100 mM KCl, 0.2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 50 mM NaF, 1 mM DAB tetrahydrochloride, 0.5 mM orthovanadate and 50 mM glycine, pH 7.4), followed by centrifugation at 10,000×g for 15 min at 4°C. The supernatant samples were then separated by 12.5% SDS-PAGE electrophoresis under reducing conditions. After gel electrophoresis and protein transfer onto a nitrocellulose membrane, the proteins were blocked in 5% non-fat dry milk at room temperature for 1 h. The membranes were then incubated overnight at 4°C with antibodies against total AMPK (Catalogue number: 2603S; Cell Signalling; diluted 1:1,000) and phospho-

AMPK^(Thr172) (Catalogue number: 2535S; Cell Signalling; diluted 1:1,000). Immunoreactivity was detected via the sequential incubation of the membranes with an anti-mouse secondary antibody for 1 h at room temperature, which was visualised using a chemiluminescence detection system. The level of AMPK was estimated relative to the level of the 37 kDa GAPDH protein (Catalogue number: 5174; Cell Signalling; diluted 1:1,000).

Quantitative RT-PCR

Total RNA from the liver tissue was extracted with the TRIZOL[®] reagent (Invitrogen) according to the manufacturer's instructions. The quality of the RNA samples was examined at 260 and 280 nm with a UV spectrophotometer (Nanovue Spectrophotometer 28923215 Ge BioSciences, USA). cDNA was produced using a high-capacity cDNA reverse transcription kit (Applied Biosystems[®], USA) containing the Multiscribe™ Reverse Transcriptase. cDNA synthesis was performed using 3 μg of RNA at 42°C. Finally, the cDNA was diluted 1:2 before use in quantitative PCR. Real-time reactions were performed using standard methods (ABI Prism 7500 Sequence Detection System; Applied Biosystems, Foster City, USA), and the real-time PCR analysis was normalised to beta-actin. The following genes were evaluated via real-time PCR: phosphoenolpyruvate carboxykinase (PEPCK) (Sigma; forward primer 5'-AATAATGGGACATTGGCTG-3' and reverse primer 5'-AAAACACCATCTTACCAAC-3'), glucokinase 2 (Sigma; forward primer 5'-ATCATCGGTGGGAAGTATATTG-3' and reverse primer 5'-CTTAGGATGTTGTGGATCTG-3'), glycogen synthase 2 (Sigma; forward primer 5'-TACGATGGGTTATTAAGAGGAG-3' and reverse primer 5'-CGTCGATCATATTGTGAGTG-3'), lactate dehydrogenase (LDHA) (Sigma; forward primer 5'-TGGGGTTGGTGGCTGTTGGCATG-3' and reserve primer 5'-ACGTTTCGCTGGACCAAATTGAGCC-3') and citrate synthase (Sigma; forward primer 5'-GGGAGCTCATCTATGAAGAC-3' and reserve primer 5'-TGAAGTGGAGTTCTGTGTAG-3').

Statistical analyses

The results are shown as the mean ± standard deviation, and all data were analysed using the Graph Pad Prism 6.0 software (Graph-Pad Software, Inc.). For comparisons among multiple groups (e.g., C, W, L and LW), the data were evaluated with analysis of variance (two-factor ANOVA) followed by the post-hoc Bonferroni's test [34].

Results

Morphological and histological parameters

Walker-256 tumour growth led to cachexia, as indicated by significant weight loss, represented by the carcass weight, in both tumour-bearing groups (W and LW). On the other hand, skeletal muscle mass loss was found only in the W group, and we observed no differences between the LW and L groups. The relative tumour weight ratios were similar in groups W and LW. The relative liver weight was greater in groups W and LW than in groups C and L (Table 1).

Walker-256 tumour growth leads to some alterations in liver morphology. The higher liver weight in

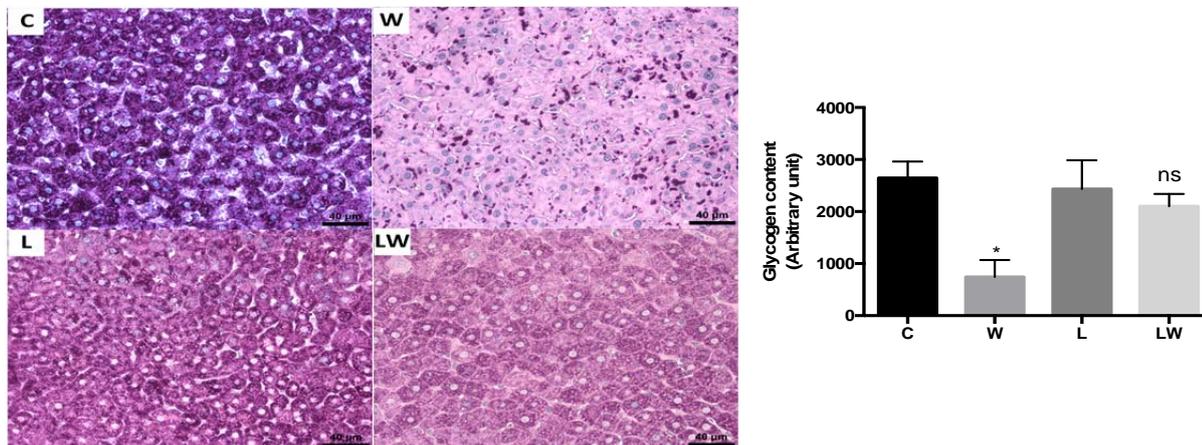
the W and LW groups (Table 1) was associated with a greater number of hepatocyte nuclei, as well as a greater nuclear hepatocyte area, in both tumour-bearing groups, with statistical significance only for W rats. The leucine-rich diet scheme led to changes in these parameters in the L group in comparison to the C group. The liver glycogen content was lower in the W group than in the control group (C). However, the glycogen content was preserved in the LW group, with no difference between the LW and L groups (Figure 1).

Table 1 Morphometric parameters

	C	W	L	LW
Morphometric parameters				
Weight gain (%)	115.1±4.8	106.8±5.6 ^a	115.1±4.8	109.1±7.1
Relative carcass weight (%)	95.1±6.8	58.0±11.9 ^a	97.8±5.7	66.9±16.4 ^{a,c}
Relative tumour weight (%)	-	14.7±4.7	-	14.7±2.4
Relative liver weight (%)	3.0±0.2	3.7±0.1 ^a	3.2±0.1	3.5±0.2 ^{a,c}
Relative muscle weight (%)	0.67±0.06	0.46±0.04 ^a	0.67±0.15	0.59±0.08
Histological parameters				
Hepatocyte area (µm ²)	13601±499	14176±624	15750±182	13706±271
Nuclear Hepatocyte area (µm ²)	25.1±2.0	28.4±2.5 ^a	27.6±2.5 ^a	27.5±2.4
Number of hepatocyte nuclei	22.7±4.8	70.0±12.2 ^a	49.6±13.2 ^a	33.7±6.9 ^b

Data are expressed as the mean ± SD. Legend: C control; W, tumour-bearing (fed a control diet, 18% protein); L, control; LW, tumour-bearing (fed a leucine-rich diet, 18% protein + 3% leucine). Relative tissue weights correspond to the ratio of the specific tissue and body weights, expressed as a percentage. For details, please see the Material and Methods section.^a P < 0.05 in comparison with the C group; ^b P < 0.05 in comparison with the W group and ^c P < 0.05 in comparison with the L group.

Figure 1. Liver histology of tumour-bearing rats in the presence and absence of a leucine-rich diet. Periodic acid-Schiff (PAS) staining of the liver in adult female rats from different experimental groups that were killed post-prandial state (40× magnification). For details, see the Material and Methods section. * P < 0.05 for comparison with the C group; ns=non-significant difference in comparison with the L group.



Serum and liver tissue biochemical analyses

Both tumour-bearing groups had significantly lower total serum protein concentrations (W and LW) than the control groups (Table 2). The glucose level was lower in the W group than in the control group (C), but no difference was observed between the LW and L groups (Table 2). The serum bilirubin concentration was greater in both tumour-bearing groups (W and LW). Additionally, serum ALP activity was higher in groups W

and LW than in their respective control groups, and the serum γGT content was greater in the tumour-bearing groups, but no differences in GPT and GOT content were observed, independent of tumour status or nutritional scheme (Table 2).

The liver tissue analysis showed that hepatic enzyme activities, such as γGT, GOT and GPT, had the same values among the groups (Table 2). However, liver alkaline phosphatase activity was higher in both tumour-bearing groups. In addition, while the liver cholesterol

concentration maintained similar values in the C, W and L groups, this value was higher in the LW group (Table 2).

Molecular analyses

Glycogen synthase, phosphoenolpyruvate carboxykinase (PEPCK) and lactate dehydrogenase (LDHA) gene expression levels were higher in the W group than in the other groups. Therefore, these enzyme expression levels were unchanged in group LW in

comparison to the respective control group (L). In addition, these genes were expressed at significantly lower levels in group LW than in group W (Figure 3B-C). On the other hand, glucokinase and citrate synthase gene expression did not vary among the experimental groups (Figure 3A and E). However, AMP-activated protein kinase (AMPK) activation was higher in both tumour-bearing groups than in the control groups (Figure 3F).

Table 2. Serum and liver tissue biochemical analyses.

	C	W	L	LW
Serum				
Total protein (g/dL)	5.0±0.4	3.0±0.4 ^a	4.7±0.1	3.2±0.3 ^c
Glucose (µM)	87.6±33.4	51.4±7.5 ^a	95.4±20.3	65.2±26.1
Bilirubin (µM)	2.3±0.7	19.8±10.1 ^a	0.7±0.3	19.4±5.0 ^c
ALP activity (U/L/min)	0.030±0.009	0.161±0.097 ^a	0.023±0.010	0.149±0.077 ^c
γGT (U/mL)	6.64±1.91	14.01±1.08 ^a	5.48±2.99	12.63±1.88 ^c
GPT (U/mL)	12.6±1.3	11.4±3.4	13.2±1.8	11.6±3.2
GOT (U/mL)	10.5±1.4	10.9±1.0	11.6±0.5	10.6±1.1
Liver Tissue				
ALP activity (U/L/min)	0.028±0.001	0.188±0.092 ^a	0.019±0.004	0.305±0.105 ^c
γGT (U/mL)	13.1±2.6	23.1±4.5	26.2±10.2	27.7±3.7
GPT (U/mL)	22.6±2.5	20.5±1.4	22.7±2.1	24.0±2.2
GOT (U/mL)	18.6±0.9	17.1±2.6	19.0±0.6	22.0±4.6
Cholesterol (mg/dL)	331.0±29.7	227.2±50.0	379.7±63.4	507.2±142.3 ^b

Data are expressed as the mean ± SD. Legend: C control; W, tumour-bearing (fed a control diet, 18% protein); L, control; LW, tumour-bearing (fed a leucine-rich diet, 18% protein + 3% leucine). For details, please see the Material and Methods section ^aP < 0.05 in comparison with the C group, ^bP < 0.05 in comparison with the W group and ^cP < 0.05 in comparison with the L group.

Figure 2. Liver gene and protein expression. A: Glucokinase gene expression (Real Time-PCR), B: Glycogen Synthase gene expression (Real Time-PCR), C: Phosphoenolpyruvate carboxykinase (PEPCK) gene expression (Real Time-PCR), D: Lactate dehydrogenase (LDHA) gene expression (Real Time-PCR), E: Citrate Synthase gene expression (Real Time-PCR) and F: AMPK (AMP-activated protein kinase) protein expression (western blot images). The graphics express the results as the mean ± SD. For details, see the Material and Methods section. ^aP < 0.05 for comparison with the C group; ^bP < 0.05 in comparison with the W group; ^cP < 0.05 for comparison with the L group.

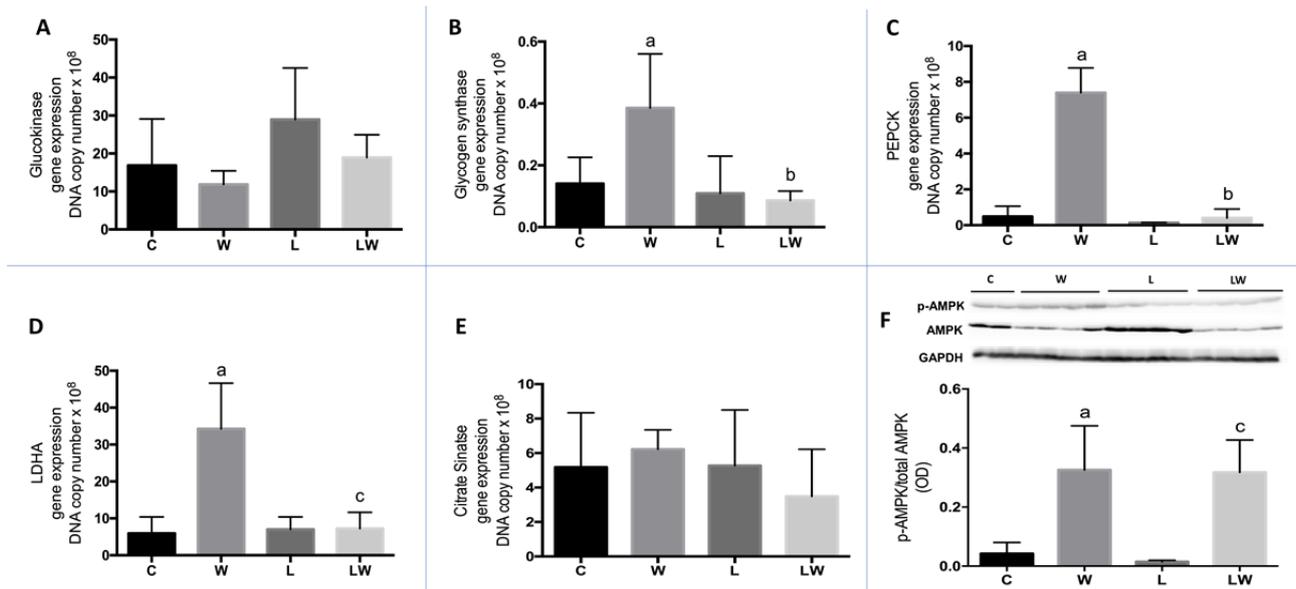
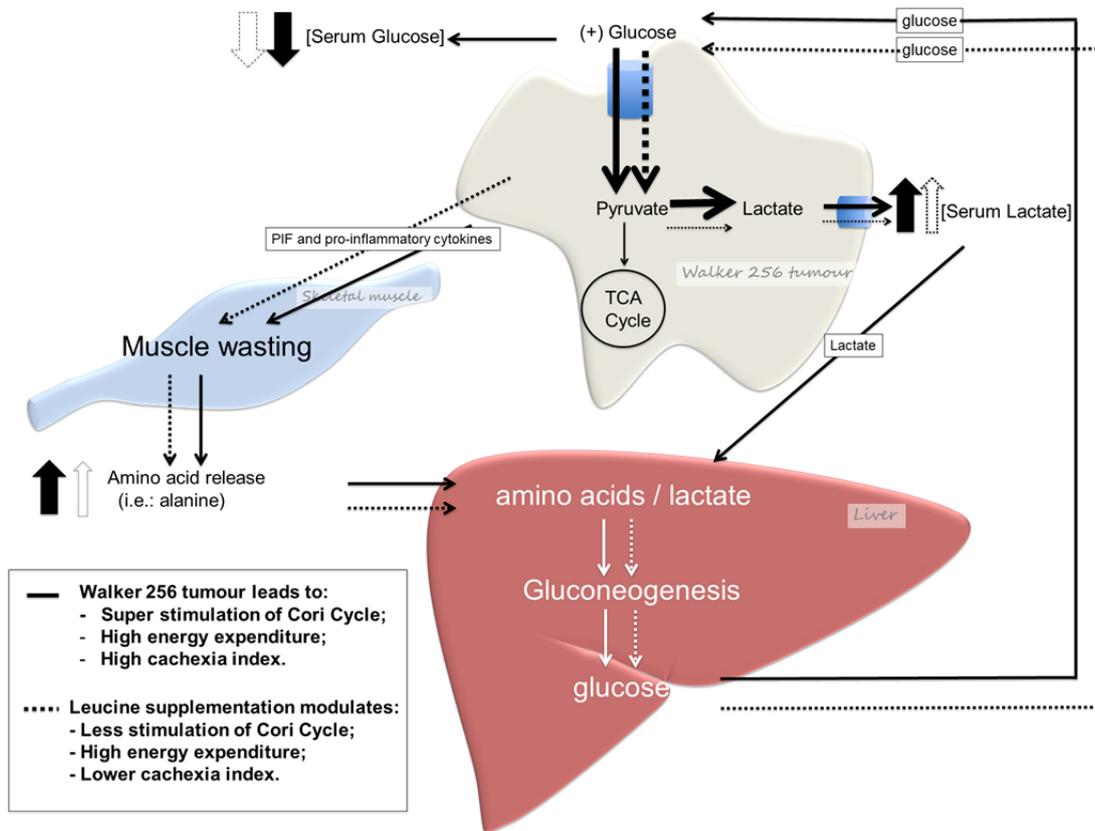


Figure 3. Illustration of the proposed mechanism by which a leucine-rich diet improves cachexia in Walker 256 tumour-bearing rats. TCA: tricarboxylic acid cycle. Dashed arrows and lines represent less stimulated pathways, with the hypothesis supported by the effects of leucine. Solid thicker lines and arrows represent more stimulated pathways, seen in the tumour-bearing group (W).



Discussion

Cachexia is responsible for 20-30% of all cancer-related deaths and also leads to a poor quality of life in these patients. Thus, understanding the mechanisms involved in cancer cachexia and the effects of co-adjuvant treatments is essential. In this study, we investigated the damage caused by Walker 256 tumour growth in the liver tissue and also showed the effects of a leucine-rich diet on the liver tissue in these cachectic rats.

Cachexia leads to significant skeletal muscle loss. On the other hand, some visceral organs, such as the heart, spleen, and liver, maintain their mass or even exhibit hypertrophy during cachexia [4]. Corroborating the literature, the increased liver mass that was found here in both tumour-bearing groups likely reflected the possibly related chronic inflammatory state that is present in cachexia hosts, which is caused primarily by an increase in circulating pro-inflammatory cytokines, such as IL-6, IL-1 and TNF- α [28]. Some studies reported that chronic exposure to IL-6 induces hyperplasia in hepatic tissue [4, 35]. In our previous studies, we found that serum levels of pro-inflammatory cytokines, especially IL-6 and TNF- α , were higher in Walker 256 tumour-bearing rats [36] and in MAC 16 tumour-bearing mice (another widely used experimental cancer cachexia model) [28].

Liver hypertrophy is speculated to contribute to cachexia progression in cancer patients by elevating resting energy expenditures [37]. The liver tissue is partly responsible for controlling the systemic metabolic rate by regulating a variety of pathways involved in the transport, storage, breakdown and utilisation of glucose. In these processes, some liver glycolytic enzymes could be changed during tumour evolution, as we have observed here, especially in the W group. The stronger expression of glycogen synthase in tumour-bearing rats in this study was not consistent with reduced glycogen stores. This observation could be explained by the fact that the glycogen produced by glycogen synthase in the liver might be immediately mobilised to provide glucose to meet the growing neoplastic tissue demands. Although enhanced liver lactate dehydrogenase expression could be related to greater lactate content (observed in our previous study [14]), this fact likely suggests higher liver gluconeogenesis.

Cancer cells preferentially use glucose as an energy source, leading to an increased glycolysis rate in tumour tissues and a consequent reduction of the blood glucose concentration [38], even with higher liver gluconeogenesis. Additionally, some cancer cells exhibit disturbed glucose metabolism, known as the Warburg effect, in which the cells prefer glycolysis over oxidative phosphorylation, even in the presence of oxygen [39]. In addition, liver gluconeogenesis is enhanced in cachexia

because the gluconeogenic precursors (amino acids from proteolysis, glycerol from lipolysis and lactate from glycolysis) are converted to de novo glucose that probably feeds the tumour (this process is called a “futile energy cycle” and was first described by Cori and Cori [40]). Since the Cori cycle is maintained by intense phosphoenolpyruvate carboxykinase (PEPCK) activity, the higher PEPCK gene expression observed here likely represented an enhanced gluconeogenesis process in the W group, which could lead to greater energy expenditures and more muscle wasting, as observed in the present study. Conversely, liver PEPCK expression exhibited no differences between the L and LW groups. This result could probably be explained by the fact that tumour tissues might use leucine directly as an energy source. Using leucine as an energy source, the tumour needs less glucose; consequently, the gluconeogenesis process is less important, and less energy is spent, reflecting the preserved skeletal muscle tissue in the LW group. In our recent study [30], which used the same experimental protocol used in this study, we found a lower cachexia index in Walker 256 tumour-bearing rats that were fed a leucine-rich diet. This outcome is likely related to the reduced gluconeogenesis that was observed in these animals supplemented with leucine. In this same study [30], we found higher lactate levels (19.2%) in the W group than in the C group, which were significantly lower (15.3% lower; [27]) in tumour-bearing rats fed a leucine rich-diet (LW) [30]. This result suggests that leucine supplementation reduced tumour lactate release and also decreased skeletal muscle wasting, leading to lower amino acid levels. Consequently, the liver receives less lactate and amino acids, leading to a lower gluconeogenesis rate in the LW group. Reduced gluconeogenesis results in reduced energy expenditures, which can contribute to the lower cachexia index that was previously observed in tumour-bearing rats fed a leucine-rich diet [30] (Figure 3).

Although a leucine-rich diet benefited some aspects of energy metabolism, the activation of hepatic AMPK (activated under conditions of nutrient stress [41]) was higher in both tumour groups, suggesting a reduced energy supply. Thus, despite having lower similar blood glucose levels (LW = W), corroborating greater AMPK activation, the hepatic glycogen was mobilised in the W group but preserved in leucine-treated Walker 256-tumour-bearing rats. This fact suggested the partially modulatory effect of leucine nutritional supplementation. Although we evaluated some liver parameters, such as bilirubin, and serum enzymes, such as alkaline phosphatase (ALP) and gamma-glutamyltransferase (γ GT), that are associated with liver damage in cancer patients and tumour-bearing rats, a leucine rich-diet could not prevent changes in these parameters. Serum ALP activity provides a sensitive indicator of obstructive and space-occupying lesions of the liver [42]. Therefore, leucine was not able to prevent this kind of liver damage. On the other hand, the leucine-

supplemented tumour group had the same γ GT levels as the W group. As an oxidative stress marker, γ GT can give rise to pro-oxidant reactions that can produce endogenous reactive oxygen species (ROS) in tumour cells and play a major role in tumour formation, cell proliferation and apoptosis [43]. Thus, the leucine rich-diet had no effect on the oxidative stress observed in tumour-bearing hosts. While leucine was not effective in protecting against the elevation of these parameters that leads to impaired liver function, leucine treatment resulted in some positive effects, such as the maintenance of skeletal muscle mass, glycogen stores and normal levels of genes related to gluconeogenesis, as shown in Figure 3.

Because the liver has the potential to contribute to several wasting-associated mechanisms, further studies are now underway in our laboratory to understand the role of the liver in cancer cachexia progression and investigate how the effects of co-adjuvant treatments can benefit this tissue.

Conclusion

Our results suggest that during Walker 256 tumour growth, a leucine-rich diet is effective in protecting against host skeletal muscle tissue wasting and leads to benefits in the liver tissue. The leucine-rich diet induces no protection against the elevation of some hepatic damage markers but maintains energy stores and likely decreases the futile Cori cycle in the liver, reducing energy expenditures in tumour-bearing rats.

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Authors' contributions

LRV designed the study; performed the experimental work, most of the biochemical/molecular assays and the statistical analyses; interpreted and discussed the results; and prepared the manuscript. ACPL performed *in vivo* experiments and biochemical assays

and contributed to discussions of the results. CMS performed some of the histological analyses. BCFS contributed to the molecular assays and discussions of the results. MCCGM was responsible for the experimental design, the interpretation and discussion of the results, and the final manuscript revision. Laís Rosa Viana, Anna Caroline Perina Luiz, Carla de Moraes Salgado, Bianca Cristine Favero-Santos and Maria Cristina Cintra Gomes-Marcondes declare that they have no conflicts of interest.

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