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"*Atividade física associada ao crescimento tumoral e suplementação nutricional de leucina: Estudo do metabolismo de proteína e carboidratos no músculo de ratos implantados com carcinossarcoma de Walker 256*"

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Orientador(a): Prof(a). Dr(a). Maria Cristina Cintra Gomes Marcondes

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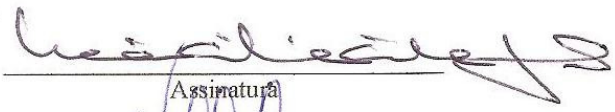

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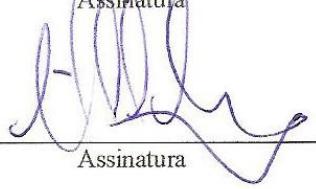

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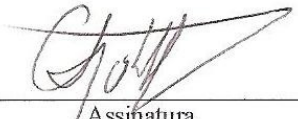

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RESUMO

A intensa mobilização de substratos dos tecidos da carcaça do hospedeiro, em função do crescimento neoplásico, promove no organismo o estado caracterizado como caquexia. Presente na maioria dos pacientes com câncer, a caquexia promove intensa perda involuntária de peso decorrente, preferencialmente, da depleção da proteína muscular em função do aumento da degradação e/ou da diminuição da síntese protéica, culminando na redução da qualidade e expectativa de vida. Sabendo-se que a leucina (BCAA) é utilizada como fonte energética pelo músculo esquelético, podendo ser transaminada e oxidada para produzir acetil-CoA, caracterizada, também, como precursora da gliconeogênese, a partir da sua degradação e formação de alanina no músculo e age, principalmente, como sinalizadora celular e que o exercício físico promove aumento do consumo de glicose, diminuindo assim os níveis de glicose e insulina circulantes, conseqüentemente, reduzindo a oferta desse substrato às células tumorais. Temos por principal interesse minimizar as alterações metabólicas do tecido hospedeiro frente ao crescimento do carcinossarcoma de Walker associado ao exercício físico aeróbio de intensidade leve a moderada e suplementação nutricional de leucina, avaliando o metabolismo protéico, através de analise do processo de síntese e catabolismo protéico, e o metabolismo glicídico, analisando-se a via glicolítica e receptor de glicose muscular, bem como a concentração sérica de citocinas, em ratos Wistar. Desse modo nossos resultados verificamos: 1) Diminuição do ganho de peso corpóreo após o implante tumoral. 2) Diminuição da síntese e aumento da degradação protéica muscular acompanhada com menor conteúdo de proteína e expressão de miosina. 3) Consequente, aumento da expressão das subunidades ubiquitina-proteossomo. 4) Aumento das citocinas pró-inflamatórias. 5) Redução da concentração sérica de glicose, insulina e aumento de glucagon. 6) Redução da expressão gênica do transportador de

glicose muscular, Glut-4. 7) Diminuição do diâmetro da fibra muscular esquelética. Já a suplementação nutricional com leucina e/ou exercício físico, a longo prazo, promove nos animais com tumor: 1) Melhora no ganho de peso corpóreo, com redução do peso tumoral em alguns grupos. 2) Diminui a degradação muscular esquelética, associada a maior expressão de miosina muscular. 3) Melhora a concentração de citocinas pró-inflamatórias. 4) Melhora a expressão gênica de transportador de glicose muscular, Glut-4. 5) Melhora, também, o diâmetro da fibra muscular.

ABSTRACT

The intense nutrients mobilization of host tissues, due to neoplastic growth, leads to host cachexia state. Most cancer patients have cachexia, which is characterized by involuntary weight loss through increase in protein muscle depletion and decrease in protein synthesis process. In these cases, there is reduction in quality and life expectancy. Leucine (BCAA) is used as an energy source by skeletal muscle, precursor of gluconeogenesis or acts as cell signaling and physical exercise promotes increased consumption of glucose, reducing the levels of circulating glucose and insulin, providing less supply of substrate to tumour. In this work, our main interests were to minimize the metabolic alterations in tumor-bearing host associated with nutritional supplementation of leucine and moderate aerobic exercise during Walker 256 tumour growth and whether this association could avoid or protect muscular depletion, analyzing protein metabolism through protein synthesis and catabolism process, and muscle carbohydrate metabolism through analysis of glucose metabolism, by evaluating glycolytic pathway and muscle glucose receptor in rats. Thus our results found: 1) Decrease in body weight gain after tumor implantation. 2) Decreased synthesis and increased muscle protein degradation accompanied with lower protein content and expression of myosin. 3) Accordingly, increased expression of ubiquitin-subunits proteosome. 4) Increase of pro-inflammatory cytokines. 5) Reduction of serum glucose, insulin and glucagon increase. 6) Reduction of gene expression of muscle glucose transporter, Glut-4. 7) Decrease in diameter of skeletal muscle fibers. Since dietary supplementation with leucine and / or exercise in the long run, promote the animals with tumor: 1) Improvement in body weight gain, reducing the tumor burden in some groups. 2) Decrease in skeletal muscle degradation, associated with increased expression of muscle myosin. 3) Improve the concentration of pro-inflammatory cytokines. 4) Improve

the gene expression of muscle glucose transporter, Glut-4. 5) It also improves the diameter of muscle fiber.

INTRODUÇÃO

Apresentação geral

O câncer é considerado o maior problema de saúde pública em diversos países, sendo a segunda maior causa de morte na Europa e Estados Unidos (Argilés, 2005) e também no Brasil (INCA, Instituto Nacional do Câncer, 2005). Por um lado, as intervenções terapêuticas promovem grande impacto no prognóstico da doença, por outro, o estado nutricional afeta grandemente o prognóstico e qualidade de vida do paciente com câncer.

O desenvolvimento neoplásico causa alterações dos processos homeostásicos e metabólicos do hospedeiro, desencadeando a caquexia, que pode levar à morte na maioria dos casos (Bacurau *et al.,* 2000). Assim, caracterizada pela perda involuntária de peso, com consequências drásticas à massa corporal e ao estado funcional do paciente, o câncer-caquexia promove redução da força muscular e da resistência cardiovascular, alterações imunológicas, dentre outras debilidades. De modo geral, a caquexia causa impacto negativo sobre a habilidade de respostas dos pacientes frente ao tratamento antineoplásico, constituindo-se no ponto crucial do sucesso do tratamento, interferindo, assim, na sua expectativa de vida (Langstein & Norton, 1991).

A anorexia, fator dentre outros que determina a caquexia em pacientes com câncer, é extremamente importante porque, nesse caso, o indivíduo incorpora menos energia do que gasta, agravando a perda de peso e as alterações do estado metabólico (Inui, 1999; Tisdale, 1999).

Dentre as alterações produzidas pelo crescimento neoplásico nos processos metabólicos, as alterações no metabolismo protéico causam diminuição do tempo de sobrevida de pacientes, pois a massa magra corporal diminui

intensamente com os efeitos do desenvolvimento da massa neoplásica (Tisdale, 2000). A redução do nitrogênio total, como consequencia do balanço nitrogenado negativo, muitas vezes em decorrência da alta demanda pelo tecido neoplásico, leva à intensa perda de proteína corpórea do hospedeiro. Alterações marcantes no metabolismo lipídico, também acarretam redução da reserva de gordura paralelamente ao aumento da massa tumoral (Tisdale, 2000). O metabolismo de glicídios, por sua vez, também está alterado devido à elevada utilização de glicose pelo tumor, a qual é sintetizada pela neoglicogênese (Holm *et al*., 1995), a partir, principalmente, da alanina proveniente do catabolismo protéico. Dessa forma, a glicose é utilizada pelas células neoplásicas instalando-se, assim, o ciclo de Cori, onde a produção de energia via glicose anaeróbia está aquém do gasto produzido pela nova síntese de glicose (Cori & Cori 1925). Nesse caso, verifica-se *déficit* energético, agravando ainda mais o estado caquético do hospedeiro.

Fadiga e perda da *performance* física caracterizam a reduzida qualidade de vida dos pacientes com câncer, antes, durante e após o tratamento (Dimeo *et al*., 2003). Por outro lado, estudos mostram que o exercício aeróbio, quando bem monitorado melhora a performance física de pacientes com câncer, proporcionando clara redução da fadiga e da perda muscular e, consequentemente, beneficiando a qualidade de vida desses indivíduos (Adamsen *et al*., 2003). Segundo Bacurau *et al.* (2000), a intervenção nutricional e exercício físico podem ajudar a reduzir a taxa de crescimento tumoral e o aparecimento de metástase em ratos, reduzindo os sintomas neoplásicos.

Estudos mostram que a suplementação de aminoácidos, particularmente a leucina, estimula a síntese de proteína muscular esquelética, porque possui efeito anabólico sobre o metabolismo protéico muscular, aumentando, assim, a

taxa de síntese e diminuindo sua degradação (Anthony *et al.*, 2001 e 2002; Ventrucci *et al.*, 2004; Garlick, 2005; Mathews, 2005).

Assim, o presente trabalho direciona o estudo da associação desses fatores: câncer, suplementação nutricional com leucina e exercício físico sobre as respostas do hospedeiro. Esse estudo leva em conta as respostas fisiopatológicas, bioquímicas e moleculares de um indivíduo regularmente exercitado e submetido à suplementação nutricional frente ao desenvolvimento de neoplasia maligna.

REVISÃO DA LITERATURA

Câncer-caquexia

Caquexia, síndrome comum em muitos tipos de cânceres, é o fator mais importante que leva à morte prematura dos pacientes. Este quadro é caracterizado pela grande perda de peso, relacionando-se à diminuição da qualidade e do tempo de vida (Tisdale, 1997).

A perda de 30% do peso corpóreo, em particular de gordura e principalmente de musculatura esquelética, diminui a *performance* física e da resposta à quimioterapia e/ou tratamentos cirúrgicos e radioterapicos dos pacientes com câncer (Tisdale, 2000).

Dentre os sintomas ou causas do crescimento neoplásico, a anorexia, decréscimo da ingestão alimentar, é a complicação mais frequente em pacientes com câncer (Inui, 2000). A síndrome anorexia-caquexia é causada por alterações metabólicas e, principalmente, pelas citocinas produzidas pelo tumor ou liberadas pelo sistema imune como resposta à presença do câncer, bem como outros produtos do tumor que promovem lipólise e proteólise tecidual desses pacientes (Inui, 2005). Desse modo, pesquisadores verificaram o efeito inflamatório crônico

na produção de citocinas pró-inflamatórias no desenvolvimento do quadro de caquexia (Al-Majid & Waters, 2008; Mantovani & Madeddu, 2008).

Pacientes com câncer, frequentemente, apresentam hipoglicemia associada ao baixo metabolismo tecidual de glicose. Porém, a glicólise anaeróbia, que ocorre nas células neoplásicas em decorrência do elevado metabolismo glicídico nessas células, favorecerá o aumento do dispêndio energético do paciente, uma vez que o ciclo de Cori, que proporciona a conversão de lactato, alanina e glicerol em glicose, eleva o *déficit* energético aumentando ainda mais o estado caquético (Cori & Cori, 1925). O aumento da síntese de glicose, a partir de alanina e glicerol, decorre do aumento da gliconeogênese hepática (Inui, 1999; Tisdale, 1997, 1999 e 2000).

A perda de tecido adiposo, originado do aumento da lipólise, é outro fator comum em pacientes com câncer. A mobilização de ácidos graxos do tecido adiposo pode ocorrer antes da perda de peso, devido à presença do fator de mobilização de lipídeos (LMF) produzido pelo tumor (Tisdale, 2000).

A massa corporal magra, em particular musculatura esquelética, diminui em proporção direta ao aumento os efeitos da massa neoplásica. Assim, redução da síntese e aumento da degradação protéica - ou seja, o desbalanço do *turnover* protéico total corpóreo - têm sido frequentemente observado nos pacientes com câncer (Inui, 1999; Tisdale, 2000), sendo o principal fator responsável pela redução do tempo de vida desses pacientes. A perda de tecido muscular leva à fadiga, fraqueza, atrofia muscular e comprometimento de diversas funções, tais como as respiratórias e cardiovasculares (Mulligan & Bloch, 1998).

Os mecanismos que levam à indução da perda muscular provocada pelo tumor ainda não foram totalmente elucidados. Sabe-se que muitos processos são

mediados pela redução da ingesta alimentar central, devido à ação de citocinas pró-inflamatórias e do fator de indução de proteólise (Al-Majid *et al*., 2001).

Exercício Físico e Câncer

A fadiga é um dos sintomas mais severos nos pacientes com câncer comprometendo, assim, a força, a resistência e a perda muscular, além de originar sequelas psicológicas (Dimeo *et al*., 1998; Al-Majid *et al*., 2001; Lucía *et al*., 2003; Thorsen *et al*., 2003). O exercício aeróbio aumenta a *performance* física, preserva as reservas corpóreas, melhorando, assim, a qualidade de vida de pacientes portadores de câncer (Crevenna *et al*., 2003).

O exercício físico de intensidade moderada em animais diminuiu a incidência de tumores transplantados, reduziu os sintomas neoplásicos, como também o surgimento de metástase e o crescimento tumoral (Bacurau *et al*., 2000). Daneryd *et al.* (1995) observaram que exercício físico voluntário em ratos implantados com tumor pode adiar o quadro de anorexia e caquexia. Além disso, o exercício físico aumentou a taxa metabólica, induziu a adaptação da biogênese da mitocôndria no músculo esquelético e aumentou a capacidade antioxidante (Daneryd *et al*., 1990 e 1995).

Modificações no estilo de vida podem afetar o estado oxidante/antioxidante. Dessa forma, o exercício físico pode aumentar os mecanismos antioxidantes do corpo (Roberts & Barnard, 2005), contribuindo para diminuir o risco de câncer de pulmão em ratos, por aumentar atividade de enzimas antioxidantes pulmonares (Duncan *et al*., 1997). Estudos mostram que o exercício físico aumenta o consumo de oxigênio - marcador da capacidade funcional - em 40%, reduzindo a náusea, depressão e a fadiga e, consequentemente, melhorando

a qualidade de vida em mulheres com câncer de mama (Al-Majid *et al.*, 2001). Por outro lado, a taxa de síntese de proteína aumenta após o exercício físico, indicando aumento na atuação de fatores de iniciação eucarióticos, que podem ser importantes na regulação global da taxa de síntese de proteína (Farrell *et al.*, 2000; Kimball *et al.*, 2002).

Além disso, o exercício físico promove o aumento do consumo de glicose, diminuindo, assim, os níveis de glicose e insulina circulantes e, consequentemente, reduzindo a oferta desse substrato às células tumorais (Bacurau *et al.*, 2000; Anthony *et al.*, 1999). A elevada concentração dos hormônios corticosteróideos e de citocinas induzida pelo exercício físico, aumenta a resposta imunológica, normalmente deprimida durante o desenvolvimento neoplásico (Bacurau *et al.*, 2000). Como efeito positivo produzido pela atividade física, a resistência periférica à insulina, verificada no hospedeiro com câncer, é também normalizada, bem como as concentrações dos hormônios catabólicos e anabólicos (Daneryd *et al.*, 1995).

O exercício físico também melhora os depósitos de glicogênio e da massa protéica muscular. Desse modo, a estimulação da enzima glicogênio sintase, observada no exercício físico, promove a síntese de glicogênio, com consequente aumento do depósito de carboidratos e disponibilidade de substrato energético durante os processos de intensa demanda (Bacurau *et al.*, 2000). Assim, o exercício físico, quando bem supervisionado, pode ser uma excelente alternativa no auxílio do tratamento e reabilitação dos pacientes com câncer, melhorando as funções fisiológicas e reduzindo a ansiedade e a depressão (Segar *et al.*, 1998; Segal *et al.*, 2001).

Suplementação de leucina e câncer

Os aminoácidos de cadeia ramificada (BCAA), valina, isoleucina e leucina são substratos essenciais e importantes reguladores da síntese de proteína corpórea, representando a maior fonte de nitrogênio para síntese de glutamina e alanina no músculo. A síntese de glutamina e alanina, a partir dos BCAAs, é ativada em doenças severas, como o câncer (Holecek, 2002). O aumento da taxa de oxidação dos BCAAs, induzido pelo câncer, é comum em respostas inflamatórias sistêmicas, contribuindo, assim, para a perda muscular (Holecek, 2002).

A leucina é utilizada como fonte energética pelo músculo esquelético, podendo ser transaminada e oxidada para produzir acetil-CoA e, também, constituir-se como uma das substâncias precursoras da gliconeogênese hepática e da formação de alanina no músculo. Além disso, a leucina participa do processo de sinalização da transcrição inibindo a degradação e/ou estimulando a síntese protéica, aumentando a expressão de fatores de iniciação eucarioticos que, normalmente, estão deprimidos durante o crescimento do tumor. (Pitkanen *et al.*, 2003; Ventucci *et al.*, 2004 e 2007). Estudo verificou que, a suplementação de leucina melhorou o balanço nitrogenado, recuperando a massa corporal magra através da preservação da massa protéica muscular e do aumento da absorção intestinal, em ratas portadoras do tumor de Walker (Ventrucci *et al.*, 2001).

Pacientes com câncer são orientados à ingestão de dieta equilibrada, rica em nutrientes e a praticar atividades físicas, para que o tratamento anticâncer atenda às expectativas de sobrevida do paciente, sendo assim necessária a mudança em seu estilo de vida. (Brown *et al.*, 2003). A suplementação de aminoácidos e exercício físico, além de aumentar a síntese protéica e o balanço nitrogenado (Wolfe, 2000), também aumenta as concentrações de glutationa no

fígado após o exercício. A enzima glutationa está envolvida no processo de detoxificação e defesa antioxidante, refletindo na habilidade do corpo em combater espécies oxigênio reativas e diminuir o estresse oxidativo (Mariotti *et al.*, 2004).

Desse modo, a partir de resultados prévios, já submetidos à publicação, verificou-se que, exercício físico e dieta rica em aminoácidos, em particular a leucina, resultaram em melhora do *turnover* protéico e conteúdo de nitrogênio na carcaça em ratos implantados com o carcinossarcoma de Walker 256, tumor experimental modelo de caquexia. Assim, esses dados sugerem que o treinamento físico, bem supervisionado, em combinação ao suporte nutricional, pode melhorar o estado caquético em ratos implantados com tumor (Salomão & Gomes-Marcondes, 2010, Salomão *et al.*, 2010).

OBJETIVOS

Mudanças nutricionais e no estilo de vida são necessárias e importantes para garantir a qualidade de vida. Além da orientação aos pacientes com câncer quanto à ingestão de dieta equilibrada, rica em nutrientes, a associação com atividades físicas pode proporcionar melhora nas repostas aos tratamentos durante e após o período de câncer, aumentando a sobrevivência ou melhorando a qualidade de vida (Brown *et al.*, 2003). Assim, analisamos os efeitos de dieta com alto teor de leucina associados ao treinamento físico aeróbio, regular, prévios ao implante do tumor Walker 256, aventando-se, assim, a hipótese de que em função do aumento do processo de síntese protéica e dos estoques energéticos promovidos pelo exercício físico e suplementação nutricional com leucina, que tem papel sinalizador celular, possibilitará a melhora do estado caquético do animal.

RESULTADOS E DISCUSSÕES

Os resultados e discussões deste trabalho serão apresentados em dois artigos científicos.

O primeiro artigo corresponde aos resultados obtidos sobre síntese e degradação protéica e expressão das subunidades do proteossomo 26S do músculo esquelético, publicado à revista *Nutrition and Cancer*.

Verificamos que, o efeito deletério do tumor conduz o hospedeiro ao estado de espoliação bastante severo. Por outro lado, a suplementação de leucina associada ao exercício físico, regular a longo prazo, promove benefícios ao hospedeiro, mostrados tanto no *turnover* protéico, como na expressão de subunidades e enzimas envolvidas na proteólise muscular.

O segundo artigo refere-se aos resultados do metabolismo de carboidratos e concentração sérica de citocinas.

Verificamos aumento das citocinas inflamatórias nos animais com tumor. Esses dados condizem com os da literatura, onde observamos o efeito inflamatório crônico na produção de citocinas pró-inflamatórias no desenvolvimento do quadro de caquexia.

Desse modo, os dados apresentados no presente trabalho mostram-nos claramente que o efeito deletério do tumor conduz o hospedeiro ao estado de espoliação bastante severo. Por outro lado, a suplementação de leucina associada ao exercício físico promove benefícios ao hospedeiro, mostrados tanto no metabolismo de glicose, quanto no metabolismo protéico e também na concentração sérica de citocinas, que conduzem ao aumento da espoliação tecidual do hospedeiro com câncer.

PRIMEIRO ARTIGO CIENTÍFICO

"Exercício físico e dieta rica em leucina modulam o metabolismo de proteínas musculares em ratos implantados com tumor de Walker".

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RESUMO

Dieta suplementada com leucina pode recuperar a massa corpórea magra, preservando a massa protéica corpórea. Além disso, o exercício físico pode ser uma excelente alternativa para melhorar a reabilitação de pacientes com câncer. Conhecendo esses fatos, examinamos os efeitos de dieta rica em leucina com ou sem exercício físico aeróbio sobre o metabolismo de proteína muscular em ratos implantados com tumor de Walker. Ratos jovens foram divididos em quatro grupos que realizaram ou não exercício aeróbio leve (natação) e foram submetidos à dieta rica em leucina ou dieta controle durante 2 meses. Após este período, esses animais foram implantados ou não com tumor de Walker 256 (sc.), receberam dieta controle ou dieta rica em leucina, de acordo com os seguintes grupos: controle, treinado, tumor e treinados implantados com tumor. Vinte e um dias após o implante, o crescimento tumoral induziu a diminuição da síntese protéica muscular e aumentou o processo catabólico, que foi associado com aumento da expressão das subunidades ubiquitina proteossomo (20S, 19S e 11S). Em contrapartida, o programa de exercícios minimizou o processo de degradação muscular e maior teor de miosina do músculo. Além disso, a suplementação de leucina também modulou as subunidades do proteossomo, especialmente a 19S e 11S. Em resumo, o exercício tem efeitos benéficos por reduzir o crescimento do

tumor, levando a melhora do *turnover* protéico, especialmente quando em conjunto com dieta rica em leucina.

Physical exercise and a leucine-rich diet modulate the muscle protein metabolism in Walker tumour-bearing rats

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ABSTRACT

Leucine-supplemented diet can recover lean body mass, preserving muscle protein mass. Additionally, physical exercise can be an excellent alternative to improve the rehabilitation of cancer patients. Knowing these facts, we examined the effects of a leucine-rich diet with or without physical aerobic exercise on muscle protein metabolism in Walker tumour-bearing rats. Young rats were divided into four groups that did or did not perform light aerobic exercise (swim training) and were on a leucine-rich diet or a control diet for 2 months. After this time, these animals were implanted or not with tumours (sc.) following groups for either control diet or leucine-rich diet fed rats: control, trained, tumour-bearing and trained tumour-bearing. Twenty-one days after implantation, the tumour growth induced a decrease in the muscle protein synthesis and increased the catabolic process, which was associated with an increase in the expression of the ubiquitin-proteasome subunits (20S, 19S and 11S). In contrast, the exercise program minimised the muscle degradation process and increased muscle myosin content. Additionally, leucine supplementation also modulated proteasome subunits, especially the 19S and 11S. In summary, the exercise has beneficial effects by reducing tumour growth, leading to an improvement in protein turnover especially when in conjunction with leucine-rich diet.

Key words: Walker Tumour, Nutritional Supplementation, Cachexia, Exercise, Leucine-Rich Diet, Protein Metabolism.

INTRODUCTION

Cachexia is characterised as a patient's involuntary weight loss, which leads to a reduction in the quality of life and survival (1). Lean body mass loss, mainly the skeletal muscle, decreases in direct proportion to the effects of tumour growth, reducing the patient's physical performance and chemotherapy response (2). Decreases in protein synthesis and/or increases in protein degradation, which change the total protein turnover balance, are the main changes that occur during tumour development (3,2). These changes induce skeletal muscle loss, fatigue, weakness and atrophy (4). Adipose tissue loss is also common in patients with cancer due to mobilisation of free fatty acids due to lipid mobilisation factor (LMF), which is largely produced by the tumour (2). Aerobic exercise can improve the physical functioning of a cancer patient and therefore, improve the quality of life (5). Fatigue is the most severe symptom in cancer patients (6,7). Moderate physical exercise in animals reduced the incidence of tumoural implants, as well as the tumour growth and metastases (8). In addition, the protein synthesis rate was enhanced after physical exercise, and there was an increase in the activity of eukaryotic initiation factors, which can be important for total protein synthesis regulation (9). In addition, the branched-chain amino acids (BCAA), valine, isoleucine and leucine, are essential substrates and important regulators of total body protein synthesis. They represent the largest sources of nitrogen for glutamine and alanine, which are synthesised in severe diseases, such as cancer. An increase in the oxidation rate of BCAAs is common in the inflammatory systemic response induced by cancer, contributing to muscle loss (10). In particular, leucine is used as an energy source by the skeletal muscle and can be transaminated and oxidised to produce acetyl-CoA. It also stimulates the cell signalling process, which can increase muscle protein synthesis and inhibit protein

degradation, preventing the depletion of muscle mass (11,12). Wolfe (13) verified that amino acid supplementation, in combination with physical exercise, can increase the protein synthesis and nitrogen balance in cancer patients. According to Segal et al. (14), supervised physical exercise can be an excellent aid during the cancer treatment and rehabilitation process by improving the physiologic and psychological state of a patient. Knowing that exercise, especially regular exercise, leads to reduced susceptibility to multiple types of disease and to a better outcome in patients with a chronic inflammation-related condition such as cancer, the main purpose of this work was to investigate whether a leucine-rich diet with or without light aerobic physical exercise, as a regular process (a long training protocol), could improve lean body mass and muscle protein metabolism in tumour-bearing rats.

MATERIALS AND METHODS

Animals and diets

Wistar male rats (21-days old), obtained from the animal facilities at the State University of Campinas, UNICAMP, Brazil, were housed in collective cages during the entire experimental period. They received diet and water *ad libitum* under control of light and darkness (12-12 hours) and temperature (22 ± 2 C). The semi-purified diets are in accordance to AIN-93M, the American Institute of Nutrition (15). Normoprotein diet (C) was comprised of 18% protein. The leucine-rich diet (L) contained 18% protein plus 3% L-leucine.

An adjustment in the amino acid-rich diet was made to reduce the amount of carbohydrates. The diets were isocaloric and had similar quantities of cornstarch (39.7%), dextrin (13.2%) and sugar (10%). Additionally, the C and L diets

contained the same amount of fat (7% soy oil), fibre (5% cellulose micro fibre), salt (3.5%) and vitamin mix (1.0%). The diets were also supplemented with cystine and choline. The amino acids (L-leucine) and cornstarch were provided by Ajinomoto Interamericana Ind. and Com. Ltda and Corn Products Brazil Ingredients, respectively.

Tumour implantation

The tumour-bearing rats received approximately 0.25×10^6 Walker 256 carcinoma cells in suspension implanted subcutaneously in the right flank (16). The rats without tumours were injected with 0.5 mL 0.9% (w.v.) NaCl solution without anaesthesia. The general guidelines of the UKCCCR (United Kingdom Co-ordinating Committee on Cancer Research, 1998) (17) for animal welfare were followed, and the experimental protocols were approved by the institutional Committee for Ethics in Animal Research (CEEA.IB/UNICAMP, protocol # 465-4).

Exercise protocol

The rats were submitted to light swim training for 8 weeks (60 days) in an 1 m^3 container at 30 ± 2^oC. The training was performed 5 days per week in the morning, starting with 5 minutes initially and progressively increasing the time until the rats could swim 45 minutes per day of exercise with no body weight overload, which was maintained until the end of the experiment. To avoid thermal stress after swimming, all exercised animals were gently dried out and kept in a warmed room. Their body temperature was measured to confirm body temperature maintenance.

Experimental protocol

The young rats were first distributed into the following 4 groups, according to the diet regimen: sedentary animals that were fed with control or leucine-rich diet (C and L groups) and exercised animals that were fed with control or leucine-rich diet (TC and TL groups). After 60 days on the regimen, the rats were redistributed into 8 groups based on whether or not they received Walker 256 tumour cells (W), corresponding to the following groups: 4 groups that received the control diet: control (C), trained (TC), tumour-bearing (W) and trained tumour-bearing (TW) and 4 groups that were fed the leucine-rich diet: rats fed the leucine-rich diet (L), trained rats fed the leucine-rich diet (TL), tumour-bearing rats fed the leucine-rich diet (WL) and trained tumour-bearing rats that were fed the leucine-rich diet (TWL). The body weights were recorded three times a week. Twenty-one days after tumour implantation, the rats were sacrificed, which was carried out 24 hours after the final exercise protocol to avoid any acute inflammatory stimulus. The gastrocnemius muscles were quickly collected to analyse the activity of proteolysis pathways and to assay protein synthesis and degradation.

Biochemical assays

Protein metabolism

Protein synthesis: After sacrifice, the gastrocnemius muscle was quickly excised, weighed and placed in Krebs-Henseleit buffer (KHB) (110 mM NaCl, 25 mM $NaHCO_3$, 3.4 mM KCl, 1mM CaCl2, 1mM KH2PO4, 1mM MgSO4, 5.5 mM glucose, 0.01% (w/v) albumin bovine, pH 7.4) followed by incubation at 37^{o}C with continuous agitation and gassing with 95% O2 and 5% CO2 (18,19). After 30 minutes, the muscles were further incubated for 2 hours in KHB supplemented

with 5µCi L-[3H]-phenylalanine per mL (Amersham). The homogenised muscle in 30% TCA was then centrifuged at 10,000 x g for 15 min at 4°C. The pellet was washed in 10% TCA, resuspended in 1N NaOH, and the protein content was measured (20). The total βemission radioactivity on liquid scintillation was assessed by using beta counting equipment (Beckman LS 6000 TA, USA). The protein synthesis rate was calculated and expressed in nmol of incorporated phenylalanine by microgram of precipitated protein per hour (18).

Protein degradation: The contralateral gastrocnemius muscle was taken to analyse protein degradation, expressed as nmol of tyrosine released by micrograms of protein per hour. After quick excision, the muscle was initial incubated in RPMI 1640 at 37°C, for 30 min. Following the incubation, the media was replaced and supplemented with cycloheximide (130 mg/mL; inhibitor of protein synthesis) for 2 additional hours of incubation with 5% CO2 and 95% O2. The tyrosine release was measured in the incubation media of the muscle using a fluorimetric assay as described by Waalkes & Udenfriend (21).

Alkaline phosphatase activity

The gastrocnemius muscle was homogenised in cold homogenising buffer (HB) (20 mM Tris, 1 mM dithiothreitol, 2 mM ATP and 5 mM $MgCl_2$, pH 7.4) and centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was analysed for total protein content (20), and the alkaline phosphatase activity was measured using 30 µL of the homogenate supernatant with 37 mM p-nitrophenylphosphate (pNPP, Sigma) as a substrate. The alkaline phosphatase activity was expressed as nM per protein content of the tissue homogenate per minute of reaction (22).

Activity of proteolysis pathways

Measurement of proteasome activity

The gastrocnemius muscle, processed as described for the alkaline phosphatase assay, was analysed for functional proteasome activity by measuring the chymotrypsin-like enzyme activity, one of the proteolytic activity core of the proteasomal subunits, according to the method of Orino and colleagues (23). Aliquots of the homogenate supernatant (50 µL) were used to determine chymotrypsin-like activity by incubating the homogenate with 100 µL of the fluorogenic substrate succinyl-Leu-Leu-Val-Try-7-amino-4-methylcoumarin (Suc LLVY-AMC, Sigma, 0.167 µg/L in Tris-HCl, Promega, pH 7.4) followed by the release of aminomethyl coumarin (AMC) from the substrate. The fluorescence was measured in a fluorimeter (excitation: 360 nm, emission: 460 nm). The chymotrypsin-like enzyme activity was expressed as fluorescent arbitrary units per µg of tissue protein per minute of reaction.

Measurement of lysosomal and calcium-dependent pathways activities

The lysosomal and calcium-dependent enzymes activities were measured in the homogenate of the gastrocnemius muscle processed as described above. The cathepsin B activity (lysosomal enzyme) was determined using 50 µL of the homogenate supernatant treated with assay buffer (352 mM KH_2PO_4, 48 mM Na_2HPO_4, 4 mM EDTA and 8 mM cystine, pH 6.0) and prewarmed at 40°C for 2 minutes. The assay was started with the addition of Z-Phe-Arg-NMec (Z-Phe-Arg 7-amido-4-methylcoumarin hydrochloride, Sigma) as the substrate, dissolved in dimethyl sulphoxide and diluted to 0.02 mM with 0.1% Brij 35. After a 10 minute incubation at 40°C, the fluorescence was measured in a fluorimeter (excitation:

360 nm, emission: 460 nm) at every minute for 30 minutes (24). The only changes made for cathepsin H (lysosomal enzyme) analysis were the substitution of the substrate Arg-NMec (L-Arginine-7-amido-4-methylcoumarin hydrochloride, Sigma) and the composition of the assay buffer (200 mM KH_2PO_4 and 200 mM Na_2HPO_4 adjusted at pH 6.8) (24). The enzyme activities were normalised to the total protein content of the samples. The cathepsins B and H activities were expressed as percentages of the control group. The calpain activity (a cytosolic protease and calcium-dependent enzyme) was measured using 50 µL of the gastrocnemius homogenate supernatant in 50 mM imidazole–HCl buffer (pH 7.5), containing 10 mM b-mercaptoethanol, 1.0 mM NaN_3 and 4 mg/mL of casein. After incubation, a final concentration of 5 mM of $CaCl_2$ was then added to activate calpain and initiate the calpain hydrolysis reaction. Time scanning at a wavelength of 500 nm was performed at each minute for 30 minutes (25); the enzyme activity was calculated by subtracting the final optical density from the initial record adjusting for the total protein content in the sample, and the activity was expressed as a percentage of the control group.

Myosin expression and proteasome system

Gastrocnemius muscle proteins were loaded and resolved by SDS-PAGE on 12% gels followed by western blotting. The muscle content of the myosin heavy chain (MHC) isoforms was assessed using an antibody against MHC at a dilution of 1:250 (Novocastra, Newcastle, UK), followed by detection with a secondary anti-mouse horseradish peroxidase (HRP) antibody (Santa Cruz Biotechnology, USA). The proteasome subunits were analysed by probing 0.45 µm nylon membranes with antibodies against the 20Sα proteasome subunit, 19S MSSI ATPase regulator subunit, 11Sα subunit and E2 subunit (all antibodies purchased from

Affinity, Newcastle, UK and diluted 1:1,500) followed by detection with an appropriate secondary HRP-labelled antibody (purchased from Santa Cruz Biotechnology, California, USA). The signal was enhanced with a chemical luminescence reagent (ECL; Amersham, GE Life Sciences, USA). Actin was used as the loading control, after probing with an anti-mouse actin antibody. A parallel gel was silver stained to ensure equal loading. Images of the gels were captured (FTI 500Image Master VDS, Pharmacia Biotech), and densitometric analyses of the bands were done with Gel Pro Analyser software (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

The results were expressed as the mean $\pm$ SEM. Data were analysed statistically by two-way ANOVA, testing the effects of diet and exercise on tumour growth, muscle parameters and proteolysis subunits pathways. Comparisons within control and tumour-bearing groups were performed using a one-way ANOVA followed by a post-hoc Bonferroni's multiple comparison test (Graph Pad Prism software, v3.00 for Windows 98, USA). Results were considered statistically when the P value was less than 5% (26).

RESULTS

Body and tumour weight

Body weight loss was verified in all tumour-bearing animals, although this decrease was minimised in rats that exercised and/or were fed a leucine-rich diet (W group reduced body weight gain by around 90%, whereas the TW was 58%, WL was 26% and TWL was 56%) (Table 1). Despite the accelerated tumour growth, exercise reduced tumour development, as tumour

weight and tumour/body weight ratio were lower than in the other groups. The tumour weight in the TW group was around 30% less than in the W group and 6% and 15% less in the WL group and TWL group compared to the W group, respectively (Table 1). Many authors, including our group, have shown that Walker tumours induce deep body weight loss associated with fast tumour growth. When tumour-bearing animals were submitted to aerobic exercise (27) or fed with a supplemented diet (28) these effects could be minimised, and some parameters, such as body weight and lean body mass, were improved in these conditions. The present work analysed the influence of exercise over a long period of time, similar to patients who have exercised since childhood. In this case, the aerobic exercise minimised the body weight loss and decreased the tumour growth but did not prevent tumour implantation or growth.

Muscle protein synthesis and degradation

Our results show that the Walker 256 tumour growth induced alterations in the protein synthesis and degradation process in the gastrocnemius muscles of the W group. These parameters could be improved with a combination of a leucine-rich diet and exercise program. Protein metabolism during cancer-induced cachexia is completely changed (2) as the growth of the tumour induces protein waste, which increases protein degradation and inhibits protein synthesis (12,29). This same pattern was found in the present work as the protein synthesis (Fig. 1A) was significantly decreased in all tumour-bearing rats (around 50% less than the control group) despite being trained and/or fed a leucine-rich diet. On the other hand, the muscle protein degradation was higher, especially in the W group (2-fold increase), whereas the other tumour-bearing groups showed a slight increase in muscle tyrosine release (Fig. 1B) corresponding to 1.5 fold in the TW and TWL

groups and 1.4 fold in the WL group. Moreover, total muscle protein content (Table 1) was decreased only in the W group compared to the other groups. Muscle myosin expression (Fig. 1D) was significantly reduced in the W group compared to the other tumour-bearing groups, which showed an increase or maintenance in the total protein content and myosin heavy chain protein expression (Table 1 and Fig. 1D, respectively). Total net protein (Fig. 1C) decreased in all tumour-bearing animals, but the TW and TWL groups had a higher total net protein than the W group.

Proteolysis pathways

The protein degradation process involves the following three pathways: the ubiquitin-proteasome system, which includes the chymotrypsin-like enzyme, the lysosomal system, which involves many enzymes including cathepsin B and H and the calcium-dependent pathway, which includes cytosolic enzymes such as calpain. The cathepsin B activity was increased in all groups (up to 60%), except the leucine group (L), when compared to the control group (Fig. 2A). On the other hand, the cathepsin H activity (Fig. 2B) was decreased in the trained (TW; 72% less) and leucine-supplemented tumour-bearing groups (TWL; 40% less) when compared to the other groups. Despite the exercise program-induced benefits to the host, tumour growth promoted an increase in the cathepsin B activity independently of the nutritional support, as the leucine-rich diet (L group) maintained the level of cathepsin B activity. However, the other lysosomal enzyme, cathepsin H, did not show high activity and expression was generally reduced, especially in the TW and TWL groups. The different patterns in both lysosomal enzymes suggest that the lysosomal pathway is involved in protein

degradation in both a beneficial process such as exercise and a detrimental process such as cancer. The cellular activity, especially in the liver (22), placenta (30) or muscle (12), can be assessed by measuring the alkaline phosphatase activity. Although tumour growth can alter the activity in many tissues, in the present work, the cell activity did not change in the skeletal muscle as the activity remained similar in all groups compared to the control group, except in the TW group, which showed a decrease in this enzyme's activity (Fig. 2C). With respect to the calcium-dependent pathway, the calpain activity (Fig. 2D) increased in the W and TW groups (30% and 22% higher, respectively). In all groups fed the leucine-rich diet, the calpain activity was reduced, especially in the tumour-bearing groups (50% less in WL and 40% less in TWL).

Ubiquitin-proteasome subunits

Protein degradation is primarily carried out by the ubiquitin-proteasome system (31,32), which contains a principal catalytic core composed of proteases, including the chymotrypsin-like enzyme associated with the structural subunits composing the 20S core particle. The 19S subunit includes an ATP-dependent regulatory particle that regulates the 20S subunit by guiding the polyubiquitin protein chain to the catalytic centre. The 11S subunit is also an ATP-independent regulatory core of the 20S subunit. In the present work, tumour growth led to an increase in the chymotrypsin-like activity (higher around 70% in the W group) (Fig. 3A) when compared to the control group. Muscle proteolysis in cancer-induced cachexia results from increased stimulation of the 19S proteasome subunit as observed in the W, TW, WL and TWL groups (Fig. 3B), although leucine-rich diet groups had a lower increase in 19S expression compared to the other tumour-bearing groups (47% less in WL and 22% in TWL). A similar increase in the 11S proteasome

subunit was observed in the W (5-fold higher) and in all groups submitted to the leucine-rich diet (L, TL, WL, TWL) when compared to the control groups C and CT (Fig. 3C); the TW group had similar values compared to the control group. E2, an ubiquitin-conjugating enzyme, which is one of the key enzymes that promotes the formation of the polyubiquitin chain, was reduced in the TW and TWL group when compared to the other tumour-bearing groups (Fig. 3D). The 20S proteasome subunits (alpha subunits 33, 32 and 31 kDa bands – Fig.4) increased in the W and TW groups compared to the control group. In the WL and TWL groups, only the 31 and 32kDa subunits expression was enhanced when compared to the control groups (C and L).

DISCUSSION

The present work analysed the impact of exercise and leucine supplementation on the tumour growth and host response to the effects of cancer. The results of this work showed that Walker 256 tumour growth induced alterations in protein synthesis and the protein degradation process in the gastrocnemius muscle in rats, which could be partially attenuated with aerobic exercise and nutritional supplementation. Knowing that exercise, when well conducted, can improve the quality of life in cancer patients (33) and minimise wasting of lean body mass, this study is the first that analysed the effects of the association of amino acid supplementation and a long training protocol as the exercise protocol continued from the weanling age until the adulthood (rats were exercised for 60 days, plus the additional 21 days allowed for tumour growth). We used this method to analyse how the trained rats would respond to the tumoural effects because the exponential tumoural growth period is too short of a time period to establish a training protocol. Although the long-term training exercise and leucine-rich diet

treatment could not prevent the decrease in muscle protein synthesis, our results show that the trained and/or leucine-supplemented tumour-bearing rats had an improvement in lean body mass that was due to a modulation in the protein degradation, maintenance of total muscle protein content and increase in the muscle myosin content.

In response to prolonged illnesses, such as cancer, the loss of lean body mass may be so severe that it is accompanied by a decrease in protein synthesis and an increase in protein degradation (34). Because proteolysis in the body is an extremely versatile and complicated process, it is controlled as precisely as protein synthesis. The protein degradation process involves the following three pathways: the ubiquitin-proteasome system, which accounts for 60% of the total protein breakdown (35), the lysosomal system, which involves the enzymes related to autophagy protein waste (36) and the calcium-dependent pathway, which includes cytosolic enzymes such as calpain (37,38). The classical protein degradation pathway is the lysosomal system called autophagy, which results in endogenous protein degradation (36) when the lysosomal enzymes are activated in normal or pathological situations. Autophagy is an intracellular membrane-mediated and non-selective process and can be regulated by physiological effectors such as insulin, glucagon and amino acids. The process degrades not only proteins but also all of the other cellular constituents, such as RNA, sugars, lipids and phospholipids, and it is a type of cell restructuring process (39,40). In the present work, we verified that some enzymes of the lysosomal pathway, such as the cathepsins B and H, were increased or modulated by the presence of the Walker tumour. This suggests that these enzymes act not only on protein degradation but also in other host responses, which needs further study. Another major pathway is the ubiquitin-proteasome system, which is also ubiquitous

throughout the body, ATP-dependent and degrades ubiquitin-conjugated proteins via the 26S proteasome (35). It is involved in many biologically important processes, such as transcriptional regulation, cell cycle control, antigen processing, apoptosis and DNA repair. The proteins degraded by this system turnover quite rapidly. Both the autophagic and proteasome systems are thought to have completely different mechanisms and act in physiological roles or in some pathological cases, such as cancer, in a completely independent manner or even in a compensatory manner in cells, especially in skeletal muscle cells. The primary mechanism for protein catabolism in cancer-induced cachexia has been attributed to an increase in the expression and activity of the ubiquitin–proteasome proteolytic pathway (41). The higher proteolysis seen in cancer-wasting conditions (especially in the W group, as verified in the present study) is attributed to the enhanced expression of the ATPase subunit of the 19S complex and 20S and the increase in chymotrypsin-like activity; these facts could facilitate the fast proteolysis of muscles in Walker tumour-bearing rats because the 19S subunit provides energy for the breakdown of ubiquitinylated proteins by the 26S proteasome (12,35,42,43). We also verified high expression of the 11S subunit, which is not dependent of ATP but can be induced by some cytokines, such as γ-IFN (12,42). In this case, we can also suggest that the main proteolysis process involved is the ubiquitin-proteasome system, including activation of both regulatory proteasomic subunits.

The exercised group (TW) showed reduced tyrosine release, indicating lower protein degradation than the W group as a benefit of aerobic exercise. Moreover, further results in the TW group support this suggestion as the reduction in the 11S subunit and E2 expression coincide to a reduction in the chymotrypsin-like activity, the lysosomal enzyme (decrease in cathepsin H activity) and the calcium-

dependent proteolytic pathway (reduced increase in calpain activity) when compared to the W group. Exercise is an essential component of a healthy and balanced lifestyle in humans (5,44). In the skeletal muscles the derivation of benefit depends on the intensity and duration of the exercise. The muscle mass depends on the regulated protein anabolic and catabolic pathways (45). Conversely, a loss of muscle mass has been observed in many disease states, including cancer-induced cachexia (46), and exercise can be used to counter this increase in proteolytic activity (45). As presented in this work, despite having cancer, the TW group showed some benefits induced by exercise, such as an increase in myosin content and a lower tumour weight ratio (30% less). Lira and colleagues (47) also showed that tumour weight decreased 10-fold after training. These observations implicate that exercise can be beneficial to health and quality of life, especially in this situation where the Walker tumour grows quickly and is considered an experimental cachexia model (48). Al-Majid and Waters (34) reviewed data from a few existing studies that demonstrated that progressive resistance exercise training might increase skeletal muscle mass and strength, improving physical functioning and enhancing the quality of life in cancer patients.

Nutrients, such as amino acids and fatty acids, have been shown to have effects on proteasome-mediated protein degradation. Leucine, a BCAA, is known to stimulate cell signalling leading to muscle protein synthesis and anabolism, mainly when it is associated with physical exercise (49-51). Similarly, leucine can also modulate the skeletal muscle proteolysis by inhibiting the ubiquitin-proteasome (12) or by the transamination of leucine, converting it to α-ketoisocaproate acid (52). In contrast, because leucine is an active regulator in the liver where there is not substantial transamination activity, there are some hypotheses that amino acids, including leucine, control proteolysis not after being metabolised but directly

from extracellular sites in the liver (39,40,53) and probably in the skeletal muscle (12,29). Because protein degradation is controlled by the ubiquitin-proteasome system and involves intracellular and myofibrillar protein breakdown (42), these parameters could be improved with a leucine-rich diet (12) and exercise (34). Leucine stimulated an increase in muscle myosin synthesis in tumour-bearing groups especially in conjunction with exercise (TW, WL and TWL groups). Additionally, this amino acid led to a reduction in tyrosine release and in chymotrypsin-like activity, which maintained the total muscle protein leading to a higher total net protein. Taking all these data together, the leucine-induced improvement in gastrocnemius myosin content probably improved the cell signalling and/or modulated the protein degradation by adapting the activity of some proteasomal enzymes, such as chymotrypsin-like and subunits of the Ub-proteasome, This result was enhanced in conjunction with physical exercise (49-51). Although the present data showed that the trained groups had an increase in the activity of most proteolytic enzymes even from the lysosomal system and the ubiquitin-proteasome pathway, these processes could be related to the specific type of the exercise used in the study or an adaptation to the effects of exercise before its main protein-synthesis effect (54,55). In accordance with observations made in muscle-wasting conditions, (i.e., cancer) some types of exercises, such as eccentric exercise, result in increased levels of Ub conjugates in muscle biopsies in humans (56,57). Accordingly, Féasson and colleagues (54) showed that proteasome enzyme activities increased after 14 days of eccentric exercise in muscle biopsies from healthy volunteers. In accordance with the literature, the tumour-bearing rats that exercised and/or consumed a leucine-rich diet exhibited fewer benefits than expected, and thus future investigations of how the exact process of leucine modulation act on the protein degradation are warranted.

Further studies are underway in our laboratory to determine if and how leucine supplementation, in combination with aerobic exercise, could counteract host wasting during cancer-induced cachexia.

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LEGENDS

FIG. 1: Effects of a leucine-rich diet in conjunction with aerobic exercise program on protein synthesis (A), protein degradation (B), total net protein (C) and myosin expression (western blotting image and densitometry analysis; D) in gastrocnemius muscle of tumour-bearing rats compared to the control groups. The images from myosin and actin (as a loading control) were the best representative data from different animals per group. The minimal number of animal used per group was eight. Legend: C–control rats, TC–trained, W-tumour-bearing, TW–trained tumour-bearing, L-rats fed leucine-rich diet, TL–trained rats fed leucine-rich diet, WL–tumour-bearing rats fed leucine-rich diet, TWL–trained tumour-bearing rats fed leucine-rich diet. The columns represent the means ± SEM. * $P < 0.05$ different from C group. **[a]** $P < 0.05$ different from W group.

FIG. 2: The lysosomal enzymes activities [cathepsin B (A) and cathepsin H (B)], cell activity [alkaline phosphatase activity (C)] and proteolytic Ca^{++}-dependent activity [calpain activity (D)] in gastrocnemius muscle of tumour-bearing rats under effects of leucine supplementation and/or an aerobic exercise program. For the legend, see Figure 1. The minimum number of animals per group was eight. The columns represent the means ± SEM. * $P < 0.05$ different from the C group. **[a]** $P < 0.05$ different from the W group.

FIG. 3: The ubiquitin-proteasome subunits, chymotrypsin-like enzyme activity (A), 19S subunit (B), 11S subunit (C) and E2 subunit (D) expression in gastrocnemius muscle from tumour-bearing rats submitted to leucine supplementation and/or aerobic exercise. For the legend, see Figure 1. Western blot images are

representative from a minimum of eight rats per group. The columns represent the means ± SEM. * $P < 0.05$ different from the C group. [a] $P < 0.05$ different from the W group.

FIG. 4: The proteasome 20S α subunit expression in gastrocnemius muscle of tumour-bearing rats under effects of leucine supplementation and/or exercise. The western blot image is representative data of the best assay from a minimum of eight rats per group. For the legend, see Figure 1. The columns represent the means ± SEM. * $P < 0.05$ different from C group.

Table Title

Table 1: Leucine-rich diet and aerobic exercise affected the body, tumour, and gastrocnemius muscle weight and total muscle protein content in tumour-bearing groups.

Table note:

Legend: C–control rats, TC–trained, W-tumour-bearing, TW–trained tumour-bearing, L-rats fed leucine-rich diet, TL–trained rats fed leucine-rich diet, WL–tumour-bearing rats fed leucine-rich diet, TWL–trained tumour-bearing rats fed leucine-rich diet.

The data are the mean ± standard error.

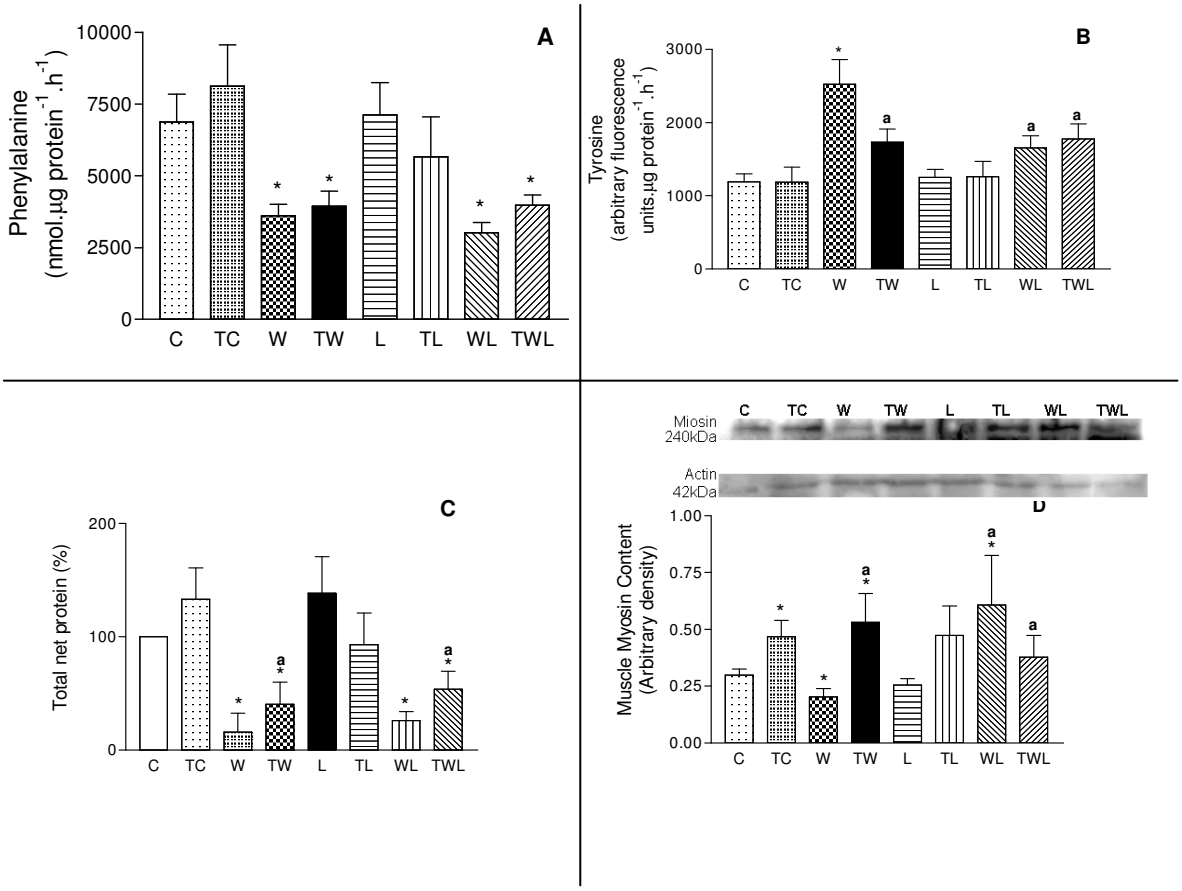
[*] Body weight gain (g) was calculated as the final body weight minus the body weight after tumour implant.

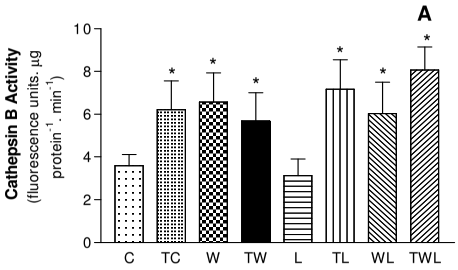
[#] Rate of gastrocnemius muscle weight/ body weight was calculated as the muscle weight percentage of the body weight.

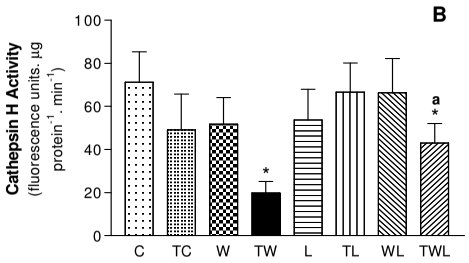
[**] The tumour / body weight ratio was calculated as the tumour weight percentage of the body weight.

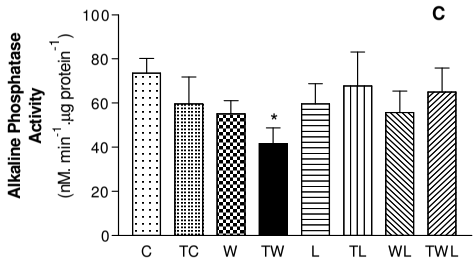
Significant differences are shown in each column by different superscript letters ($P < 0.05$, two-way ANOVA followed by Bonferroni's test).

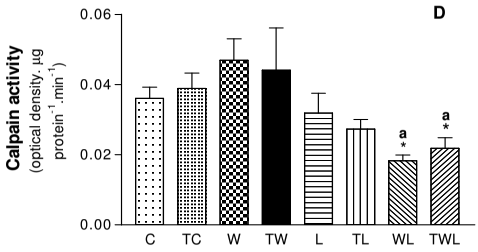
Groups	Initial Weight (g)	Final Weight (g)	Weight Gain after tumour* (g)	Gastrocnemius muscle/body weight rate# (%)	Total muscle Protein (µg/µL)	Tumour Weight (g)	Tumour/body Weight rate** (%)
C (13)	69.3 ± 4.7 [a]	361.8 ± 22.4 [a]	28.6 ± 6.3 [a]	0.58 ± 0.03 [a]	3.63 ± 0.15 [a]		
TC (12)	73.1 ± 4.0 [a]	346.5 ± 25.1 [b]	44.7 ± 1.8 [b]	0.50 ± 0.02 [b]	3.15 ± 0.33 [a]		
W (9)	67.2 + 3.1 [a]	401.0 + 10.3 [c]	3.0 + 5.2 [c]	0.46 + 0.04 [b]	2.87 + 0.20 [b]	57.5 + 5.8 [a]	19.4 + 2.3 [a]
TW (8)	60.0 ± 2.6 [a]	293.6 ± 14.9 [d]	11.8 ± 7.1 [d]	0.53 ± 0.04 [a.c]	3.15 ± 0.19 [a]	40.0 ± 5.5 [b]	15.5 ± 2.4 [b]
L (16)	62.2 ± 2.3 [a]	333.0 ± 18.6 [b]	35.1 ± 3.4 [a]	0.54 ± 0.02 [a]	3.40 ± 0.12 [a]		
TL (13)	62.4 + 2.7 [a]	311.7 + 17.9 [b]	33.0 + 6.0 [a]	0.57 + 0.05 [a]	4.00 + 0.31 [a]		
WL (11)	63.6 ± 2.8 [a]	329.5 ± 15.4 [b]	21.1 ± 8.3 [a]	0.50 ± 0.02 [b.c]	4.21 ± 0.33 [a]	53.9 ± 5.0 [a.c]	19.6 ± 1.7 [a]
TWL (11)	66.7 ± 5.0 [a]	320.3 ± 17.1 [b]	12.5 ± 4.7 [d]	0.46 ± 0.05 [b]	4.18 ± 0.35 [a]	49.0 ± 4.7 [c]	18.6 ± 1.5 [a]

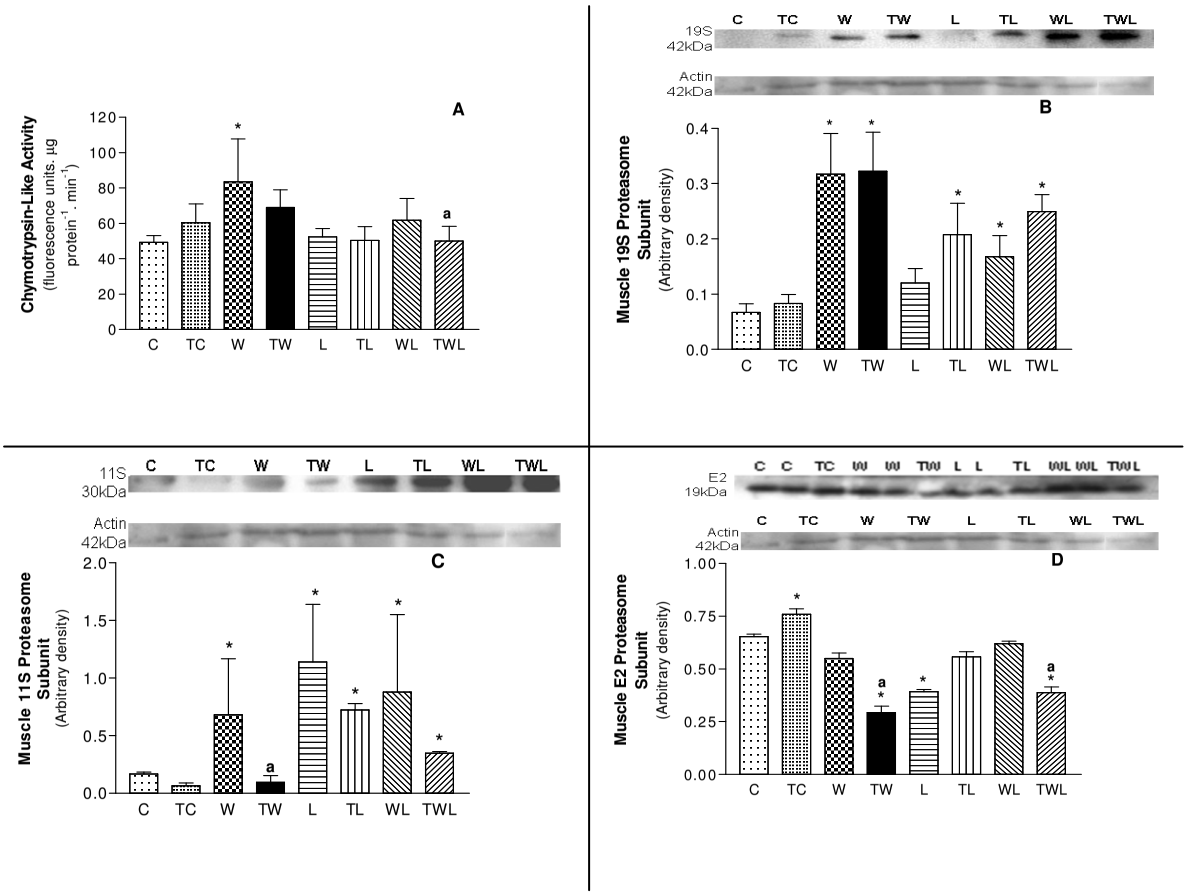

A
Phenylalanine (nmol.µg protein-1.h-1)
10000
7500
5000
2500
0
C TC W TW L TL WL TWL
B
Tyrosine (arbitrary fluorescence units.µg protein-1.h-1)
3000
2000
1000
0
C TC W TW L TL WL TWL
C
Total net protein (%)
200
100
0
C TC W TW L TL WL TWL
C TC W TW L TL WL TWL
Miosin 240kDa
Actin 42kDa
D
Muscle Myosin Content (Arbitrary density)
1.00
0.75
0.50
0.25
0.00
C TC W TW L TL WL TWL

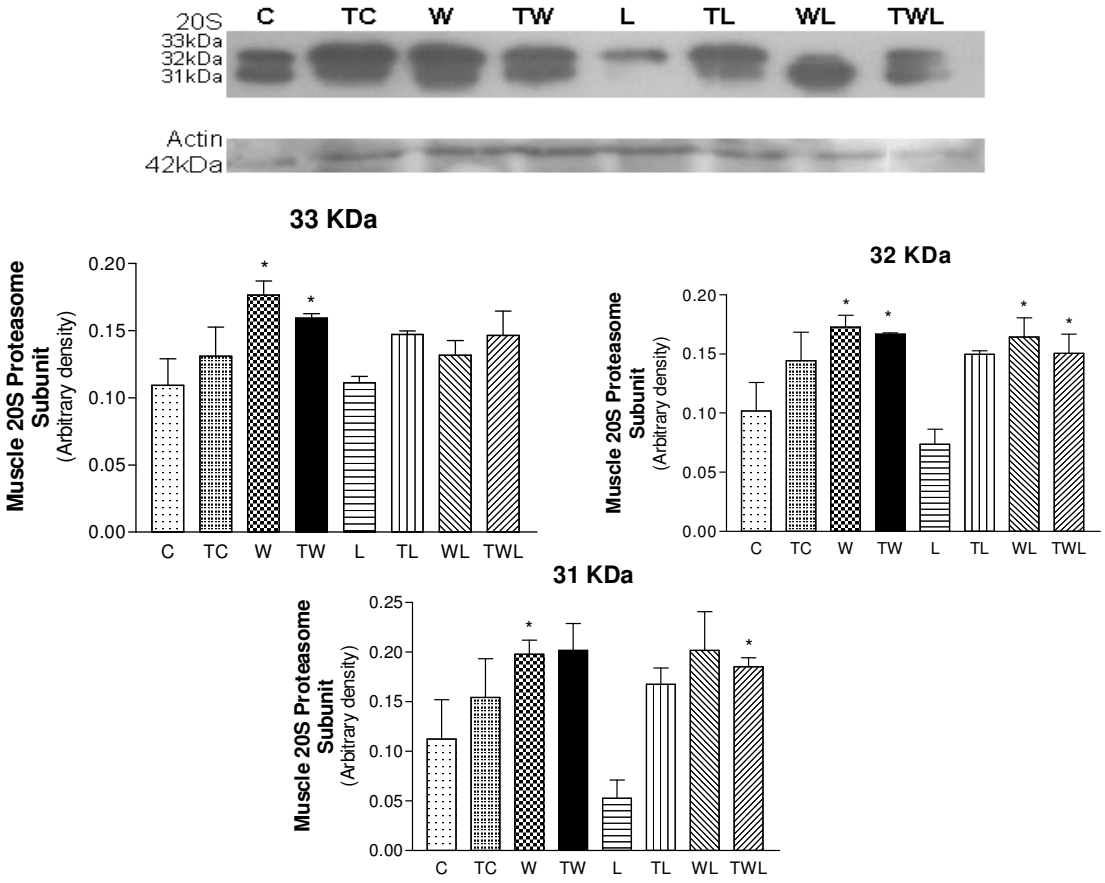

A
Cathepsin B Activity
(fluorescence units. µg protein^{-1}. min^{-1})
10
8
6
4
2
0
C TC W TW L TL WL TWL


B
Cathepsin H Activity
(fluorescence units. µg protein^{-1}. min^{-1})
100
80
60
40
20
0
a
C TC W TW L TL WL TWL


C
Alkaline Phosphatase Activity
(nM. min^{-1} .µg protein^{-1})
100
80
60
40
20
0
C TC W TW L TL WL TWL


D
Calpain activity
(optical density. µg protein^{-1} .min^{-1})
0.06
0.04
0.02
0.00
a
a
C TC W TW L TL WL TWL


A
Chymotrypsin-Like Activity
(fluorescence units. µg protein⁻¹. min⁻¹)
120
100
80
60
40
20
0
C TC W TW L TL WL TWL
a
B
19S
42kDa
Actin
42kDa
C TC W TW L TL WL TWL
Muscle 19S Proteasome Subunit
(Arbitrary density)
0.4
0.3
0.2
0.1
0.0
C TC W TW L TL WL TWL
C
11S
30kDa
Actin
42kDa
C TC W TW L TL WL TWL
Muscle 11S Proteasome Subunit
(Arbitrary density)
2.0
1.5
1.0
0.5
0.0
C TC W TW L TL WL TWL
a
D
E2
19kDa
Actin
42kDa
C TC W TW L TL WL TWL
Muscle E2 Proteasome Subunit
(Arbitrary density)
1.00
0.75
0.50
0.25
0.00
C TC W TW L TL WL TWL
a


20S
33kDa
32kDa
31kDa
C
TC
W
TW
L
TL
WL
TWL
Actin
42kDa
33 KDa
32 KDa
31 KDa
Muscle 20S Proteasome Subunit
(Arbitrary density)
0.00
0.05
0.10
0.15
0.20
0.25
*

SEGUNDO ARTIGO CIENTÍFICO

"Dieta rica em Leucina em combinação com treinamento físico, a longo prazo, alterou a expressão gênica das enzimas glicolítica no músculo dos grupos implantados com tumor".

(Artigo a ser submetido na *Journal of Applied Physiology)*

RESUMO

A intensa mobilização de substratos dos tecidos da carcaça do hospedeiro, em função do crescimento neoplásico, promove no organismo o estado caracterizado como caquexia. Presente na maioria dos pacientes com câncer, a caquexia promove intensa perda involuntária de peso decorrente, preferencialmente, da depleção da proteína muscular em função do aumento da degradação e/ou da diminuição da síntese protéica, culminando na redução da qualidade e expectativa de vida. Sabendo-se que a leucina (BCAA) é (1) utilizada como fonte energética pelo músculo esquelético, podendo ser transaminada e oxidada para produzir acetil-CoA, (2) é caracterizada como precursora da gliconeogênese, a partir da sua degradação e formação de alanina no músculo e (3) age, principalmente, como sinalizadora celular e que o exercício físico promove aumento do consumo de glicose, diminuindo assim os níveis de glicose e insulina circulantes, consequentemente, reduzindo a oferta desse substrato às células tumorais, temos como principal objetivo avaliar a suplementação de leucina associado ao exercício físico, na hipótese de prevenir a depleção muscular, preservando as reservas energéticas, e possivelmente o estado caquético do animal. Ratos portadores de tumor foram submetidos ao exercício aeróbico longo, em combinação com dieta suplementada com leucina, avaliando-se o metabolismo

de carboidratos do músculo, através da avaliação da via glicolítica e receptor de glicose muscular nesses ratos. Os dados aqui apresentados mostram-nos claramente que o efeito deletério do tumor conduz o hospedeiro ao estado de espoliação bastante severo. Por outro lado, a suplementação de leucina associada ao exercício físico promove benefícios ao hospedeiro, mostrados tanto no metabolismo de glicose, quanto na concentração sérica de citocinas, que conduzem ao aumento da espoliação tecidual do hospedeiro com câncer.

Leucine-rich diet in combination with long training exercise changed gene expression of glycolitic enzymes in muscle of tumour-bearing groups.

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ABSTRACT

The intense mobilization of substrates occurred during tumour growth induces waste of carcass tissues leading to body state characterized as cachexia. Present in most patients with cancer, cachexia promotes intense involuntary weight loss resulting mainly from depletion of muscle protein due to increase in proteolysis and/or decreased protein synthesis. This fact results in reduced quality and life expectancy. Knowing that leucine, branched chain amino acid (BCAA), is used as an energy source by skeletal muscle or a precursor of gluconeogenesis or acts as a cell signalling and the physical exercise promotes increased consumption of glucose, reducing the levels of circulating glucose and insulin, which promotes less supply of substrate to tumour cells, the main objective of the present work was to minimize metabolic changes in host tissue against the effects of Walker carcinoma growth. Tumour-bearing rats were submitted to long aerobic exercise in combination with leucine-supplemented diet assessing the muscle carbohydrate metabolism through analysis of glucose metabolism, by evaluating glycolytic pathway and muscle glucose receptor in these rats. The results showed tumour growth produced deleterious effect in tumour-bearing host leading to the cachectic state and the leucine supplementation combined with exercise benefit host tissues, counteracting the hypoglycaemia and low GLUT-4 receptor imposed by tumour growth, and improved the glucose uptake increasing the hexokinase and decreasing the pyruvate dehydrogenase enzymes expression, and improved the inflammatory response in these animals, reducing the pro-inflammatory and increasing the anti-inflammatory cytokines. These results suggests that there are some improvement of host tissue in detriment of tumour growth.

Key Words: Leucine; exercise; Walker 256; glucose metabolism; cachexia.

INTRODUCTION

Cachexia syndrome is common in many types of cancers and is the most important factor which leads to premature death of cancer patients. This syndrome is characterized by great weight loss in relation to decrease of the life quality and survival (1).

Among the symptoms produced by neoplastic growth, the food intake decrease, called anorexia, is the most frequent complication in patients with cancer (2). The anorexia-cachexia syndrome is caused by metabolic changes and, especially, by cytokines produced by tumour or released in response of host immune system, these cytokines produced by tumour also promote lipolysis and proteolysis in these cancer patients (2).

Patients with cancer often have hypoglycaemia associated with low tissue glucose metabolism. However, the anaerobic glycolysis that occurs in neoplastic cells, due to the high glucose metabolism, leads to increase in waste and energy expenditure in host tissues, converting lactate, alanine and glycerol in glucose, by Cori cycle, which increases the energy deficit, resulting in cachectic state (1, 3). The hepatic gluconeogenesis from lactate, alanine and glycerol increases in approximately 40% in cancer patients (1,4,5). Studies also observed association between hypoglycemia and lactic acidosis in patients with lymphoma (6), but this fact is more often observed in animal models (7-9,10). Reduced synthesis and increased protein degradation, which represent increase in total body protein turnover has been widely observed in cancer patients (2,5). The loss especially of skeletal muscle leads to fatigue, weakness and muscle atrophy (11). Fatigue is one of the most severe symptoms in cancer patients, compromising strength and endurance with consequent impairment of life quality (12-15). Aerobic exercise

improves physical performance and when well supervised to cancer patients it might increase the quality of life (16). In animals, the moderated exercise reduced the incidence of tumours, neoplastic symptoms, and also the incidence of metastases and tumour growth (17). Physical activity promotes higher consumption of glucose, reducing the levels of circulating glucose and insulin, leading low supply to tumour cells (18). Additionally, high concentration of corticosteroid and cytokines induced by physical exercise increase immune response, usually depressed during tumour development (19). Conversely, the positive effect produced by physical activity improves insulin resistance, present in host, changing the balance between anabolic and catabolic hormones (20). Therefore, physical exercise, when properly supervised, can be an excellent alternative to improve treatment and rehabilitation of patients with cancer (21, 22).

The branched chain amino acids (BCAA) - especially leucine - are essential substrates and important regulators of body protein synthesis and represent the largest source of nitrogen for synthesis of glutamine and alanine in muscle (23). The increased of leucine oxidation rate in skeletal muscle produces acetyl-CoA, which is common in inflammatory responses normally induced by the cancer, contributes to muscle loss (23). In addition, leucine participates in signalling process of transcription and can inhibit the degradation and / or stimulate protein synthesis. Therefore, leucine prevents the host waste, preserving body protein mass, as previously observed in tumour-bearing rats (9, 24). In previous studies leucine-supplemented diet improved nitrogen balance, restoring lean body mass through the preservation of muscle protein mass and increasing the intestinal absorption in Walker tumour-bearing rats (7).

Knowing that exercise, especially regular exercise, leads to reduced susceptibility of multiple types of disease and a better outcome in patients with a chronic

inflammation-related condition such as cancer, the main purpose of this work was to investigate whether a leucine-rich diet with or without light aerobic physical exercise, as a regular process (a long training protocol), could improve metabolism of glucose and serum cytokines in tumour-bearing rats.

MATERIALS AND METHODS

Animals and diets

Wistar male rats (21-days old), obtained from the animal facilities at the State University of Campinas, UNICAMP, Brazil, were housed in collective cages during the entire experimental period. They received diet and water *ad libitum* under control of light and darkness (12-12 hours) and temperature (22 ± 2 C). The semipurified diets are in accordance to AIN-93M, the American Institute of Nutrition (25). Normoprotein diet (C) was comprised of 18% protein. The leucine-rich diet (L) contained 18% protein plus 3% L-leucine.

An adjustment in the amino acid-rich diet was made to reduce the amount of carbohydrates. The diets were isocaloric and had similar quantities of cornstarch (39.7%), dextrin (13.2%) and sugar (10%). Additionally, the C and L diets contained the same amount of fat (7% soy oil), fibre (5% cellulose micro fibre), salt (3.5%) and vitamin mix (1.0%). The diets were also supplemented with cystine and choline. The amino acids (L-leucine) and cornstarch were provided by Ajinomoto Interamericana Ind. and Com. Ltda and Corn Products Brazil Ingredients, respectively.

Tumour implantation

The tumour-bearing rats received approximately 0.25 × 10^6 Walker 256 carcinoma cells in suspension implanted subcutaneously in the right flank (26). The rats without tumours were injected with 0.5 mL 0.9% (w.v.) NaCl solution without anaesthesia. The general guidelines of the UKCCCR (United Kingdom Coordinating Committee on Cancer Research, 1998) (27) for animal welfare were followed, and the experimental protocols were approved by the institutional Committee for Ethics in Animal Research (CEEA.IB/UNICAMP, protocol # 465-4).

Exercise protocol

The rats were submitted to light swim training for 8 weeks (60 days) in 1 m^3 container at 30 ± 2°C. The training was performed 5 days per week in the morning, starting with 5 minutes initially and progressively increasing the swim time until 45 minutes per day of exercise with no body weight overload, until the end of the experiment. To avoid thermal stress after swimming, all exercised animals were gently dried out and kept in a warmed room. Their body temperature was measured to confirm body temperature maintenance. To guarantee the training protocol the cardiac frequency were recorded and was observed bradycardia especially in leucine-treated rats (data not shown).

Experimental protocol

The young rats were first distributed into the following 4 groups, according to the diet regimen: sedentary animals that were fed with control or leucine-rich diet (C and L groups) and exercised animals that were fed with control or leucine-rich diet (TC and TL groups). After 60 days on the regimen, the rats were redistributed into 8 groups based on whether they received or not implant of Walker 256 tumour

cells (W), corresponding to the following groups: 4 groups that received the control diet: control (C), trained (TC), tumour-bearing (W) and trained tumour-bearing (TW) and 4 groups that were fed the leucine-rich diet: rats fed the leucine-rich diet (L), trained rats fed the leucine-rich diet (TL), tumour-bearing rats fed the leucine-rich diet (WL) and trained tumour-bearing rats that were fed the leucine-rich diet (TWL). The body weights were recorded three times a week. Twenty-one days after tumour implantation, the rats were sacrificed, which was carried out 24 hours after the final exercise protocol to avoid any acute inflammatory stimulus. The serum samples were collected to assay glucose, insulin and glucagon and pro and anti-inflammatory cytokines content and gastrocnemius muscles were quickly collected and kept in -80^{o}C to analyse the muscle glucose metabolism.

Glucose metabolism in the gastrocnemius muscle

Reverse-transcription polymerase chain reaction (RT-PCR)

To investigate the gene expression of glycolytic enzymes in muscle of different animals, we used analysis of reverse-transcription polymerase chain reaction (RT-PCR).

mRNA extraction was performed with Trizol reagent - Sigma, following the manufacturer's specifications. The RNA was quantified by spectrophotometer at 260 nm and purity determined by gel electrophoresis on 1% agarose Tris borate, pH 8.3 and stained with ethidium bromide (28). The complementary DNA copy of mRNA was obtained by synthesis of cDNA using primers for:

Hexokinase: 5'-GACCAAGTCAAAAAGATTGA-3' (sense), 5'-TCTTCTCGTGGTTCACCTGC-3' (antisense) (29).
Pyruvate dehydrogenase: 5'-GTGATGGTGCTGCTAACCA-3' (sense), 5'-

AACTGGGCAGCATCCTCGAT-3' (antisense) (30).

Glucose transporter:

Glut-4: 5'-GGTTCCATCCATGAGTTATGTGTC-3' (sense), 5'-CTAAGAGAGAAGGTGTTCCGTCG-3' (antisense) (31).

The amplification of mRNA was used competitive and co-amplified using RT-PCR (32), using 1.5 mM $MgCl_2$, 40 pmol of each primer, 4 pmol of each nucleotide and 0.5U Tag DNA enzyme polymerase. The amplification was performed in thermal cycler Mastercycler gradiant (Eppendorf, Wien, Austria), initial denaturation at 95℃ for 5 minutes followed by 40 cycles of 95℃ for 30 seconds hybridization 65℃ for 30 seconds and extension 72℃ for 30 seconds. Negative control was used mix containing all reagents except RNA sample, amplified with the reaction test to ensure that no contamination. The positive control was a constitutive primer β-actin 5'-GGACTTCGAGCAGGAGATGG-3' (sense) and 5'-GCACCGTGTTGGCGTAGAGG-3' (antisense). All amplification products were evaluated in 3% agarose gel electrophoresis, using 5 µL of each PCR product against 1µL of DNA molecular marker of 100 bp, diluted (1:4) in sample buffer. The electrophoresis gels were visualized under UV light, photographed and quantified using Gel-Pro Plus software (1.0 version, Silver Spring, Cybernetcs USA).

Histology and Morphometry

The gastrocnemius muscle was dissected and fractional tissue was fixed in paraformaldehyde and then placed in paraffin for histological sections 3µm thick hematoxilin/eosin stained (33).

Photomicrographs were obtained using a digital camera attached to Leica microscope, and images were captured and analyzed with Image Pro Plus Sofware (1.0 version for Windows, Cybernetics, Silver Spring, USA).

Biochemical Analysis of Cytokines, Hormones and Glucose

The analysis of interleukins 4, 6 and 10, tumour necrosis factor alpha (TNFa), interferon gamma (INFγ) and insulin and glucagon were made by binding antigen anti-body, through multiplex immunoassay kits Millipore (Linco / Millipore) assay in cytometry flow for fluorescence, using the Luminex equipment (Millipore, USA).

Aliquots of the serum were evaluated for glucose content, through specific glucose - enzymatic kit, following the manufactures specifications.

Statistical analysis

The results were expressed as the mean $\pm$ SEM. Data were analysed statistically by two-way ANOVA, testing the effects of diet and exercise on tumour growth, muscle parameters and genes expressions. Comparisons within control and tumour-bearing groups were performed using a one-way ANOVA followed by a post-hoc Bonferroni's multiple comparison test (Graph Pad Prism software, v3.00 for Windows 98, USA). Results were considered statistically when the P value was less than 5% (34).

RESULTS

Cytokines

The pro-inflammatory cytokine, tumour necrosis factor (TNFα) (Fig. 1A) changed significantly as an effect of tumour growth, with a pronounced increase in tumour-

bearing groups W, LW and TWL, when compared with their respective controls C, TC, L and TL. Another proinflammatory cytokine, interferon-γ (INF-γ) (Fig. 1B), showed a significant increase in group W, compared to control groups and experimentals groups TW, LW and TWL. Except in TW and LW groups, the other groups presented decrease in serum INF-γ. The interleukin 4 (IL-4), cytokine related to defense response, an anti-inflammatory cytokine (Fig. 1C) showed significant increase in group W, compared to the groups supplemented with leucine tumours WL and TWL. The serum concentration of interleukin-6 (IL-6), a pro-inflammatory cytokine (Fig. 1D), increased significantly in group W, compared to groups C, TC, TW, L and TL. IL-6 is also a myokine and is produced from muscle especially during contraction. The trained-rats and leucine-fed groups showed increase in IL-6 compared to control group (C), but not as higher as verified in W group. The anti-inflamatory cytokine, IL-10 (Fig. 1E), increased in all groups with tumour, with significance in W and TWL groups when compared with their respective controls (C, TC, L, TL). In L and TL groups, the level of IL-10 decreased compared to C and TC rats.

Glucose Metabolism

The content of serum glucose (Fig. 2A) reduced in tumour-bearing groups W, LW and TWL, when compared to control groups C and TC. Despite having cancer the TW had similar glycaemia as the control group. We also observed a significant reduction in plasma insulin (Fig. 2B) and a significant increase in glucagon levels (Fig. 2C) in all tumour-bearing when compared to control groups, although, this increase was lower in LW and TLW groups. These results suggested a

compensatory balance between anabolic and catabolic hormones, since the glycaemia in TW was maintained under normal values.

The gene expression of glucose transporter Glut-4 in the gastrocnemius muscle (Fig. 3A) showed significant reduction in tumour groups W and TW, suggesting low glucose uptake by muscle cell.

Hexokinase, an enzyme that phosphorylates uptaked glucose into glucose-6-phosphate, in the glycolytic pathway, showed a significant increase of gene expression in trained groups and in all tumour groups except in TWL, when compared to C group (Fig. 3B).

The pyruvate dehydrogenase converts pyruvate (product of glycolysis in the cytosol) into acetyl (acetyl-CoA), which is then oxidized in mitochondria inner membrane to produce energy via Krebs cycle. Significant reduction of pyruvate dehydrogenase was observed in TWL group when compare to controls TC and L (Fig. 10). Despite having tumour, W and LW groups tended to increase the gene expression of pyruvate dehydrogenase, suggesting that pyruvate decarboxilation process contributes to linking the glycolysis metabolic pathway to the citric acid cycle, increasing energy disposal to muscle cells in response to lower glucose uptake.

Morphology of the gastrocnemius muscle

The muscle morphometry showed that muscle weight was reduced in W and TWL groups (data not shown), but the morphology analysis showed a significant reduction in fibre diameter of gastrocnemius muscle (Fig. 4) in W and LW groups compared to C, TC, L and TL groups. The trained-rats from TC and TL groups

tended to increase muscle fibre diameter, similar pattern verified in TW and TWL group, despite the accelerated tumour growth.

DISCUSSION

Knowing that exercise can improve the quality of life in cancer patients (21,22) when well conducted and minimise wasting of lean body mass (20,35,36), the results of this work showed that leucine-rich diet in combination or not with long training exercise promoted changes in skeletal muscle responses, including the whole host which modified gene expression related to glucose metabolism.

Walker 256 tumour growth induced changes not only in protein synthesis (inhibition) and protein degradation (stimulation) (our previous results) but also led changes in glucose metabolism in gastrocnemius muscle in rats. Despite having cancer, these changes were partially attenuated by the aerobic exercise and nutritional supplementation.

The present work analysed the impact of long training exercise and leucine supplementation considering therapeutic interventions promote major impact on the prognosis of cancer disease, and the nutritional status greatly affects the prognosis and quality of life of cancer patients.

The pathophysiology of cancer cachexia is not fully understood; however, studies have shown that cytokines are important in the alteration of carbohydrate, lipid, and protein metabolism. A number of cytokines, including tumour necrosis factor-α, interleukins 1 and 6, interferon gamma, and leukemia-inhibitory factor, have been proposed as mediators of the cachectic process (1,37), High plasma concentrations of cytokines were observed in patients with cancer and seem to be involved with weight loss by a mechanism not related to anorexia (38). Splenic macrophages and non-adherent splenic lymphocytes from tumour-bearing mice

showed an increased production of IL-6, TGF-beta, IFN-gamma, IL-2R and VEGF during the late tumour-bearing stage, which could be implicated in the differential regulation of tumour growth in a tumour stage dependent manner (39).

In accordance with data from the literature, we found significant increases in proinflammatory cytokines, IL-6, TNF-α and INF-γ in the group implanted with tumour (W) when compared with control groups, corroborating with literature, these cytokines are directly related to lean body mass wasting. Probably, these facts could be related with changes in carbohydrate metabolism, especially in gastrocnemius muscle. These data are consistent with the literature, where there are reports of the effect of chronic inflammation in the production of proinflammatory cytokines in the development process of cachexia (40,41). TNF-α induces IL-6 secretion and synergizes with it in many of its actions, e.g., both stimulate other cytokines in a cascade, which has both proinflammatory and anti-inflammatory components (37). In contrast, IL-6 seems to be beneficial for insulin-regulated glucose metabolism in muscle, since during contraction serum levels of IL-6 elevated preceding the appearance of other cytokines. This fact was observed in trained-rats as showed in the present work. Meanwhile, the effects of IL-6 are seemingly influenced by whether it is present acutely or chronically; the latter (42), as observed in cancer state, is associated with insulin resistance and also with reduced insulin levels, also observed in tumour-bearing rats.

The changes in balance between proinflammatory and anti-inflammatory cytokines, especially when in the direction of proinflammation state, potentially causes or exacerbates the health complications found in pathologies as cancer-cachexia (43,44). Meanwhile, the action of cytokines alone is unable to explain the complex mechanism of wasting in cancer cachexia (37). Argilés and colleagues (45) proposed the adoption of the balance of pro-and anti-inflammatory cytokines

as markers of the severity of cancer cachexia. Recently, the IL-10/TNF-a ratio has been adopted as a marker of the intensity of the inflammatory condition in obese individuals and animals (44). Increase in serum levels of IL-10 was observed in all tumour-bearing groups, similar to results found by other researchers (46).

Important and marked alterations in carbohydrate metabolism are observed in tumour-free tissues of patients with cancer (5, 37, 47). The neoplastic cell preferentially uses glucose as energy substrate, 10 to 50-times more compared to normal cells, indicating that the presence of the tumour increases consumption of glucose (3, 4, 47). Conversely, we observed decrease in serum glucose in all tumour-bearing groups, justifying the high glucose utilization preferentially by neoplastic cells (Fig. 2A). In experimental animals, hypoglycaemia is common since glucose is the main energy substrate used by the tumour for its growth (1,48). On the other hand, the exercised tumour-bearing group (WT) trended to maintain the glucose level, because the exercise diverted the substrate from tumour cells toward to skeletal muscle, decreasing the supply to neoplastic growth. Maybe the higher proinflammatory cytokines levels found in W group led to inflammatory process which is responsible for oxidative stress and muscle wasting, justifying the lower diameter of muscle fibre and high gene expression of hexokinase and pyruvate dehydrogenase. In contrast, the other tumour-bearing rats (TW, LW and TWL) have some parameters recovered probably due to exercise or leucine-rich supplementation. Several studies point to peripheral resistance to insulin in the presence of cancer (49,50). Physical exercise, on the other hand, greatly improves tissue responsiveness to insulin (18,20), especially in diabetic patients submitted to physical exercise, or in experimental animals with tumour (49,50).

There is an increase in hepatic gluconeogenesis from substrates such as alanine and lactate both from muscle (5,37). Glucose is degraded to lactate by tumour cells and the lactate is converted back into glucose in the liver - Cori cycle (3,5). This conversion results in consumption of six molecules of ATP, leading to energy waste, focusing on the "futile energy cycle" of glucose, which agrees with the tissue degradation and loss of weight and body mass in these patients. Patients with advanced cancer and progressive weight loss show the activity of Cori cycle about 2 to 3-times higher than that measured in cancer patients without weight loss or healthy volunteers (1,4,5,51). This increased turnover of glucose has been reported in patients with gastrointestinal tumours, in proportion to the extent of disease.

Glucose intolerance due to increased insulin resistance, both exogenous insulin and the endogenous and inadequate release of insulin, occurs in almost 60% of patients with malignant tumours. Glucose intolerance is caused by decreased sensitivity of beta receptors on pancreas cells, while resistance to insulin action is caused by decreased sensitivity of peripheral tissues, in some case produced by proinflammatory cytokines, as verified here. Pyruvic acid, an intermediate metabolite of glucose and effective scavenger of reactive oxygen species (ROS), inhibits tumour necrosis factor-alpha production and reduces IL-6 (interleukin-6) and its mRNA expression in liver. According to Das (52), some studies suggest that pyruvate has potent anti-oxidant and anti-inflammatory actions. Since insulin influences the production of pyruvate by its action on glucose metabolism and pyruvate is an insulin secretagogue (52), maybe the similar gene expression of PDH and low serum levels of TNF-α, IL-6 and INF-γ could improve pyruvate level released from muscle in TW and TWL assuring lower waste process during tumour growth. This suggests that pyruvate plays an important role in metabolic

pathologies as cancer (where insulin resistance is common due to enhanced TNF-alpha production) (52). Additionally, the non-tumour-bearing animals showed increase in gene expressions of GLUT-4 transporter (suggesting glucose uptake), and hexokinase (suggesting higher glucose phosphorilation to hexose) and PDH enzymes (conversion of pyruvate to acetil-CoA) indicating that these genes leading to better energy supply to muscle tissue in these groups. In contrast, the lower gene expression of PDH associated with similar GLUT-4 and hexokinase gene expression could be related not only to energy supply but better adaptation to convert pyruvate into acetil-CoA.

As mentioned above, several studies with experimental tumours, including Walker 256 tumour, have shown decreased insulin secretion as one of the main effects of the tumour, providing glucose supply to cancer cells, as well as increased peripheral resistance to insulin (50,53). The hormonal changes related to the effects of tumour growth showed a significant decrease in serum insulin levels in groups W, TW, LW and TWL, associated to increase in serum levels of glucagon. These hormonal changes were probably correlated to increase in anaerobic glucose utilization by tumour cells, which could reduce the translocation of glucose transporters under low insulin-sensibility by peripheral tissue, such as skeletal muscle. Thus, a decrease of gene expression of glucose transporter Glut-4 in groups W and WT may exacerbates the metabolic imbalances observed in advanced stages of neoplastic process (18,20), as found in these groups. Furthermore, the increased glucagon levels in the W, TW, LW and TWL corroborates with studies in the literature that the counterregulatory hormones provide greater availability of nutrients to neoplastic tissue (54). On the other hand, according to the data presented here, we observed reduction in the diameter of gastrocnemius muscle fibres only in W and LW groups, suggesting

that, in TW and TWL, the leucine and exercise could minimize the muscle response to tumour effects. These data are consistent with our previous report, that the long training exercise protocol could benefit the protein synthesis rather than protein degradation, especially when associated to leucine treatment (36).

Therefore, the data presented here clearly show tumour growth produced deleterious effect in tumour-bearing host leading to the cachectic state and the leucine supplementation combined with exercise benefit host tissues, as shown in metabolism of glucose and protein metabolism and also in serum cytokines, improving host tissue in detriment of tumour growth. Further studies are underway in our laboratory to determine if and how leucine supplementation in combination with exercise could counteract host carcass wasting during cancer cachexia.

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Legends

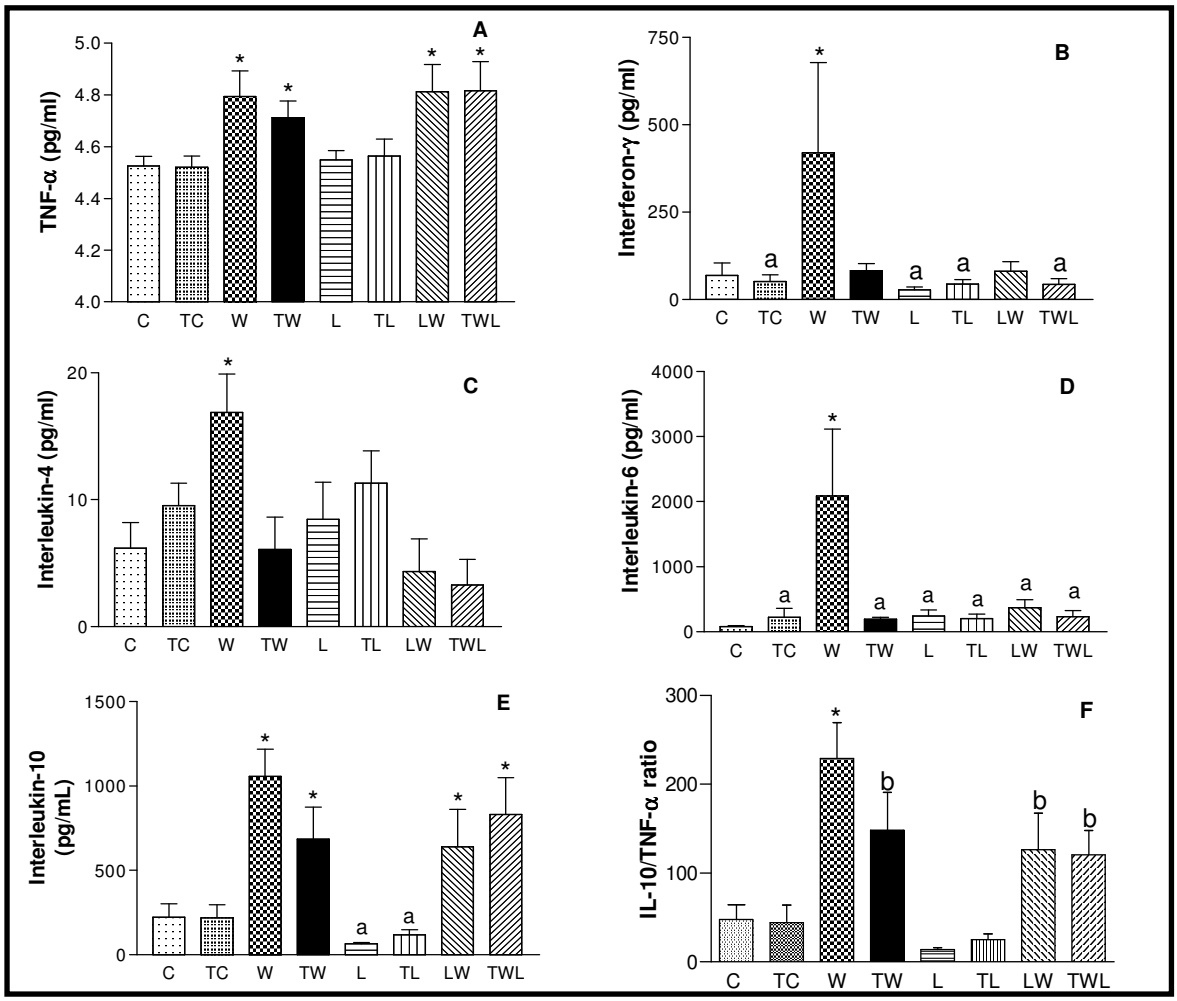
Fig. 1. Serum cytokines levels of TNF-α (A), INF-γ (B), IL-4 (C), IL-6 (D), IL-10 (E) and the TNF-α/IL-10 ratio (F) of tumour-bearing rats under effects of leucine supplementation in combination of not with aerobic exercise program. The minimal number of animals used per group was eight. Legend: C–control rats, TC–trained, W-tumour-bearing, TW–trained tumour-bearing, L-rats fed leucine-rich diet, TL–trained rats fed leucine-rich diet, WL– tumour-bearing rats fed leucine-rich diet, TWL–trained tumour-bearing rats fed leucine-rich diet. The columns represent the means ± SEM. (A) * $P < 0.05$ different from the C and L groups. [a] $P < 0.05$ different from C group. [b] $P < 0.05$ different from W group

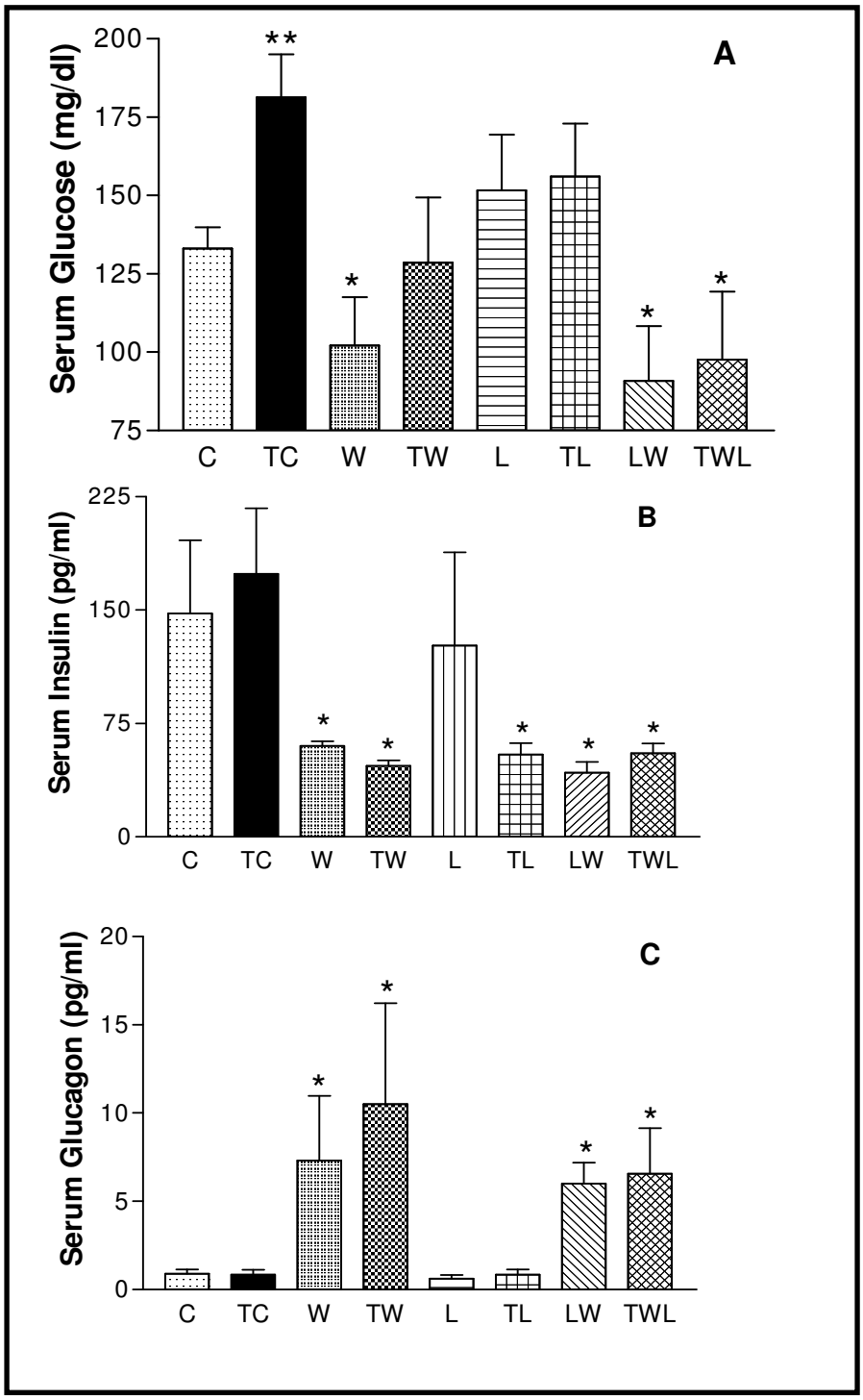
Fig. 2. Blood glucose concentration (A), serum levels of insulin (B) and glucagon (C) in tumour-bearing rats under effects of leucine supplementation in association or not with aerobic exercise program. The minimal number of animal used per group was eight. Legend: C–control rats, TC–trained, W-tumour-bearing, TW–trained tumour-bearing, L-rats fed leucine-rich diet, TL–trained rats fed leucine-rich diet, WL– tumour-bearing rats fed leucine-rich diet, TWL–trained tumour-bearing rats fed leucine-rich diet. The columns represent the means ± SEM. * $P < 0.05$ different from C group. ** $P < 0.05$ different from W, LW and TWL group.

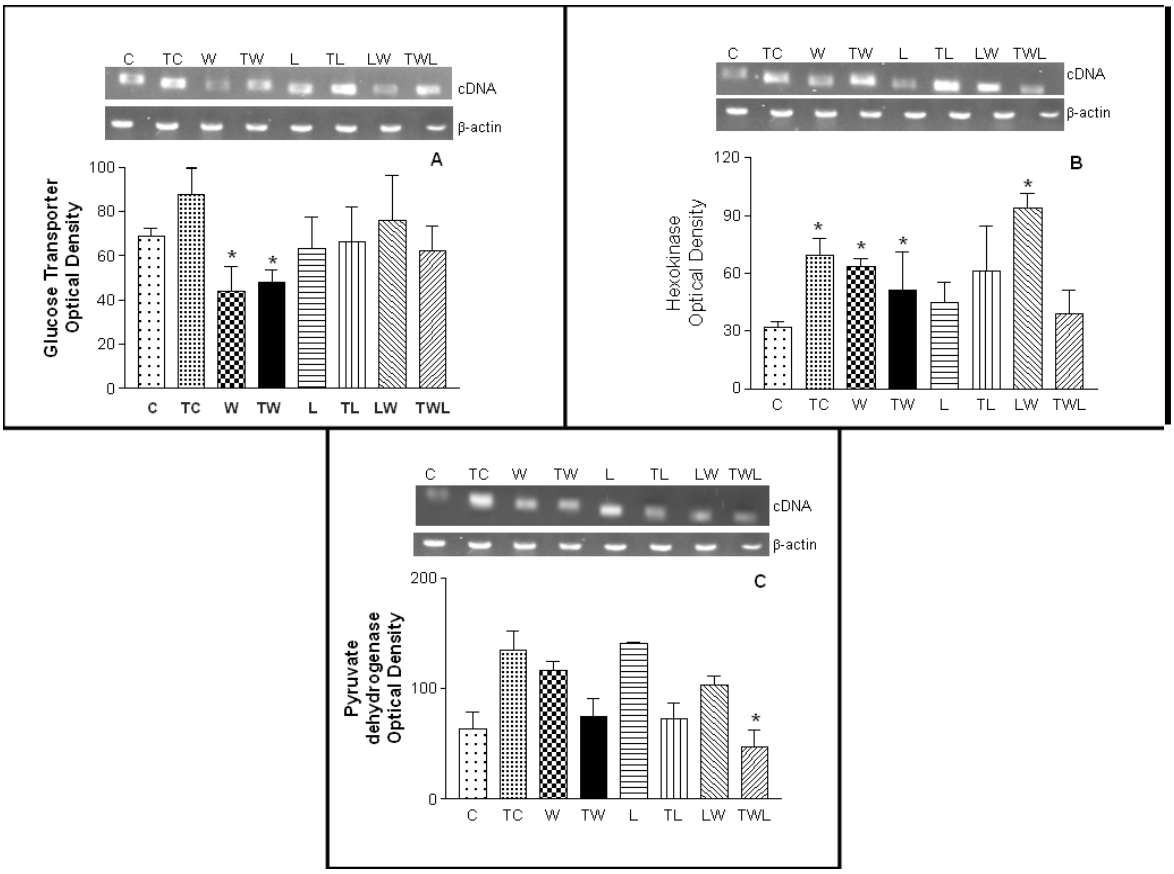
Fig. 3. Gene expression of glucose transporter Glut-4 (A), enzyme hexokinase (B) and enzyme pyruvate dehydrogenase (C) in gastrocnemius muscle of tumour-bearing rats under effects of leucine supplementation and/or aerobic exercise program. The minimal number of animal used per group was eight. Images of gene expression are representative of 8 muscle samples from each group. Legend: C–control rats, TC–trained, W-tumour-bearing, TW–trained tumour-bearing, L-rats fed

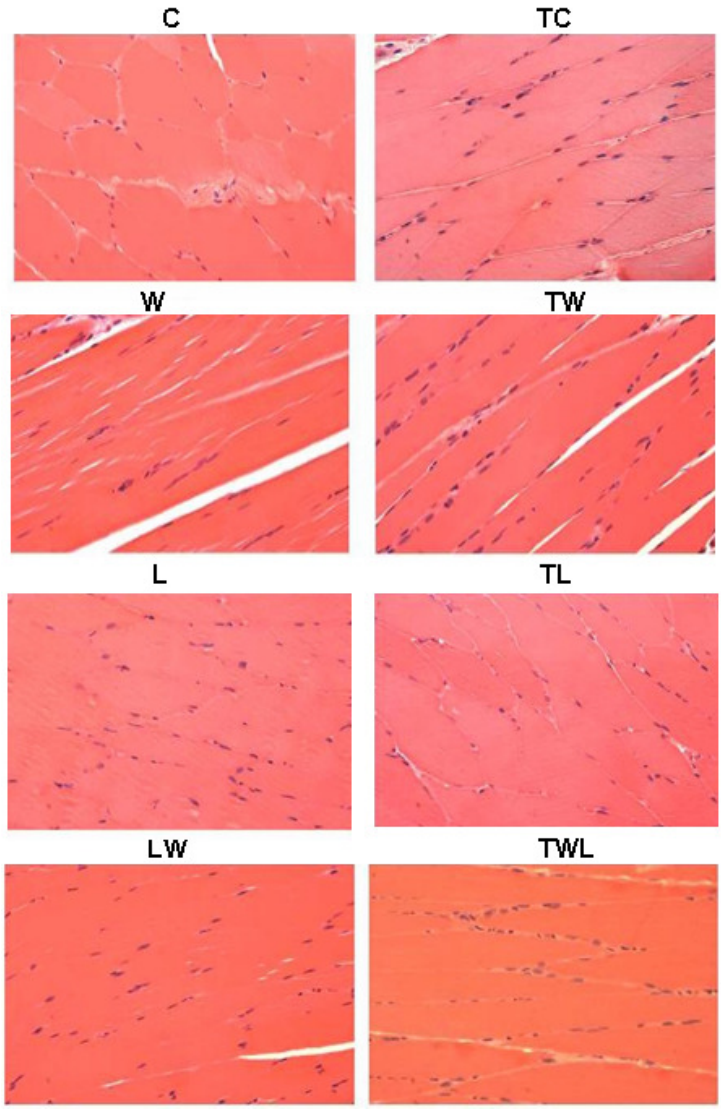
leucine-rich diet, TL–trained rats fed leucine-rich diet, WL– tumour-bearing rats fed leucine-rich diet, TWL–trained tumour-bearing rats fed leucine-rich diet. The columns represent the means ± SEM. Graphics (A) and (B) * $P < 0.05$ different from the C and TC groups. Graphic (C) * $P < 0.05$ different from the TC and L groups.

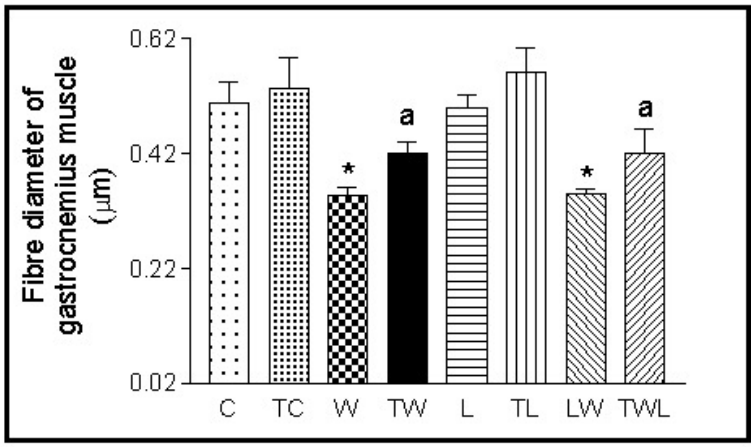
Fig. 4. Histological sections of the gastrocnemius muscle stained with HE (A) and diameter of gastrocnemius muscle (3µm) (B) in tumour-bearing rats under effects of leucine supplementation in combination or not with long training exercise program. The minimal number of animal used per group was eight. Histology magnification is 200x. Legend: C–control rats, TC–trained, W-tumour-bearing, TW–trained tumour-bearing, L-rats fed leucine-rich diet, TL–trained rats fed leucine-rich diet, WL–tumour-bearing rats fed leucine-rich diet, TWL–trained tumour-bearing rats fed leucine-rich diet. The columns represent the means ± SEM. * $P < 0.05$ different from the C, TC, L e TL groups. [a] P<0,05 different from W and LW group.


A
TNF-α (pg/ml)
5.0
4.8
4.6
4.4
4.2
4.0
C TC W TW L TL LW TWL
B
Interferon-γ (pg/ml)
750
500
250
0
a
C TC W TW L TL LW TWL
C
Interleukin-4 (pg/ml)
20
10
0
C TC W TW L TL LW TWL
D
Interleukin-6 (pg/ml)
4000
3000
2000
1000
0
a
C TC W TW L TL LW TWL
E
Interleukin-10 (pg/mL)
1500
1000
500
0
a
C TC W TW L TL LW TWL
F
IL-10/TNF-α ratio
300
200
100
0
b
C TC W TW L TL LW TWL


A
Serum Glucose (mg/dl)
200
175
150
125
100
75
**
*
*
*
C TC W TW L TL LW TWL
B
Serum Insulin (pg/ml)
225
150
75
0
*
*
*
*
*
C TC W TW L TL LW TWL
C
Serum Glucagon (pg/ml)
20
15
10
5
0
*
*
*
*
C TC W TW L TL LW TWL


C TC W TW L TL LW TWL
cDNA
β-actin
A
Glucose Transporter Optical Density
100
80
60
40
20
0
C TC W TW L TL LW TWL
B
Hexokinase Optical Density
120
90
60
30
0
C TC W TW L TL LW TWL
C
Pyruvate dehydrogenase Optical Density
200
100
0
C TC W TW L TL LW TWL


C
TC
W
TW
L
TL
LW
TWL


Fibre diameter of gastrocnemius muscle (μm)
0.62
0.42
0.22
0.02
*
a
*
a
C
TC
W
TW
L
TL
LW
TWL

CONCLUSÕES

No presente trabalho concluímos que, o crescimento tumoral promove alterações no metabolismo dos animais implantados com tumor, tais como: 1) Diminuição do ganho de peso corpóreo após o implante tumoral. 2) Diminuição da síntese e aumento da degradação protéica muscular acompanhada com menor conteúdo de proteína e expressão de miosina. 3) Consequente, aumento da expressão das subunidades ubiquitina-proteossomo. 4) Aumento das citocinas pró-inflamatórias. 5) Redução da concentração sérica de glicose, insulina e aumento de glucagon. 6) Redução da expressão gênica do transportador de glicose muscular, Glut-4. 7) Diminuição do diâmetro da fibra muscular esquelética.

Concluiu-se que a suplementação nutricional com leucina e/ou exercício físico, a longo prazo, promove nos animais com tumor: 1) Melhora no ganho de peso corpóreo, com redução do peso tumoral em alguns grupos. 2) Diminui a degradação muscular esquelética, associada a maior expressão de miosina muscular. 3) Melhora a concentração de citocinas pró-inflamatórias. 4) Melhora a expressão gênica de transportador de glicose muscular, Glut-4. 5) Melhora, também, o diâmetro da fibra muscular.

Diante desses resultados podemos concluir claramente que o efeito deletério do tumor conduz o hospedeiro ao estado de espoliação bastante severo e que a suplementação de leucina associada ao exercício físico promove benefícios ao hospedeiro, mostrados tanto no metabolismo de glicose, quanto no metabolismo protéico e também na concentração sérica de citocinas, que conduzem ao aumento da espoliação tecidual do hospedeiro com câncer.

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CERTIFICADO

Certificamos que o Protocolo nº **465-4**, sobre **"CRESCIMENTO TUMORAL ASSOCIADO AO EXERCÍCIO FÍSICO E DIFERENTES ESQUEMAS NUTRICIONAIS"** sob a responsabilidade de **Profa. Dra. Maria Cristina Cintra Gomes Marcondes / Emilianne Miguel Salomão** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 13 de setembro de 2005.

CERTIFICATE

We certify that the protocol nº **465-4**, entitled **"EFFECTS OF AEROBIC EXERCISE ON TUMOR GROWTH IN RATS SUBMMITED TO LEUCINE SUPPLEMENTED DIET"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on September 13, 2005.

Campinas, 13 de setembro de 2005.

Profa. Dra. Ana Maria A. Guaraldo
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