FLÁVIA ALESSANDRA GUARNIER

ESTRESSE OXIDATIVO EM MÚSCULO ESQUELÉTICO DE RATOS COM CAQUEXIA INDUZIDA POR TUMOR DE WALKER-256

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Dissertação apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito final à obtenção do título de Mestre em Patologia Experimental.

Orientador: Dr. Rubens Cecchini

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Londrina, 23 de fevereiro de 2006.

Este é um trabalho realizado no laboratório de Fisiopatologia de Radicais Livres, da Universidade Estadual de Londrina, formado pelo artigo:

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RESUMO

A caquexia é definida como uma síndrome de progressiva perda de peso, e persistente perda de massa magra. É característica freqüente de pacientes portadores de câncer e se caractreiza por perda maior que 10% da massa corporal total, sendo responsável por cerca de 22% dos óbitos em pacientes com doença maligna avançada. A perda maciça de massa muscular é responsável pela maior parte da perda de peso total e ocorre independentemente da ingesta alimentar ou absorção de nutrientes. Alguns grupos têm demonstrado que o estresse oxidativo moderado pode aumentar a degradação proteica através do superexpressão dos componentes da via ubiquitina-proteassoma. Entre as modificações induzidas pelas ERO, se incluem a carbonilação proteica, a peroxidação lipídica, e o consumo das defesas antioxidantes solúveis. Esse trabalho se propôs a esclarecer o envolvimento do estresse oxidativo no desenvolvimento da caquexia relacionada ao câncer, em músculo esquelético de ratos com a forma subcutânea do tumor de Walker-256. Para isso, foram utilizados ratos Wistar machos de 200-250g, com água e ração *ad libitum*. O primeiro grupo (n=6) recebeu inoculação subcutânea, no flanco direito, de 0,5 mL de PBS; o segundo grupo (n=18) recebeu implantação subcutânea no flanco direito de uma supensão células tumorais em 0,5 mL de PBS; e um terceiro grupo (n=6) foi inoculado com PBS e recebeu a mesma quantidade de ração previamente ingerida pelos animais inoculados com tumor, durante 14 dias. Nos dias 5, 10 e 14, após a inoculação do tumor, os animais foram pesados, decapitados, e o tumor cuidadosamente dissecado e pesado. O músculo gastrocnêmio esquerdo de cada animal foi rapidamente retirado para pesagem e armazenamento. Os músculos foram homogeneizados em tampão gelado, e submetidos às reações com o TBA, quimiluminescência estimulada por tert-butil, e quantificação das defesas antioxidantes solúveis. A implantação do tumor de Walker-256 levou à diminuição progressiva do peso corporal no 5º e 10º dias, que foi acompanhada pela progressiva perda de massa do músculo gastrocnêmio (2,9%, 12,3%, e 15,8% no 5º, 10º e 14º dias, respectivamente). Os animais que receberam a mesma quantidade de ração consumida pelos animais inoculados com o tumor apresentaram, em 14 dias, a mesma diminuição encontrada no grupo 5 dias, quando comparados ao controle (10.1 ± 1.55%). Os índices de massa muscular/massa corporal total observados foram: um aumento de 0,56 no grupo pair-fed, e diminuições de 0,27 no grupo 5º dia, 0,86 no grupo 10º dia, e 0,81 no grupo 14º dia. A lipoperoxidação, avaliada pela formação de TBARS, mostrou um aumento significativo no 10º dia, chegando a aproximadamente 2,5 vezes os valores encontrados no grupo controle. A QL estimulada por *t*-butil hidroperóxido mostrou aumento altamente significativo ($p<0,001$) em todos os grupos experimentais avaliados (5º, 10º e 14º dias), mostrando maior evidência no 5º dia após inoculação (pico de 6,23 Unidades Relativas de Luz) das células tumorais.

Palavras-chave: caquexia. estresse oxidativo. perda de massa muscular. proteólise. câncer.

GUARNIER, Flávia Alessandra. **Oxidative stress injury in rat skeletal muscle with cachexia induced by solid Walker-256 tumor.** 2007. 50f. Dissertation (Master`s Degree in Experimental Pathology) – Londrina State University, Londrina, 2007.

ABSTRACT

Cachexia is a wasting syndrome, characterized by progressive weight loss, generally asssociated to the cancer and other inflammatory diseases. Studies revealed increases on TBARS and CuZn SOD on hypothalamus of rats with cachexia induced by Walker-256 tumour. Other studies showed that moderate oxidative stress in skeletal muscle of tumour bearing rats enhanced protein degradation by the ubiquitin-proteasome proteolytic pathway. This work wanted to clarify the involvement of oxidative stress on skeletal muscle of rats with Walker-256 carcinosarcoma. For this purpose, male Wistar rats were inoculated subcutaneously with a suspension of tumour cells. On days 5, 10 and 14 after tumour implantation, the animals were weighed, killed by decapitation, and the tumour carefully excised and weighed. The gastrocnemius muscle was rapidly excised, weighed and stored at liquid nitrogen until use. Then, the muscles were homogenized in cold buffer and oxidative stress evaluated by the tiobarbituric acid reactive substances (TBARS), chemiluminescence induced by t-butyl (CL), and quantification of antioxidants defenses (TRAP, SOD, Glutathione). In addition, the oxidation of proteins was evaluated by amounts of carbonyl proteins. Our results revealed a progressive loss of body weight on 5^{th} and 10^{th} days after tumour implantation, what was accompanied by muscle mass loss. The levels of TBARS enhanced 2.5 times on 10^{th} day. The CL increased significantly ($p<0.001$) in all experimental groups, showing proeminent elevation of lipid peroxidation on 5^{th} day. The TRAP values decreased on 5^{th} day, returning to control levels on 10^{th} day. The Stress Index, calculated through total and oxidized glutathione concentrations, showed 2 and 3 times of enhacement on days 5 and 14, respectivelly. The SOD activity increased from 0.195 ± 0.01 USOD/mg of protein on 5^{th} day, to 0.240 ± 0.03 USOD/mg of protein on 10^{th} day. Carbonyl proteins increased either, although this enhacement has appeared only on 10^{th} day, declining on day 14. The results revealed the involvement of oxidative stress on weight loss process of skeletal muscle with cachexia induced by Walker-256 tumour. In addition, the enhancement of carbonyl proteins could feed the proteolysis process and consequent promote reduction of muscle mass.

Keywords: Cachexia. oxidative stress. Muscle loss. Proteolysis. cancer.

SUMÁRIO

1 INTRODUÇÃO

A caquexia é definida como uma síndrome de progressiva perda de peso, anorexia, e persistente perda de massa magra. É característica freqüente em pacientes portadores de câncer. A manifestação da caquexia é caracterizada por perda maior que 10% da massa corporal total, e é responsável por cerca de 22% dos óbitos em pacientes com doença maligna avançada. A perda maciça de massa muscular é responsável pela maior parte da perda de peso total, e pode ocorrer independentemente da diminuição na ingesta alimentar ou má absorção de nutrientes. Essa perda muscular significativa é conseqüência do desequilíbrio entre síntese e degradação de proteínas, e provoca astenia (perda de força muscular), vista ainda no estágio inicial da doença. Recentemente, alguns estudos demonstraram o envolvimento da via proteolítica ubiquitina-proteasoma na perda de massa muscular na caquexia. Algumas citocinas pró-inflamatórias, produzidas por células tumorais, também têm demonstrado envolvimento na patogênese da perda de massa muscular, como IL-1, IL-6, e TNF-α, que foi originalmente chamada de "caquectina". O estresse oxidativo é definido como uma produção excessiva das espécies reativas de oxigênio (ERO) que não podem ser neutralizadas por defesas celulares antioxidantes. Recentemente, o estresse oxidativo tem conseguido especial atenção por seu possível envolvimento na caquexia do câncer. Alguns estudos têm demonstrado que o estresse oxidativo moderado pode aumentar a degradação protéica através do aumento na expressão dos componentes da via ubiquitina-proteassoma. Entre as diferentes modificações induzidas pelas ERO em resíduos de aminoácidos, a carbonilação protéica consiste em um dos melhores marcadores de lesão de proteínas em vários estados patológicos. A peroxidação lipídica representa um dos mais significativos processos que precedem a degeneração celular e necrose, sendo extensivamente representada pela produção de malondialdeído (MDA). Estudos mais recentes utilizam um método mais sensível para detecção de lipoperoxidação, por quimiluminescência.

Considerando todas essas informações, e também a existência de poucos dados na literatura a respeito de lesões por estresse oxidativo, em músculo esquelético na caquexia do câncer, esse trabalho se propôs esclarecer o envolvimento do estresse oxidativo no desenvolvimento da caquexia relacionada ao câncer, em músculo esquelético de ratos com a

forma subcutânea do tumor de Walker-256, um modelo experimental que vem sendo utilizado para indução de caquexia experimental. Para esse propósito, foram explorados alguns dos mais importantes sistemas antioxidantes e a injúria às proteínas e aos lipoperóxidos de membrana, em diferentes tempos da progressão do tumor.

2 MATERIAIS E MÉTODOS

Foram utilizados ratos Wistar machos de 200-250g, com água e ração comercial (Nuvilab®) oferecidas ad libitum. Verificou-se o consumo de ração diariamente. Os ratos foram divididos em dois grupos. O primeiro (n=6) recebeu inoculação subcutânea, no flanco direito, de 0,5 mL de PBS (controle). O segundo grupo (n=18) recebeu implantação subcutânea no flanco direito de uma supensão de 8,0 x 10^7 células tumorais em 0,5 mL de PBS. Realizou-se também um controle paralelo, onde 6 animais inoculados com PBS receberam a mesma quantidade de ração previamente ingerida pelos animais inoculados com tumor, durante 14 dias (pair-fed). Nos dias 5, 10 e 14, após a inoculação do tumor, os animais (6 por dia de sacrifício) foram pesados, decapitados, e o tumor cuidadosamente dissecado e pesado.O músculo gastrocnêmio esquerdo de cada animal foi rapidamente retirado para pesagem e armazenagem em nitrogênio líquido, até o momento do uso. O índice de porcentagem de massa muscular/ porcentagem de massa corporal total perdida também foi calculado, para que se pudesse ter uma clara idéia da quantidade de massa muscular perdida, em relação ao total.

Para os ensaios de Quimiluminescência induzida por hidroperóxido de t-butil (QL) e Teste das Substâncias Reativas ao Ácido Tiobarbitúrico (TBARS), os músculos foram homogeneizados em banho de gêlo [10 mg de tecido/mL de tampão Fosfato de Potássio Monobásico (KH_2PO_4/K_2HPO_4),120 mM KCl, pH 7.4]. Para os ensaios de atividade da Superóxido Dismutase (SOD), Capacidade Antioxidante Total (TRAP), e para atividade do sistema glutationa, utilizou-se do sobrenadante de um homogenato 50 mg/mL do mesmo tampão, centrifugado a 11000 x g por 15 minutos, a 4º C. Para obtenção de proteínas carboniladas, utilizou-se homogenato total 50mg/mL.

A avaliação da lipoperoxidação de membranas celulares de músculo esquelético foi realizada através do teste das substâncias reativas ao ácido tiobarbitúrico (TBARs) e Quimiluminescência induzida por t-butil hidroperóxido (QL). A atividade da superóxido dismutase (SOD) e a quantificação das glutationas reduzida e oxidada, assim como o índice de estresse, foram obtidos através de procedimentos padronizados. A Capacidade Antioxidante Total (TRAP) foi avalida por luminescência, utilizando trolóx como padrão. Para detecção de proteínas carboniladas, utilizou-se o método colorimétrico, através da reação com a Dinitrofenilhidrazina (DNFH).

Os resultados representam a média e erro padrão de 6 animais por grupo. A análise de significância foi realizada por teste t de student, para dados não-pareados. Para os resultados de QL, utilizou-se teste t de Student para dados pareados. Consideraram-se significativos os resultados quando $p < 0,05$.

3 RESULTADOS

Como principais resultados, obtivemos:

1) A implantação do tumor de Walker-256 levou à diminuição progressiva do peso corporal no 5º (10,55% de perda em relação ao controle) e 10º(14,27%) dias, que foi acompanhada pela progressiva perda de massa do músculo gastrocnêmio (2,9%, 12,3%, e 15,8% no 5º, 10º e 14º dias, respectivamente). Observou-se também progressivo aumento da massa tumoral e diminuição na ingesta alimentar dos animais inoculados com células tumorais, quando comparados ao grupo controle. Os animais que receberam a mesma quantidade de ração consumida pelos animais inoculados com o tumor apresentaram, em 14 dias (10.1 ± 1.55%)a mesma diminuição encontrada no grupo 5 dias, quando comparado ao controle..

2) Os índices de massa muscular/massa corporal total observados foram: um aumento de 0,56 no grupo pair-fed, e diminuições de 0,27 no grupo 5º dia, 0,86 no grupo 10º dia, e 0,81 no grupo 14º dia.

3)A lipoperoxidação, avaliada pela formação de TBARS, mostrou um aumento significativo no 10º dia, chegando a aproximadamente 2,5 vezes os valores encontrados no grupo controle. Os níveis de TBARS retornaram aos do grupo controle no 14º dia.

4)A QL estimulada por t-butil hidroperóxido mostrou aumento significativo ($p<0,001$) em todos os grupos experimentais avaliados (5º, 10º e 14º dias), mostrando maior evidência no 5º dia após inoculação (pico de 6,23 Unidades Relativas de Luz) das células tumorais. Os valores do V_0 mostraram-se progressivamente aumentados até o 14º dia: 58 x 10^{-3} no grupo controle, e 62,5 x 10^{-3}; 92,5 x 10^{-3}, e 103,10^{-3} Unidades Reativas de Luz por minuto, respectivamente).

5)Constatou-se uma diminuição significativa na concentração de TRAP, já no 5º dia (0,357 ± 0,03 μM de trolóx) após a implantação do tumor, que reverteu-se completa e progressivamente no 10º dia (1,277 ± 0,08 μM de trolóx). No 14º, ultrapassaram (2,186 ± 0,19 μM de trolóx), de forma significativa, os níveis do grupo controle (1,217 ± 0,13 μM de trolóx).

6)As concentrações de glutationa reduzida (GSH) apresentaram queda progressiva até o 14º dia (4,040 ± 0,1 no grupo controle, 1,965 ± 0,2 no 5º, 1,490 ± 0,1 no 10º, 1,267 ± 0,05 μM/mg de proteína no 14º dias), o que não acarretou em aumento nas concentrações de GSSG (0,345

± 0,04 no grupo controle, e 0,310 ± 0,2; 0,172 ± 0,02; e 0,378 ± 0,03 μM/mg de proteína respectivamente Além disso, o índice de estresse revelou-se da seguinte forma: 8,71 ± 2,7 no grupo controle, 18,98 ± 1,9 no 5º dia, 11,94 ± 1,0 no 10º dia, e 20,03 ± 2,5 no 14º dia.

7)A atividade da SOD mostrou-se significativamente aumentada no 5º (0,240 ± 0,03 U SOD/mg de proteína) e 10º (0,195 ± 0,01 U SOD/mg de proteína) dias após a implantação do tumor, recuperando a atividade dos níveis controle (0,131 ± 0,006 U SOD/mg de proeína) no 14º dia (0,101 ± 0,01 U SOD/mg de proteína).

8)Os níveis de proteínas carboniladas mostraram um aumento progressivo (2,176 ± 0,23 proteínas carbonílicas/mg de proteínas totais no grupo controle, 2,608 ± 0,19 no 5º dia), atingindo o seu máximo no 10º dia (3,046 ± 0,19), e retornando aos níveis encontrados no grupo controle no 14º dia (2,478 ± 0,26 proteínas carbonílicas/mg de proteínas totais).

4 DISCUSSÃO

A) A presença do tumor causou perda progressiva de massa total nos animais, com a máxima relação perda muscular/corporal sendo atingida no 10º dia. Estes dados coincidem com os dias em que foram apresentados os maiores níveis de MDA, indicando que a peroxidação lipídica acontece concomitantemente à diminuição da massa muscular. Como os grupos pair-fed não apresentaram perda significativa de massa muscular e corporal em relação ao controle, estes resultados provavelmente devem-se à ação sistêmica do tumor.

B) A análise total da curva de QL revelou que os maiores níveis de emissão foram alcançados já no 5º dia após a inoculação, se mantendo significativos até o 14º dia. Estes resultados apontam para um rápido consumo das defesas antioxidantes não-solúveis antes que níveis significativos de lipoperóxidos se estabeleçam, o que foi reforçado pela determinação dos valores de V_0 , que se elevaram no 10º dia após a implantação do tumor.

C) Os níveis de proteínas carboniladas dos animais com caquexia induzida por tumor apontaram para o mesmo padrão de lesão mostrado nos testes de TBARS e QL, além de coincidir com a maior relação entre perda de massa muscular e massa corporal. Considerando o fato deradicais livres reagem prontamente com proteínas, e que, como consequência, modificações de proteínas podem ocorrer através da reação de radicais secundários com aldeídos de baixo peso molecular, podemos então sugerir que o estresse oxidativo possa estar envolvido na regulação do mecanismo proteolítico.

D) O padrão dos três sistemas antioxidantes investigados no músculo de ratos caquéticos foi significativamente diferente dos músculos controle. Estes resultados apontam para mobilização do sistema antioxidante, contra a lesão, num estado precoce do avanço da síndrome da caquexia.

5 CONCLUSÕES

Este estudo mostra claras evidências sobre a existência de uma associação entre a caquexia induzida por câncer experimental, lipoperoxidação e ataque maciço a proteínas. Os principais mecanismos antioxidantes foram explorados e apontados, o que provavelmente indica lesão durante a progressão da doença.

ARTIGO

OXIDATIVE STRESS INJURY IN RAT SKELETAL MUSCLE WITH CACHEXIA INDUCED BY SOLID WALKER-256 TUMOR.

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Abreviations: Tumor Necrosis Factor – TNF; Malondialdehyde – MDA; Reactive Substances of Thiobarbituric Acid – TBARS; Chemiluminescence – CL; Total Antioxidant Capacity – TRAP; Relative Light Units – RLU; Reduced Glutathione – GSH; Oxidized Glutathione – GSSG; Stress Index - SI; Superoxide Desmutase— SOD, Reactive Oxygen Species – ROS, RNS – Rective Nitrogen Species.

ABSTRACT

Cachexia is a wasting syndrome, characterized by progressive weight loss, generally asssociated to the cancer and other inflammatory diseases. Studies revealed increases on TBARS and CuZn SOD on hypothalamus of rats with cachexia induced by Walker-256 tumour. Other studies showed that moderate oxidative stress in skeletal muscle of tumour bearing rats enhanced protein degradation by the ubiquitin-proteasome proteolytic pathway. This work wanted to clarify the involvement of oxidative stress on skeletal muscle of rats with Walker-256 carcinosarcoma. For this purpose, male Wistar rats were inoculated subcutaneously with a suspension of tumour cells. On days 5, 10 and 14 after tumour implantation, the animals were weighed, killed by decapitation, and the tumour carefully excised and weighed. The gastrocnemius muscle was rapidly excised, weighed and stored at liquid nitrogen until use. Then, the muscles were homogenized in cold buffer and oxidative stress evaluated by the tiobarbituric acid reactive substances (TBARS), chemiluminescence induced by t-butyl (CL), and quantification of antioxidants defenses (TRAP, SOD, Glutathione). In addition, the oxidation of proteins was evaluated by amounts of carbonyl proteins. Our results revealed a progressive loss of body weight on 5^{th} and 10^{th} days after tumour implantation, what was accompanied by muscle mass loss. The levels of TBARS enhanced 2.5 times on 10^{th} day. The CL increased significantly ($p<0.001$) in all experimental groups, showing proeminent elevation of lipid peroxidation on 5^{th} day. The TRAP values decreased on 5^{th} day, returning to control levels on 10^{th} day. The Stress Index, calculated through total and oxidized glutathione concentrations, showed 2 and 3 times of enhacement on days 5 and 14, respectivelly. The SOD activity increased from 0.195 ± 0.01 USOD/mg of protein on 5^{th} day, to 0.240 ± 0.03 USOD/mg of protein on 10^{th} day. Carbonyl proteins increased either, although this enhacement has appeared only on 10^{th} day, declining on day 14. The results revealed the involvement of oxidative stress on weight loss process of skeletal muscle with cachexia induced by Walker-256 tumour. In addition, the enhancement of carbonyl proteins could feed the proteolysis process and consequent promote reduction of muscle mass.

Keywords: cachexia, Walker-256, skeletal muscle, oxidative stress, lipid peroxidation, chemiluminescence.

1.INTRODUCTION

Cachexia is a complex and multifatorial syndrome, responsible for 22% of patients death [1]. About 30% of body mass loss weight is invariably fatal [2]. Muscle wasting accounts for the majority of the muscle loss, which may occur independently on the decrease of food intake or malabsorption of nutrients [3]. Thus, asthenia (lack of muscular strength) reflects the important muscle waste that takes place in the cachectic cancer patient, and it is one of the most relevant characteristics generally associated to this syndrome [4].

Loss of skeletal muscle in patients and animals is a consequence of protein synthesis and degradation inbalance, as indicated by a variety of metabolic alterations [5]. During the past decade, several studies have been performed in order to clarify the contribution of different proteolytic pathways to muscle wasting, and the mechanisms responsible for their activation and regulation. Of the proteolytic pathways contained in the skeletal muscle, the lysosomal and the protesomal systems can operate a degradative proteolysis, while the calcium-dependent (like calpains) and the caspase systems only operate limited or partial proteolysis [6]. The ubiquitin-proteasome system, which is crucially involved in the degradation of regulatory and abnormal cellular proteins, is believed to provide most, at least, of the proteolytic activity required for the degradation of muscle ptotein [7]. The expression of genes pertaining to this system and the amount of ubiquitin-protein conjugates are increased in atrophying muscles in cancer conditions [6]. Further evidences show that muscle proteasomal activity, as assayed with peptide substrates, is enhanced in experimental models of cancer cachexia [8], as well as in some cancer patients [9]. Several cytokines have been implicated in the pathogenesis of muscle wasting, such as TNF-α, IL-1 e IL-6. TNF-α, that was first called “caquectin”, and IL-1, bind to its receptors and induce

the activation of the NF-κB transcription factors [10]. It has been recently demonstrated recently that activation of the NF-κB transcription pathway, activated by cachectic factors as TNF-α, is sufficient to induce skeletal muscle atrophy, and that occurs in part via NF-κB [11]. The involvement of oxidative stress on the ubiquitin-proteasome proteolytic pathway has been suggested [12].

Oxidative stress is defined as an imbalance between production of reactive oxygen/nitrogen species, and antioxidant defense. Since this state can cause damage to all types of biomolecule, including, proteins and lipids [13], it has gained attention for its possible involvement on muscle damage of cancer cachexia. Gomes-Marcondes and Tisdale [12] showed that muscle wasting in cancer cachexia is asociated with increased levels of malondialdehyde in gastrocnemius muscles, and that mild input in ROS generation can increase protein degradation in skeletal muscle by causing a greater expression of the major components of the ubiquitin-proteasome system [14], as proteins are one of the major targets of oxidative stress-derived effects in tissues [1]. Similarly, Buck and Chojkier [14] demonstrated that muscle wasting and dedifferentiation could be prevented by treatment with α-tocopherol or BW755c antioxidants, reverting the cachectic status caused by TNF-α. Freitas et al [15] revealed increased indices of lipid peroxidation and antioxidant enzymatic activity in brain regions of rats bearing solid tumor. On the other hand, muscles of rats bearing an ascitic form of tumor presented no significative differences on antioxidant enzymatic activity, after tumor implantation [1]. Some studies have pointed out to the involvement of glutathione system through regulation of protein ubiquitinylation [16-18], and of hydrogen peroxide through the phosphorilation of Iκb, a part of NFκB that feeds the proteolytic pathway [10, 12].

The most used method to demonstrate lipid peroxidation levels is the production of MDA [1, 12]. In early work, the measurement of oxidative damage on lipids in skeletal muscle by a very sensitive chemiluminescence procedure, was shown [19-21]. Proteins Among the different modifications induced by ROS in amino acid residues, protein carbonylation consists one of the best characterized markers of protein damage in several conditions and disease states [1, 22].

Considering all this information, and since there are poor evidences about the oxidative stress damage in skeletal muscle of cancer cachexia, we proposed to clearify the involvement of oxidative stress in the development of cancer-related cachexia in muscles of rats bearing the subcutaneous form of Walker-256 carcinosarcoma, a tumor that has been extensively used as an experimental model to induce cachexia in rats [23-25]. For this purpose we explored muscle levels of several antioxidant, protein and lipoperoxidative injury in three different time courses of tumor progression.

2. MATERIAL AND METHODS

2.1. Animals

Adult male Wistar rats, obtained from the Animal House of the Biological Sciences Center at the Universidade Estadual de Londrina, weighing 200-250g, were used (n=6/group). The animals had water and comercial food (Nuvilab CR1, Nuvital Nutrients Ltda., Curitiba, Brazil) ad libitum. The food intake was measured daily. All animals were carefully monitored and maintained in accordance with ethical recommendations for animal experimentation.

2.2. Tumor inoculation

Rats were divided into two groups, named controls and tumor hosts. The former received 0.5 mL of a PBS solution injection and the latter received a Walker-256 cell suspension (8,0 x 10^7 cells in 0,5mL of PBS), subcutaneously injected on the right flank. Tumor cells were maintained in our laboratory as an ascitic intraperitoneal tumor, after 1 week of the injection of 2.0 x 10^6 cells /0.5 mL of PBS.

A food intake control group was carried out, where 6 animals innoculated with PBS were fed with the same amounts of food consumed by tumour group, during 14 days (pair-fed). On days 5, 10 and 14 after subcutaneous tumour implantation, the animals were weighed, killed by decapitation, and tumor was carefully excised and weighed. The cachectic index was determined by the following formula and should be above 10% to characterize cachexia.

$$\% \text{ loss of body weight} = \frac{[ibm - fbm + (tw) + gbm]}{(ibm + gbm)} \times 100\%,$$

where, *ibm* = inicial body mass of the tumour bearing animal, *fbm* = final body mass of the tumour bearing animal, *tw* = tumor weight, and *gbm* = mean of gain of control group body mass. The *ratio % of muscle mass loss / % of total body mass loss* was calculated in order to obtain a pattern of general waste.

The contralateral gastrocnemius of tumour bearing animals were rapidly excised, weighed, and stored at liquid nitrogen until use (at most 60 days of storage). The animals of the control group that received PBS subcutaneous injection were treated at the same manner, and compared with experimental groups.

2.3. Tissue prepare

Muscles were placed on ice and homogenized five times for 30s periods cycles with 60s intervals in an Ultraturrax homogenizer, containing 10 mg of tissue/mL of 30mM KH_2PO_4/K_2HPO_4 buffer and 120 mM KCl, at pH 7.4. This homogenate was used for the tert-butil hydroperoxide-stimulated chemiluminescence, and TBARS assays. The supernatant of the homogenate was obtained by centrifugation at 11,000 x g for 15 min at 4ºC, from a homogenate containing 50 mg of tissue/mL of the same buffer, and used for the TRAP, glutathione, and SOD assays. For total protein carbonylation, tissues were specially treated, according to Reznick and Parker [26], with some adaptations, as described later.

2.4. Determination of Thiobarbituric Acid Reactive Substances (TBARS)

The lipoperoxidation of muscle cells of control, 5, 10 and 14 days of tumor homogenates were determined by TBARS reaction, where MDA levels were measured and the resulta were expressed in nanomoles MDA/g tissue, as described by Oliveira and Cecchini [27].

2.5. Measurement of tert-butil hydroperoxide-initiated chemiluminescence of muscle homogenates

Reaction mixtures were placed in 2mL luminescence tubes, containing the following at the respective final concentration: total muscle homogenate from contralateral gastrocnemius of tumour bearing rats (10 mg/mL), 30 mM KH_2PO_4/K_2HPO_4 buffer (pH 7,4, 120 mM KCl), and 3 mM tert-butil hydroperoxide, in a total volume of 1 mL. The tert-butil hydroperoxide-initiated chemiluminescence (CL) reaction was measured in a TD/20 20 luminometer (Turner Designs), with a response range from 300-650 nm. The tubes were kept in the dark up to the moment of assay, which was carried out in a dark room at approximately 28ºC [28, 29]. Results are expressed in Relative Light Units/g tissue (RLU/g tissue). The entire curve was used as an indicator of lipid peroxidation. V_0 values were obtained by linear regression of the ascending part of the CL curve.

2.6. Measurement of the total antioxidant capacity of muscle (TRAP)

Total antioxidant capacity of the muscle homogenate was measured by CL, in a reaction medium containing 20μM 2,2-azo-bis-(2-amidinopropano) and 200 μM luminol. The addition of 70 μL of each supernatant (control, 5, 10 and 14 days - 50 mg/mL) decreased the CL to basal levels for a period (induction time) proportional to the antioxidant content of the sample until reaching the CL regeneration level [30, 31]. The system was calibrated with equal concentration of trolox.

2.7. SOD activity

The oxidative stress interference on the SOD activity was determined according to Marklund and Marklund (1974) [32], based on the inhibition of pirogalol autoxidation in acquous

solution. SOD inhibits pirogalol oxidation by catalyzing O_2^- to H_2O dismutation. This oxidation is accompanied by yellow color formation in the reaction medium, monitored at 420 nm. In the reaction, crescent volumes (125, 150, 175 and 200 μL) of contralateral gastrocnemius supernatant are diluted in 100μL of TRIS buffer 1M HCl 5mM EDTA, pH 8.0, and, after that, 15μL of pirogalol is added. The reaction was monitored for 5 minutes, and the absorbance, in the beginning and in the end of the assay, was registered. The auto-oxidation of pirogalol was used as control. The amount of SOD that is able to inhibit pirogalol autoxidation in 50%, is defined as the enzymatic activity unit (U). Final results were expressed in U SOD/ mL .mg protein $^{-1}$. min $^{-1}$

2.8. Glutathione Assay

The levels of reduced glutathione (GSH) were determined by titration with 5,5'-dithio-bis (2-nitrobenzoic acid), evidenced by a yellow color formation. Oxidized glutathione (GSSG) was determined at the same manner, in the supernatant previously incubated with 4-vinylpyridine for 60 min at room temperature according to the method proposed by Tietze (1969) [33]. Volumes of supernatant were adjusted for the assay with muscle homogenate, containing 50mg/mL. The results were expressed in μmol/mg protein. The stress index was calculated, by the equation *[(GSSG/GSH-GSSG) x 100]*.

2.9. Carbonyl proteins content

For access carbonyl proteins content, we used the method described by Reznick and Parker (1994) [26], with adaptations. About 200 mg of contralateral gastrocnemius muscles from control and tumor animals were placed on homogenization glass tubes containing 4 mL of homogenizing buffer (50 mM phosphate buffer, 1 mM EDTA, pH 7.4). Tissues samples were finally homogenized and incubated for 15 min at room temperature. Thus, samples were

centrifuged at 3,000 x g for 10 min at room temperature to remove debris, and 1 mL of each extracted protein was placed on glass tubes. About 4 mL of 2,4- Dinitrophenylhydrazine (DNPH) diluted in 2.5 M HCl solution was added in each tube and left for 1 h of incubation at room temperature, vortexed every 15 min. Then, samples were washed with 5 mL of 20% TCA (w/v) solution and centrifuged for 10 min to collect the protein precipitates. Another wash was performed using 10% TCA, and protein pellets were broken mechanically. Finally, the pellets were washed 3 times with 4 mL of ethanol-ethylacetate (1:1, v/v) to remove free DNPH and lipid contaminants. The final precipitates are dissolved in 2 mL of 0.6 M guanidine hydrocloride solution and any insoluble materials are removed by additional centrifugation. Carbonyl content was calculated by obtaining the peak of absorbance on a spectra at 355-390 nm of the DNPH-treated samples, against samples treated only with 2.5 M HCl. To calculate the concentration of carbonyls, we used the described formula: C = Abs(355-390) x 45.45 nmol/mL, where C is the concentration of DNPH/mL, and 45.45 its absortion coefficient [26]. The procedures were made on ice bath until TCA wash. Results were expressed in nanomoles of carbonyls. mL^{-1}.mg total protein $^{-1}$.

2.10. Protein concentration

Protein was determined by the method of Lowry et al [34], modified by Miller [35], except on protein carbonyl contents, when we used a spectra at 280 nm of each sample to determine total protein content. Both methods used bovine serum albumin (BSA) as standard. A calibration curve to determine the concentration of BSA was made.

2.11. Statistical analysis

The results are shown as means ± SEM of six animals. Data were evaluated using a non-paired Student's t test. Correlation analysis was used to determine V_0. $p < 0,05$ was considered significant.

3. RESULTS

3.1. Characterization of cachexia

Table 1 shows the characterization of cachexia on tumor-bearing animals. Walker-256 tumor leads to a progressive decrease in body weight during the course of 14 days. General body weight loss was accompanied by loss of contralateral gastrocnemius weight (2.9, 12.35 and 15.88% when compared to control). Pair fed group did not present significant differences in muscle and body weight, when compared to control group. Figure 1 demonstrates the rate of muscle mass loss compared with total body mass loss. On 10^{th} day, muscle (12.33 ± 3.00%) represented the major part of general body loss (13.1 ± 1.75%), which was mantained on day 14 (15.88 ± 3.36% and 20.01 ± 3.86%, respectively). We also observed progressive increase in tumor weight and decrease in food intake at the same period, when compared with control group.

3.2. Lipoperoxidative damage

Lipoperoxidation in cell membranes of skeletal muscle was determined by the progressive formation of TBARS (Figure 2) in the reaction mixture, showing a significant increase of approximately 2.5 times on day 10 after tumor inoculation when compared with control group (from 0.391 ± 0.022 on control, to 0.900 ± 0.1 nanomoles MDA/g tissue on Day 10). However, when TBARS was measured in day 14, the levels returned to the ones observed on the control groups (0.468 ± 0.09 nanomoles MDA/g tissue), representing no significant differences.

Tert-butyl hydroperoxide-initiated chemiluminescence was used to analyze the integrity of non-enzymatic antioxidant defenses, and the levels of lipid peroxides in muscle cells of animals exposed to tumor action. This assay indicates that the increase in CL is closely related to

the oxidative stress previously suffered by the tissue, inducing the consumption of antioxidant defenses such as vitamin E and the formation of lipoperoxides resulting in an increase in photon emission [27, 30, 36]. Figure 3 shows a significant increase of total CL on all days of inoculation ($p < 0.001$ for all curves, when compared to control). We found, however, increased values of V_0. This value represents the initial velocity of the reaction, pointing to the concentration of lipoperoxides present on tissue. It was observed to be time-dependent, being more representative on day 10 (58 x 10^{-3} URL/min on control group; ^{3}62.5 x 10^{-3} URL/min on 5th day; 92.5 x 10^{-3}. URL/min on 10th day; and 103 x 10^{-3} URL/min on 14th day after tumour implantation)

3.3. Total antioxidant capacity of the muscle

Figure 4 show the values of total antioxidant capacity (TRAP) of contralateral gastrocnemius muscle of the animals. Control values were 1.217 ± 0.13 μM trolox equivalents; a significant decrease in antioxidant capacity was observed on day 5 ($p<0.001$), which was completely and progressive reversed on days 10 (1.50 ± 0.1 μM trolox) and 14, which showed to be higher than the control levels.

3.3. Antioxidant enzymes

GSH levels were progressive and significantly decreased on day 5, 10 and 14 (51%, 63%, and 68%, respectively, of reduction, when compared to control), which was not accompanied by the increase on GSSG levels. GSSG tended to decrease already on day 5 (10% of reduction), achieving significant difference in day 10 (50% of reduction, $p< 0.05$). On day 14, GSSG returned to level similar to control, but not presented significant difference when compared to control. Yet, stress index (SI), that represents cellular oxidative stress, has demonstrated

significant increased differences, when compared to control, on days 5 and 14 (increasing about 54 and 56%, respectively). Absolute values (in means ± SE) can be seen on Table 2.

Figure 5 shows that the activity of superoxide dismutase was significantly enhanced on days 5 and 10 (from 0.131 ± 0.01 U SOD/mL/mg protein/min on control group, to 0.240 ± 0.03 on day 5, and 0.196 ± 0.01 on day 10), recovering control levels on day 14 (0.108 ± 0.08 U SOD/mL/mg protein/min), showing again activity against oxidative damage on days 5 and 10.

3.4. Protein Carbonylation

For the carbonylated proteins, it is evidenced that only the day 10 there is a significant increase (Figure 6) on carbonyl proteins levels, (approximately 33,3% on day 10), which returns to control levels on day 14. Reflecting, at this point, not only damages to muscle cell lipids membranes, but also to proteins.

4. DISCUSSION

To investigate the role of oxidative stress on gastrocnemius muscle induced by cancer cachexia, we chose an experimental model that has notable cachectic response. Walker-256 tumor is a rat tumor that grows exponentially, becoming ulcerative when implanted subcutaneously. Its growth promotes a survival time about 14±1 days with concomitant reduction of food intake [8, 37]. In addition, the presence of tumor causes rapid and progressive loss of body weight and tissue waste, particularly in skeletal muscle [38]. Body and muscle wasting occurred 5 days after tumour inoculation, where cancer development was evident only after 10 days. The muscle/body rate was 0.27 upon the day 5 and increased to 0.86 and 0.81 on the days 10 and 14 respectively. These data suggest a marked effect of cancer development on muscle damage after 10 days of inoculation. The control group which was carried out with food restriction (*pair fed*) did not induce any significant body weight loss and muscle wasting. Studies using TNF or Walker-256 tumor inducing cachexia, revealed reduction in animal food intake [1,14].

Previous studies have suggested that ROS could have a central role in muscle wasting [1, 14, 15, 23]. Decreased weight and muscle wasting were accompanied by increased MDA levels in skeletal muscle of rats injected with TNFα. These parameters were reverted by α-tocopherol treatment [14]. MDA and 4-hydroxy-2-nonenal (HNE) adducts were found to be increased in gastrocnemius of animals bearing the ascitic form of AH-130 Yoshida hepatoma, when compared to controls [1]. Freitas et al [15], using rats bearing Walker-256 solid tumor, demonstrated enhanced TBARs in some brain regions after 14 days of subcutaneous implantation. It has been demonstrated that twenty days after cachectic tumor MAC16 transplantation in mice or in vitro

C_2C_{12} myotubes treatment with either H_2O_2 or hydroxyl radical generating system, caused a significant rise in the MDA content [12]. In addition, these authors suggested that mild oxidative stress increases protein degradation in skeletal muscle by causing up regulation in the ubiquitin proteasome proteolytic pathway. Therefore, supported by evidences obtained in the present work, we suggest that muscle waste is a result of multifatorial biochemichal alterations, in which oxidative stress has an important involvement. First, by oxidative stress as a mediator of tissue injury, including protein oxidation, and second, by as a modulator of muscle wasting process. To understand these mechanisms, the oxidative stress injury in muscle lipid was evaluated by a very sensitive chemiluminescence method besides MDA and carbonylated protein measurements. Additionally, different antioxidant systems were measured in several time after tumor implantation. Our results showed an incresing tendency in the MDA levels on 5^{th} day after tumor implantation (figure 2), reaching higher concentration on 10^{th} day, declining afterwards to control levels on 14^{th} day. These data suggest that lipid peroxidation is a continuing event during the muscle wasting process, and are coincident with the maximal muscle wasting/body mass rate, at the day 10 after inoculation. Besides of a *tert*-butil hydroperoxide-initiated chemiluminescence curve measurements, we carried out a kinetic analysis of the ascending part of the CL curves as an indicator of membrane lipid peroxidation (figure 3) [27, 28, 36]. Zamburlini et al [39] used purified lipid hydroperoxides or plasma LDL lipid hydroperoxides and found that emission obtained was proportional to the lipid hydroperoxide content of the sample. The relationship between chemiluminescence and tissue damage has been previously demonstrated [20, 21, 27, 36, 40]. In the present study, using contralateral gastrocnemius from rats hindlimbs subjected to tumor implantation, the total analysis of the CL curve revealed that the higher emission levels was achieved as early as 5 days after inoculation, maintaining significantly high until the day 14. These results show a fast consumption of non soluble antioxidant defense before significant

levels of lipid peroxides levels have been attained. Additionally, the V_0 values were elevated only in the 10th after the tumor implantation. (figure 3). These results reveal a massive free radical attack mainly on the cells membrane, promoting increased levels of tissue lipid peroxides, which agree with the maximal MDA levels and also, with wasting muscle/body mass rate found on the 10th day. Considering that: (1) the fact that proteins may be attacked whenever free radicals are generated, and, as a consequence, oxidative modification of proteins may occur by reaction with diverse primary radicals as •OH or secondary radicals as alkylperoxyl or low molecular aldehydes as MDA and HNE [41-43] and (2) that, the increased susceptibility of oxidized proteins to undergo proteolytic degradation [44, 45], we decided to detect whether carbonyl groups were increased in the cachectic muscles. Total protein carbonyl group showed the same profile of MDA and CL curve during the 14 days of tumor development. The more strike similarity occurs in the 10th day after the tumor implantation, showing higher values for all parameters analyzed. Since our results demonstrated increased levels of carbonylated proteins on day 10 with the concomitant increase on MDA levels, higher V_0 and wasting muscle values we can suggest that besides of clear participation in tissue injury, the oxidative stress should also be involved in the regulation of the proteolytic system that results in accelerated muscle proteolysis, which is the tissue that defines the cachectic state and considered to be the first cells that are subjected to metabolic alterations, specially by the activation of ubiquitin-protesome pathway, possibly through free radical production, which in turns produces more free radical, originating a vicious cycle.

The profile of the three antioxidants systems investigated in the muscle of cachectic rats was significantly different with respect to control muscle. Thus, SOD and the stress index raised significantly 5 days after tumor inoculation. On the same time, there were a marked reduction in

the total antioxidant capacity and GSH levels. The stress index is reduced in the day 10, although SOD activity remains significantly high. The TRAP returned to control levels in this time. The higher levels of antioxidant observed at the 5^{th} day with decrease to 10^{th} day of implantation agree with the onset of wasting antioxidant defense before oxidative injury has been established. Augmented SOD was previously found in brain of rats bearing Walker-256 tumor 14 days after tumor inoculation [15]. Similarly, Lawler et al. [46] found increased of SOD activity in soleus muscle subjected to immobilization for 28 days.

In conclusion, this study provides important evidences of the existence of a clear association between experimental cancer-induced cachexia in skeletal muscle, lipid peroxidative, and massive protein damages. The levels of the major antioxidant mechanisms were explored, showing activity and consumption in all of them. This indicates that the damage has different patterns of lesion during disease progression. Cancer cachexia is a severe debilitating disorder for which there are currently few therapeutic options. Over the past few years, basic science advances have begun to reveal the role of oxidative stress on the progression of cancer cachexia, and the knowledge of this pathway gives an insight to the improvement of therapeutics strategies. Further studies are needed to clarify and define the role of ROS and its part on this syndrome and, fortunately, leading to new treatment strategies, possibly involving modulation of the effects of ROS molecules on host metabolism.

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Table 1. Progressive body and muscle weight loss in tumor induced cachexia.

	Control	Pair-fed	Day 5	Day 10	Day 14
Food Intake (% of mean)*	-	-	11.77	28.87	45.02
Tumour weight (mg)	-	-	3.1 ± 0.7	14.58 ± 0.9	25.26 ± 2.1
Loss of body weight (%)*	-	10.1 ± 1.55	10.55	14.27	19.49
Gastrocnemius weight (mg)	1012.5 ± 28.9	1074.17 ± 46.36	983.33 ± 5.4	887.5 ± 74.5	851.67 ± 2.1
%	-	+5.74	-2.9	-12.3	-15.9
% muscle mass loss/%body mass loss ratio	-	-0.56	0.27	0.86	0.81

***** Percent of reduction compared to control (100%). Groups represent number of days after subcoutaneous injection of 8.0 x10^7 tumor cells. Each group consisted of 6 animals. Controls received an injection of 0.5 mL of PBS only.Values are expressed as mean ± SE or in % when specified.

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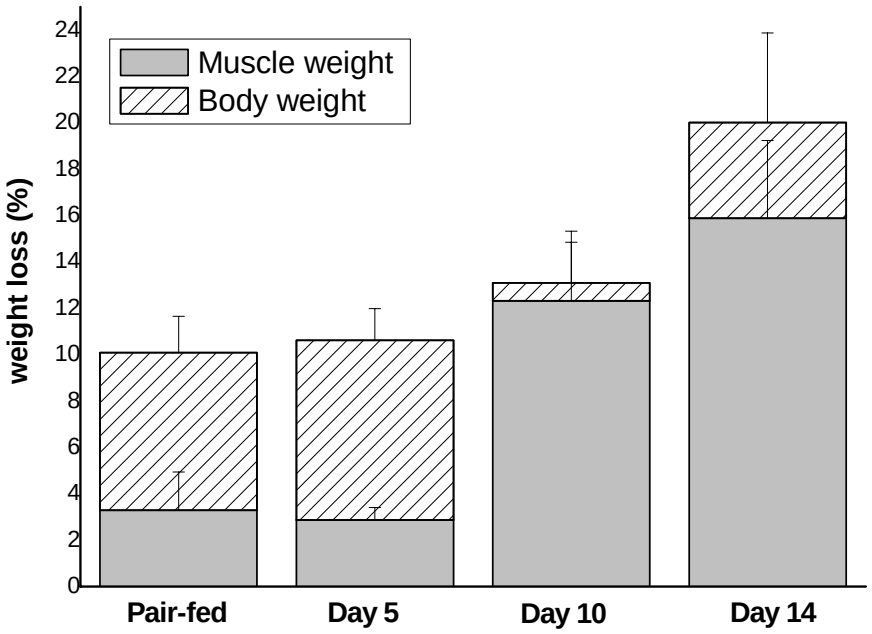

Figure 1. Relation between muscle mass loss (solid barr) and body mass loss (striped barr) when compared with control groups (100%) on the tumor progression of rats implanted with the solid form of Walker-256 tumor (5, 10 and 14 days after innoculation). Both barrs starts on graph basis.

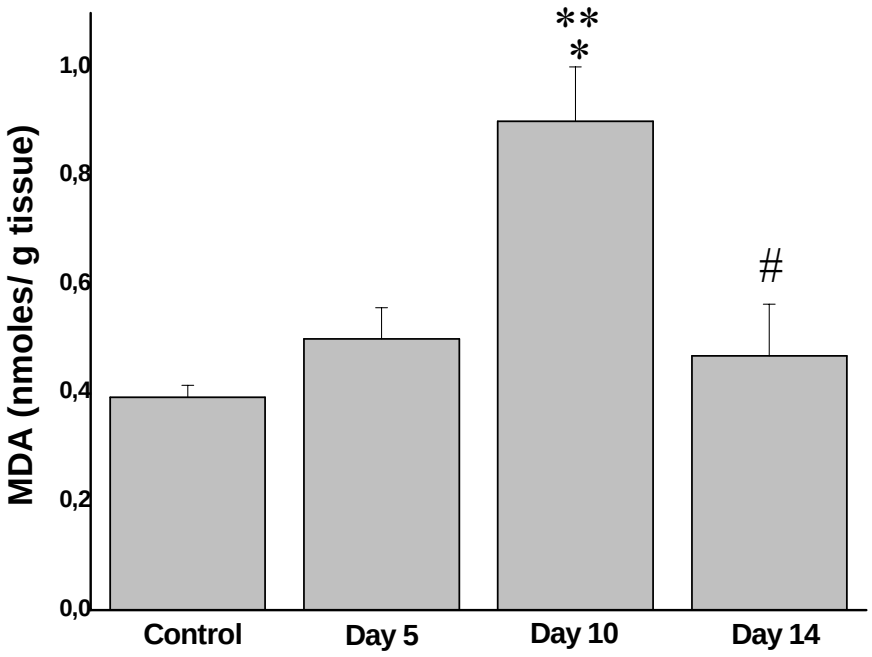

Figure 2. TBARS levels in contralateral gastrocnemius muscle homogenates of rats inoculated with Walker-256 tumor cells. The values above are expressed as mean ± SE of 6 different animals. * $p< 0.05$, when compared with Control Group; ** $p<0.05$, when compared with Day 5; and # $p<0.05$, when related with Day 10.

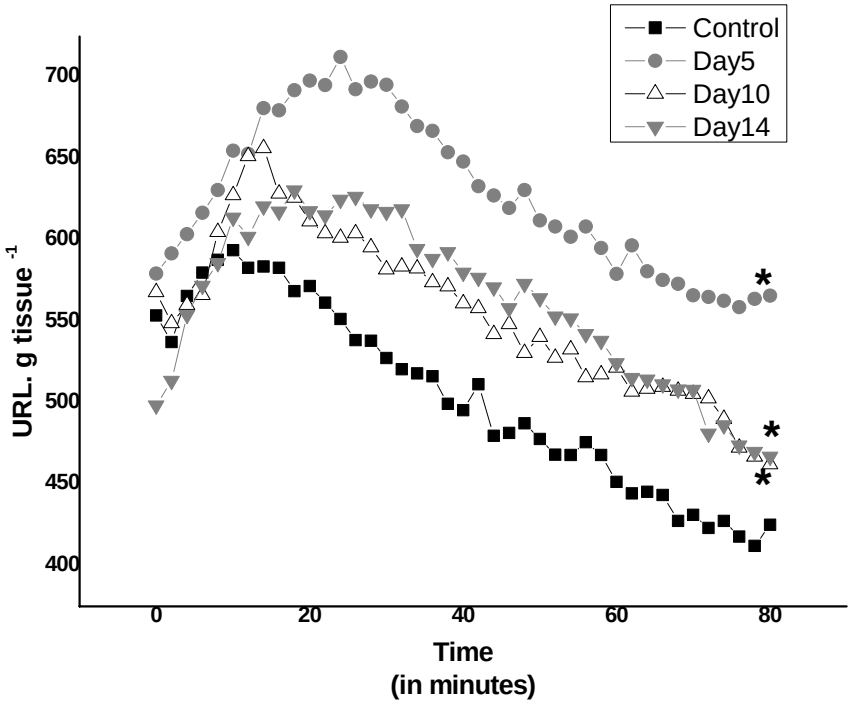

Figure 3. Effect of cachexia in contralateral gastrocnemius muscle of control and cachexia-induced rats on the time-course of hydroperoxide-initiated chemiluminescence. Curves represent means of 6 animals curves. For each animal 80 min curve, a 40 point curve was extracted. Means were compared by a paired Student's t test. * $p < 0.001$, when compared with Control group.

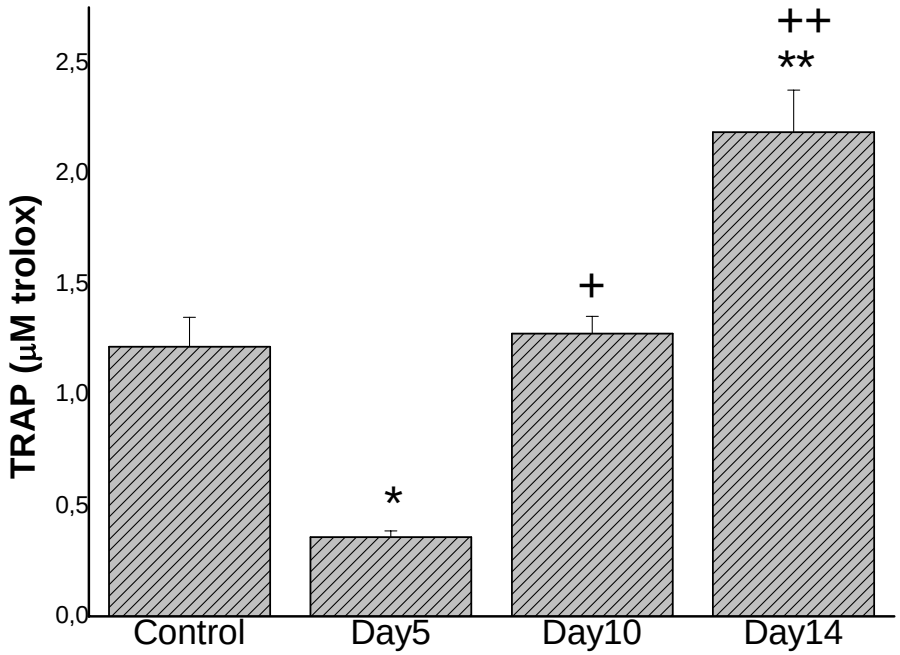

Figure 4. Effect of Walker-256 tumor inoculation on total antioxidant capacity (TRAP) in homogenate supernatant of rat muscles. Groups were compared by a paired Student's *t* test. Values are expressed as mean ± SE of 6 animals. * $p<$ 0.001, when compared with Control Group; $^{**}p<0.01$, when compared with Control Group; $^{+}p<0.001$, when compared with Day 5; and $^{++}$p< 0,001 when compared with Day 10 . Results are expressed in TRAP (µM trolóx).

Table 2. Levels of glutathione in sham and contralateral gastrocnemius of rats inoculated with Walker-256 tumor in different times.

Groups	GSH	GSSG	SI
Control	4.040±0.1	0.345±0.04	8.71±2.7
Day 5	1.965±0.2[a]	0.310±0. 2	18.98±1.9[b]
Day 10	1.490±0.1[a]	0.172±0.02[b]	11.94±1.0
Day 14	1.267±0.05[a,c]	0.378±0.03[c]	20.03±2.5[b,c]

GSH - Levels of Reduced Glutathione, GSSG - Levels of Oxidized Glutathione, and SI-Stress Index [(GSSG/GSH-GSSG)x100]. Values represent mean ± SE of 6 animals. p values, as determined by t-test (two populations) are: [a] $p < 0.001$, when compared to Control Group; [b] $p<0.05$, when compared to Control Group; and [c] $p<0.05$, when compared to Day 10. Results are expressed in μM / mg protein.

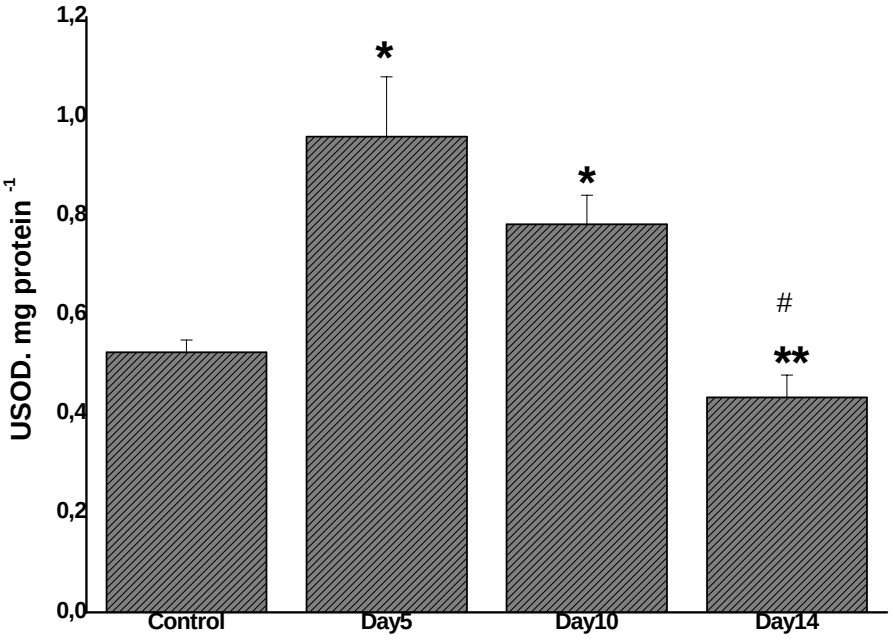

Figure 5. SOD activity in supernatant from contralateral gastrocnemius muscles of the control and tumour-bearing rats**.** These results represents mean ± SE of 6 animals**.** *p* values, as determined by a non-paired *t*-test (two populations) are: * $p< 0.05$, when related with Control Group; ** $p<0.05$, when compared with Day 5; and # $p<0.05$, when compared with Day 10.

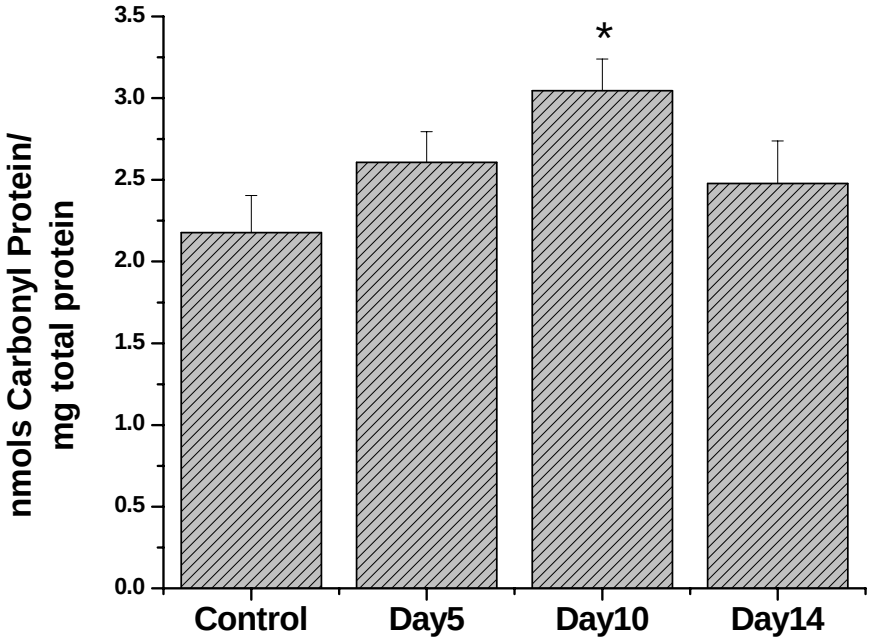

Figure 6. Levels of protein carbonylation on homogenates from contralateral gastrocnemius muscles of control and tumour-bearing rats. These results represent means ± SE of 6 animals**.** *p* value, as determined by t-test (two populations) is: * $p < 0.05$, when compared to Control Group.