SARA SANTOS BERNARDES

LESÃO OXIDATIVA NO MÚSCULO ESQUELÉTICO DE RATOS COM TIREOTOXICOSE SEVERA EXPERIMENTAL

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

Orientador: Profª. Drª. Alessandra Lourenço Cecchini Armani

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Londrina, 29 de outubro de 2010.

DEDICATÓRIA

A **Deus**, por sempre estar ao meu lado e me guiar até aqui.

Aos meus pais, Adelino e Maria, e ao meu esposo, Silvio, que sempre acreditaram nos meus sonhos e deram suporte para minha formação pessoal e intelectual.

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Se as coisas são inatingíveis... ora!
Não é motivo para não querê-las...
Que tristes os caminhos, se não fora
a presença distante das estrelas!

Mário Quintana

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BERNARDES, Sara Santos. **Lesão oxidative no músculo esquelético de ratos com tireotoxicose severa experimental.** 2010. 68f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2010.

RESUMO

Miopatia é uma manifestação inicial freqüente na tireotoxicose, caracteriza por diminuição da massa muscular, perda de força e fraqueza. O estado hipermetabólico na tireotoxicose acelera a produção de espécies reativas de oxigênio (EROs) na mitocôndria e induz mudanças na defesa antioxidante de diversos tecidos, inclusive no músculo esquelético. Apesar dessa relação, a participação de EROs na perda de massa muscular na tireotoxicose ainda não é conhecida. O objetivo desse trabalho foi avaliar a participação das EROs nas alterações musculares esqueléticas observadas na tireotoxicose severa experimental. Para isso, foram realizados dois grupos experimentais de ratos Wistar machos tratados com triiodotironina (T3), durante 3 ou 5 dias, e dois grupos experimentais tratados com T3 e alfa-tocoferol, um agente antioxidante de membrana, durante os mesmos períodos. Todos os grupos experimentais foram comparados com seu respectivo par controle. Nosso estudo mostrou que ocorre estresse oxidativo no músculo *gastrocnemius* de ratos com tireotoxicose severa experimental tratados durante 3 ou 5 dias com a T3. As EROs mostraram-se envolvidas na ativação de vias de perda de massa muscular, evidenciado pelo aumento do conteúdo de tirosina intracelular observado 5° dia. O antioxidante alfa-tocoferol melhorou os parâmetros oxidativos no músculo *gastrocnemius,* principalmente no dia 3, onde o estresse oxidativo foi mais evidente, e diminuiu a atividade hipermetabólica mitocondrial e a temperatura interna nos grupos experimentais com tireotoxicose severa. Além disso, a melhora dos parâmetros oxidativos no 3° dia protegeu parcialmente a perda de massa muscular observada no 5° dia de tratamento com a T3. Os resultados encontrados nesse trabalho sugerem que o alfa-tocoferol retarda a ativação de vias de perda de massa muscular na tireotoxicose, protegendo o músculo esquelético, provavelmente por desviar o catabolismo para a lipólise. Esses achados mostram uma participação ativa das EROs nas alterações musculares observadas na tireotoxicose.

Palavras-Chave: Triiodotironina. Tireotoxicose. Massa muscular. Alfa-tocoferol.

BERNARDES, Sara Santos. **Oxidative skeletal muscle injury in rats with experimental severe thyrotoxicosis.** 2010. 68f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2010.

ABSTRACT

Myopathy is an early manifestation of thyrotoxicosis. The hypermetabolic state in thyrotoxicosis accelerates ROS production in the mitochondria and induces changes in the antioxidant defenses in skeletal muscle, but the participation of ROS in muscle wasting loss is still unclear. The aim of the work is to comprehend the participation of ROS in the muscle alterations in rats with experimental severe thyrotoxicosis. For that, alpha-tocopherol (α-T) was administered. The experimental model consisted of two groups of rats treated with hormone triiodotironina (HT) for 3 and 5 consecutive days and two groups of rats that where treated also with α-T. Our study shows that oxidative stress is present in the skeletal muscle on 3 and 5 days hormone treatment. ROS can be involved in the activation of muscle wasting pathways, evidenced by the increase of intracellular tyrosine content by 5 days HT. α-T ameliorate antioxidant parameters, mainly on day 3, decrease mitochondrial respiration and internal temperature in HT α-T groups, protecting partially *gastrocnemius* mass loss observed on day 5. We suggest that, α-T slows down the skeletal muscle wasting pathways in thyrotoxicosis, protecting skeletal muscle mass loss, diverting catabolism to lipolysis. These findings show an active participation of reactive oxygen species on muscular alterations observed in thyrotoxicosis.

Keywords: Triiodothyronine. Thyrotoxicosis. Skeletal muscle. Alpha-tocopherol.

SUMÁRIO

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1 INTRODUÇÃO

1.1 GLÂNDULA TIREÓIDE E HORMÔNIOS TIREOIDIANOS

A importância dos hormônios tireoidianos no desenvolvimento, homeostase, proliferação e diferenciação celular tem sido bem documentada. Sabe-se que, nos mamíferos eles atuam em todos os órgãos e vias metabólicas, e seus principais efeitos incluem o desenvolvimento de vários tecidos, como o do sistema nervoso central, o consumo de oxigênio, a regulação da temperatura corporal, a freqüência cardíaca e também o metabolismo de carboidratos, proteínas e lipídeos. Além disso, participam da síntese e da degradação de fatores de crescimento e hormônios, resultando em outros efeitos secundários (RIBEIRO et al., 1995). Em adultos, a glândula tireóide normal é constituída de dois lobos castanhos claros conectados por um istmo, envoltos por uma cápsula fibrosa, pesando entre 15 e 25 gramas, localizada abaixo e anteriormente a laringe (LIVOLSI, 2002; MAITRA; ABBAS, 2005).

No exame microscópico, a tireóide é dividida em lóbulos compostos de 20 a 40 folículos uniformemente dispersos, que são as unidades funcionais da glândula. Os folículos podem ser uniformes ou variáveis em relação ao tamanho, sendo revestidos de epitélio colunar cubóide e envoltos por uma extensa rede capilar, fazendo com que o fluxo sanguíneo seja bastante intenso em comparação a outros tecidos (LIVOLSI, 2002). Os folículos são repletos de um colóide espesso contendo tireoglobulina, uma glicoproteína com cerca de 115 resíduos de tirosina, que é sintetizada, glicosilada e secretada na luz do folículo, onde sofre iodação dos resíduos de tirosina, dando origem aos hormônios tireoidianos (COOPER; GREENSPAN; LADENSON, 2007).

A síntese de 3,5,3',5'-tetraiodo-L-tironina (L-tiroxina ou T_4), e 3,5,3' triiodo-L-tironina (triiodotironina ou T_3) - os principais hormônios tireoidianos - são dependentes de iodo e do estímulo do hormônio tireoestimulante (tireotropina ou TSH). A liberação de TSH pela hipófise é estimulada pelo hormônio de liberação de tireotrofina (TRH), secretado pelo hipotálamo em resposta a diversos estímulos (COOPER; GREENSPAN; LADENSON, 2007). O TSH atua em receptores de

membrana dos folículos tireoidianos através de mecanismos que envolvem a adenosina monofosfato cíclica (AMPc) e a fosfaditilinositol-3-quinase, controlando todos os aspectos da síntese de hormônios tireoidianos, como estímulo da transcrição dos genes do transportador de iodeto, síntese e secreção de tireoglobulina, geração de peróxido de hidrogênio (H_2O_2), iodação da tirosina, fluxo sanguíneo glandular e a secreção de T_3 e T_4 (MAITRA; ABBAS, 2005). A primeira etapa da síntese desses hormônios é a captação do iodeto plasmático pela glândula, que dentro dos folículos é oxidado a iodo pelo H_2O_2 antes de se ligar a posição 3 dos resíduos tirosil da tireoglobulina, gerando a 3-monoiodotirosina (MIT). A iodação subseqüente da posição 5 do resíduo tirosil da MIT leva a formação da 3,5-diiodotirosina (DIT). Essas reações são catalisadas pela enzima tireoperoxidase (TPO) (AZEVEDO et al., 2005; KELLY, 2000). A T_4 é originada pelo acoplamento de 2 móleculas de DIT, e é sintetizada em maior quantidade, enquanto a T_3 é formada pela junção de 1 molécula de DIT e 1 molécula de MIT (Figura 1).

Em condições normais, todo T_4 e aproximadamente 20% da T_3 circulantes são produzidos diretamente pela tireóide. Ao sair da glândula, esses hormônios são conjugados às proteínas plasmáticas globulina ligadora de tiroxina (TBG), que corresponde a 70% das ligações plasmáticas da T_4 e 80% da T_3, transtirretina (TTR) e albumina (AZEVEDO et al., 2005). Acredita-se que apenas as frações livres da T_4 e T_3 são capazes de se ligar aos receptores específicos nos tecidos periféricos, sendo que normalmente cerca de 0,04% da T_4 e 0,4% da T_3 circulam livres no sangue (COOPER; GREENSPAN; LADENSON, 2007).

Cerca de 80% da T_3 circulante provém da desiodinação periférica do anel fenólico da T_4, particularmente no fígado, rins e músculo esquelético, através da ação das enzimas 5'-desiodinase do tipo 1 (D1), que é selênio dependente, e do tipo 2 (D2). Existe ainda a 5'-desiodinase tipo 3 (D3), que tem a expressão gênica aumentada pelo excesso de hormônios tireoidianos, atuando por *feedback* negativo na homeostasia dos mesmos, uma vez que converte T_4 a T_3 reversa (rT_3) e T_3 a 3,3'-diiodotironina, um subtipo de T_2, ambos metabolicamente inativos (BIANCO et al., 2002; COOPER; GREENSPAN; LADENSON, 2007; KELLY, 2000). D1 e D2 são expressas em diversos tecidos. A D1 é altamente expressa no fígado e rins, e em menores quantidades no músculo cardíaco, esquelético e outros tecidos, sendo responsável pela manutenção dos níveis séricos de T_3. A D2 é encontrada no cérebro e na glândula pituitária, garantindo níveis intracelulares ideais de T_3 nas

células neuronais (COOPER; GREENSPAN; LADENSON, 2007). A ação biológica dos hormônios tireoidianos se dá em sua maioria pela ação da T_3. Nos tecidos periféricos a T_4 é desiodinada a T_3, que se liga a receptores nucleares do hormônio tireoidiano (TR) das células alvo com uma afinidade dez vezes maior do que a T_4, apresentando uma atividade proporcionalmente maior (MAITRA; ABBAS, 2005; LIVOLSI, 2002; COOPER; GREENSPAN; LADENSON, 2007).

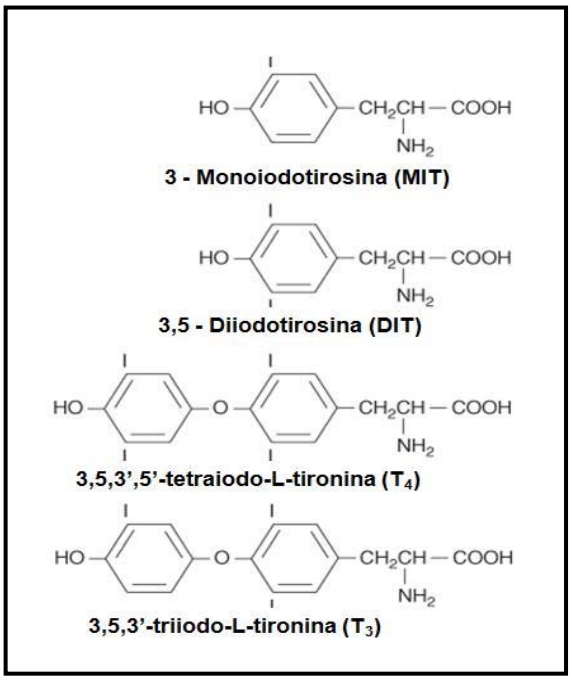

Figura 1 – Estrutura química das moléculas de 3,5,3',5'-tetraiodo-L-tironina (T_4) e 3,5,3'-triiodo-L-tironina (T_3).
A molécula de T_4 é formada pelo acoplamento de duas moléculas de 3,5-diiodotirosina (DIT), enquanto a T_3 é originada do acoplamento de uma molécula de uma molécula de 3-monoiodotirosina (MIT) e outra de DIT.

1.1.1 Mecanismos de Ação dos Hormônios Tireoidianos

Os hormônios tireoidianos possuem dois mecanismos básicos de ação: (1) genômica, através da interação da T_3 com receptores tireoidianos nucleares (TR) e (2) ação não genômica, mediada pela interação com receptores de superfície e citoplasmáticos, podendo ou não envolver resposta nuclear (COOPER; GREENSPAN; LADENSON, 2007; DAVIS; LEONARD; DAVIS, 2008).

Os TRs pertencem a superfamília de receptores nucleares, que inclui os receptores para glicocorticóides, mineralocorticóides, estrogênios,

progesterona, vitamina D e retinóides. Possuem um gene localizado no cromossomo 17 (TRα) e outro no cromossomo 3 (TRβ), que geram dois subtipos de receptores cada, distribuídos diferentemente nos tecidos (ZHANG; LAZAR, 2000, COOPER; GREENSPAN; LADENSON, 2007).

Esses receptores encontram-se ligados aos genes-alvo em regiões específicas do DNA chamadas TREs (elementos responsivos ao hormônio tireoidiano). Quando a T_3 une-se ao receptor nuclear ocorre uma modificação conformacional, remoção de proteínas co-repressoras, recrutamento de proteínas co-ativadoras e proteínas acessórias, induzindo a transcrição gênica (YEN, 2001; FLAMANT; GAUTHIER; SAMARUT, 2007). Na ausência do ligante, os TRs ligam-se ao DNA (ácido desoxirribonucleico) na forma de homo ou heterodímeros, que por sua vez, se associam a outras proteínas conhecidas como co-repressoras, e atuam reprimindo a transcrição gênica. Com a ligação do hormônio ao receptor, observa-se formação predominante de heterodímeros com o receptor de ácido retinóico X (RXR), seguida de dissociação das proteínas co-repressoras e associação com proteínas co-ativadoras, que estimulam a transcrição dos genes alvo (YEN, 2001; ZHANG; LAZAR, 2000).

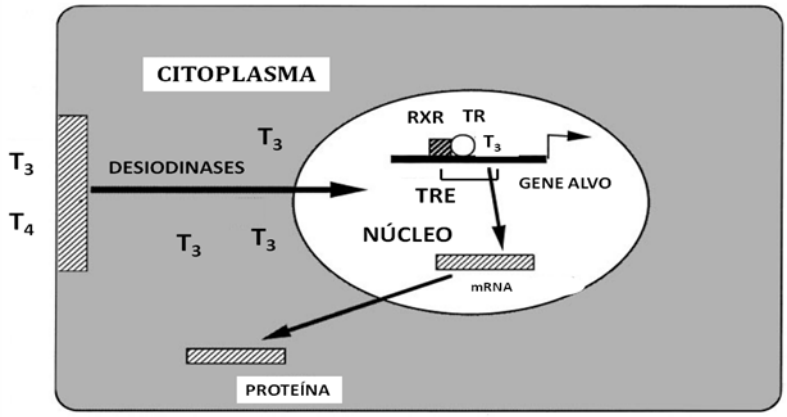

Figura 2 – Mecanismo básico de ação genômica dos hormônios tireoidianos.
Na presença de T_3, o receptor para o hormônio tireoidiano (TR) forma um heterodímero com o receptor de ácido retinóico X (RXR), que se liga a seqüências específicas do DNA (TRE). O complexo formado por TR-RXR-TRE interage com proteínas co-ativadoras e dissocia as co-repressoras, modificando a expressão gênica. Adaptado de Yen (2001).

Os efeitos genômicos dos hormônios tireoidianos tem uma latência de horas a dias. Entretanto, alguns efeitos fisiológicos da T_4 e T_3 ocorrem rapidamente e são chamados de não genômicos, apesar de poderem culminar em ativação nuclear e eventos celulares (DAVIS; LEONARD; DAVIS, 2008; HIROI; KIM;

YING, 2006). Essa via requer receptores de membrana plasmática, sendo a integrina $\alpha V\beta 3$ a mais estudada, ou receptores nucleares localizados no citoplasma, como o TRα1 e TRβ1, que podem apresentar as mesmas vias de sinalização da sua forma nuclear, porém diferentes respostas efetoras (DAVIS; LEONARD; DAVIS, 2008; MOELLER et al., 2006)**.**

As ações não genômicas dos hormônios tireoidianos incluem basicamente a modulação do tráfico de proteínas intracelulares e ação sobre canais e transportadores de membrana. Os hormônios tireoidianos podem interferir na atividade da bomba de Na^+/H^+, na inserção do canal de Na/K-ATPase na membrana e no aumento da entrada de glicose nas células de diversos tecidos, através da ação sobre transportadores de glicose de membrana (DAVIS; LEONARD; DAVIS, 2008; LEI et al., 2003; YEN, 2001). É bem descrito também a ação desses hormônios - principalmente da T_4 - sobre a motilidade celular de astrócitos, através da conversão da actina solúvel em actina fibrosa (FARWELL; TRANTER; LEONARD 1995; FARWELL; DUBORD-TOMASETTI, 1999). Receptores para hormônios tireoidianos também são encontrados nas mitocôndrias, levando principalmente a ativação e biossíntese de enzimas da fosforilação oxidativa e outras moléculas que atuam na cadeia respiratória (GOGLIA MORENO; LANNI, 1999; PSARRA; SEKERIS, 2008). Ainda na mitocôndria, a T_3 e a 3,5-diiodotironina, um subtipo de T_2 gerada perifericamente, atuam de modo não genômico, através da ativação alostérica da citocromo c oxidase (ARNOLD; GOGLIA; KADENBACH, 1998). É relatado na literatura que os hormônios tireoidianos aumentam a expressão gênica das proteínas desacopladoras mitocondriais UCP-2 (*uncoupling protein 2* ou proteína desacopladora 2) e UCP-3 (*uncoupling protein 3* ou proteína desacopladora 3) no músculo esquelético e no fígado, entretanto seu papel no desacoplamento de prótons e liberação de calor não é bem conhecido (HARPER; SEIFERT, 2008).

Em níveis normais, a T_3 e T_4 levam a ativação da biossíntese de proteínas através da ligação aos receptores nucleares específicos, a regulação da calorigênese e da temperatura corporal, e possuem importante participação no metabolismo de carboidratos, lipídeos e proteínas. Ocorre também a promoção do crescimento, da diferenciação e da maturação dos tecidos, sendo de extrema importância no desenvolvimento do sistema nervoso central intra-útero (YEN, 2001; COOPER; GREENSPAN; LADENSON, 2007).

1.1.2 Tireotoxicose

O termo hipertireoidismo ou tireotoxicose é usado para definir o excesso de hormônios tireoidianos livres na circulação sanguínea. Uma vez que nem todo excesso de hormônios tireoidianos na circulação é causado por uma hiperfunção da glândula tireóide, alguns autores definem o termo tireotoxicose como o mais correto (MITRA et al., 2008; MAITRA; ABBAS, 2005). Os hormônios tireoidianos em sua forma livre podem estar aumentados na periferia devido a diversas patologias, sendo a doença de Graves, o bócio nodular tóxico e adenoma hiperfuncionante da tireóide as causas mais comuns (AZEVEDO et al., 2005; MAITRA; ABBAS, 2005). Fontes exógenas de hormônios tireoidianos, uso de medicamentos que podem interferir na atividade da glândula tireóide (como carbonato de lítio e interferon-α), tireoidite subaguda e tumores hipofisários também podem ser observados em quadros clínicos de tireotoxicose (MITRA et al., 2008; COOPER; GREENSPAN; LADENSON, 2007).

Em excesso, esses hormônios provocam ansiedade, taquicardia, diarréia, perda de peso, fome demasiada, suor excessivo, tremores e fraqueza muscular. Metabolicamente, são hiperglicemiantes por aumentarem a gliconeogênese e glicogenólise hepática, bem como a absorção intestinal de glicose. No metabolismo lipídico, promove a lipólise no plasma e a síntese e degradação de colesterol. O excesso de hormônios tireoidianos também aumenta o *turnover* e perda de proteínas no músculo esquelético, que pode levar ao desenvolvimento de miopatia proximal (CARTER; BENJAMIN; FAAS, 1981; COOPER; GREENSPAN; LADENSON, 2007).

Tecidualmente, o excesso de T_3 e T_4 afeta principalmente as funções normais do coração, fígado e do sistema neuromuscular (BAYRAKTAR; VAN THIEL, 1997; MAITRA; ABBAS, 2005; RAMSAY, 1966). Os efeitos cardiovasculares são devido ao aumento da demanda circulatória por causa do hipermetabolismo e necessidade de dissipação de calor (DAVIES; LARSEN, 2003). Ocorre aumento da contratilidade cardíaca e da necessidade de oxigênio, aumentando o débito cardíaco e levando a taquicardia, arritmias, palpitações e cardiomegalia (MAITRA; ABBAS, 2005). No fígado, o excesso de hormônios tireoidianos altera o metabolismo de lipídeos, carboidratos e proteínas, como já mencionado. Também podem ser

encontradas uma série de alterações histológicas, como esteatose, degeneração hidrópica, hiperplasia das células de Kupffer, depleção de glicogênio nos hepatócitos, pequenos focos de necrose, infiltrado mononuclear, congestão passiva, áreas extensas de necrose - na tireotoxicose grave - e alterações crônicas que variam de fibrose portal leve até cirrose franca (FONG; MCHUTCHISON; REYNOLDS, 1992; MAITRA; ABBAS, 2005). No sistema neuromuscular, ocorre hiperatividade do sistema nervoso simpático, gerando tremores, ansiedade, dificuldade de concentração e insônia. Já no músculo esquelético, sendo mais evidente nos músculos proximais, ocorre fraqueza muscular em casos leves e miopatia tireotóxica em casos moderados a graves, com proteólise e consequente perda de massa muscular, podendo ser encontrado ao exame microscópico infiltrações localizadas de linfócitos e de gordura (DAVIES; LARSEN, 2003; O'NEAL et al., 2009; MAITRA; ABBAS, 2005).

Em um estudo onde foi induzido tireotoxicose leve em humanos, Riis e colaboradores (2008) observaram que o metabolismo de proteínas foi o primeiro a ser alterado, antes mesmo de ocorrerem mudanças no gasto de energia ou no metabolismo de açúcares e gorduras. De todos os hormônios, os tireoidianos parecem exercer mais efeitos sobre o fenótipo de fibras musculares (PETTE; STARON, 2000). Em ratos e humanos, ocorre diminuição de fibras lentas (tipo I ou vermelhas) e aumento de enzimas que atuam na via glicolítica (NICOL; BRUCE, 1981; CAPÓ; SILLAU, 1983; CELSING et al., 1985). Em ratos, as fibras oxidativas lentas e fibras glicolíticas rápidas (tipo IIb ou brancas) se convertem em fibras oxidativas glicolíticas rápidas (tipo IIa ou intermediárias brancas) (NICOL; BRUCE, 1981; CAPÓ; SILLAU, 1983). Essas alterações condizem com os achados clínicos encontrados no sistema muscular esquelético, uma vez que a perda de fibras tipo I leva a uma menor produção de ATP, contribuindo para que a contração muscular seja menos efetiva (SANTOS et al., 2002). A força muscular retorna ao normal quando a homeostasia é restaurada, porém a massa muscular leva mais tempo para ser recuperada (DAVIES; LARSEN, 2003; NORRELUND et al., 1999).

1.2 Estresse Oxidativo e Hormônios Tireoidianos

Radicais livres podem ser definidos como fragmentos moleculares com capacidade oxidante, contendo um ou mais elétrons desemparelhados em seu orbital atômico ou molecular, o que lhes confere alta reatividade (HALLIWELL; GUTTERIDGE, 2007). Basicamente, os radicais livres são constituídos de espécies reativas de oxigênio (EROs), e de espécies reativas de nitrogênio (ERNs). As EROs/ERNs podem ter tanto origem endógena, como por exemplo subprodutos do metabolismo aeróbio, quanto exógena, como toxicantes ambientais.

Em condições fisiológicas os radicais livres são neutralizados por substâncias antioxidantes, garantindo a homeostasia. Segundo Halliwell e Gutteridge (2007), antioxidante é qualquer substância que atrasa, previne ou remove o dano oxidativo a uma molécula alvo. Esta definição inclui compostos de natureza enzimática e não enzimática, como as enzimas superóxido dismutase, catalase e do sistema glutationa, substâncias queladoras de metais, que previnem o desencadeamento de reações oxidativas dependentes de ferro e cobre, e compostos fenólicos que atuam como removedores de radicais livres. Flutuações dos níveis de radicais livres possuem importante função regulatória, porém quando os níveis se encontram altos e/ou sustentados, os radicais livres podem causar diversos danos ao DNA, lipídeos e proteínas, levando a uma situação chamada de estresse oxidativo, que pode resultar de um aumento de agentes oxidantes ou de uma diminuição das defesas antioxidantes (HALLIWELL; GUTTERIDGE, 2007; SIES, 1997).

As EROs formadas endogenamente são em sua maior parte derivadas da mitocôndria, destacando-se também a produção pela enzima xantina-oxidase (FINKEL; HOOLBROOK, 2000; RAHA; ROBINSON, 2000). A maioria do oxigênio consumido pelos organismos aeróbicos é reduzido à água pela enzima citocromo c oxidase na reação terminal da cadeia respiratória mitocondrial. No entanto, uma pequena parte das moléculas de oxigênio (1-2%) são convertidas em radical superóxido ($O_2^{\bullet -}$) por outros componentes da cadeia respiratória, principalmente o Complexo I – NADH desidrogenase e o complexo III – ubisemiquinona (UQ10), sendo o último uma fonte constante de geração de $O_2^{\bullet -}$ (RAHA; ROBINSON, 2000; TURRENS, 1997).

O aumento do $O_2^{\bullet-}$ produzido é acompanhado do aumento de enzimas que participam de sua degradação e metabolismo, como a superóxido dismutase, catalase e glutationa peroxidase (FINKEL; HOOLBROOK, 2000). A enzima superóxido dismutase converte o $O_2^{\bullet-}$ em H_2O_2, que por sua vez é degradado em água pelas enzimas catalase e glutationa peroxidase. O excesso de $O_2^{\bullet-}$ provoca a liberação de íons ferro de moléculas que possuem sítios ferro-sulfúricos, como a aconitase, a succinato desidrogenase e a NADH-ubiquinona oxiredutase (RAHA; ROBINSON, 2000), e esse ferro livre participa da conversão do H_2O_2 em radical hidroxil ($^{\bullet}OH$) através da reação de Fenton ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$). As etapas descritas encontram-se ilustradas abaixo:

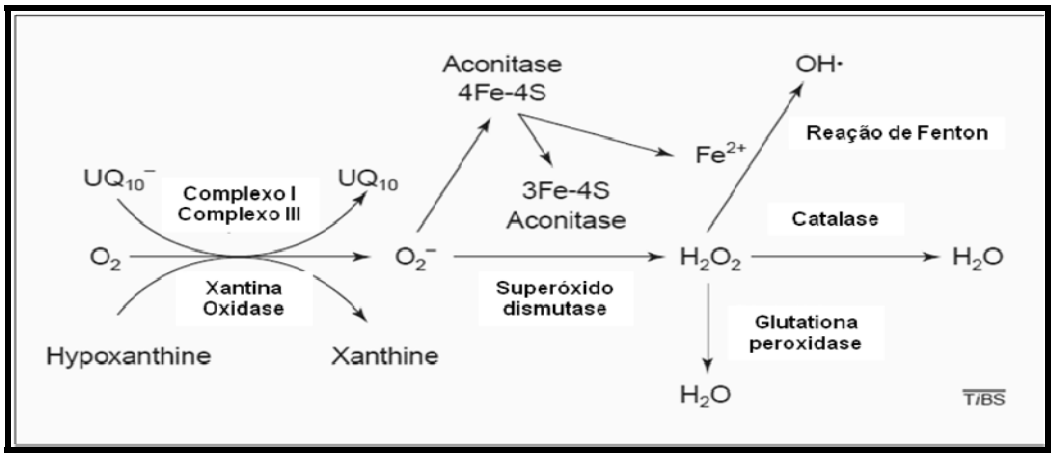

Figura 3 – Fontes endógenas de produção de espécies reativas de oxigênio (EROs). O radical superóxido ($O_2^{\bullet-}$) é produzido pela cadeia respiratória, por ação da enzima xantina-oxidase ou outras enzimas celulares. A enzima superóxido dismutase converte o $O_2^{\bullet-}$ em peróxido de hidrogênio (H_2O_2), que por sua vez é degradado em água pelas enzimas catalase e glutationa peroxidase. O excesso de $O_2^{\bullet-}$ provoca a liberação de íons ferro de moléculas que possuem sítios ferro-sulfúricos, como a aconitase, participando da conversão do H_2O_2 em radical hidroxil ($^{\bullet}OH$) através da reação de Fenton. Adaptado de Raha e Robinson (2000).

A nível celular, os EROs podem, direta ou indiretamente, modular as funções de muitas enzimas e fatores de transcrição através de uma infinidade de cascatas de sinalização. Estes sinais resultam em mudanças na expressão gênica, que influenciam a capacidade de sobrevivência ou morte da célula. A magnitude e a duração do estresse, bem como o tipo de células envolvidas são fatores importantes na determinação de quais vias são ativadas, bem como no resultado celular final (HALLIWELL, 2007; MARTINDALE; HOOLBROOK, 2002).

É bem relatado na literatura que o excesso de hormônios tireoidianos leva a um aumento da taxa metabólica basal, gerando estresse oxidativo sistêmico com participação de EROs e ERNs, bem como acúmulo de moléculas oxidativamente modificadas em roedores e humanos (ADALI et al., 1999; GREDILLA et al., 2001; MOHAMADIN et al., 2006; TAPIA et al., 2003; VENDITTI; DI MEO, 2006). É observado na tireotoxicose uma diminuição das defesas antioxidantes enzimáticas e aumento de marcadores de estresse oxidativo, como substâncias reativas ao ácido tiobarbitúrico (TBARs), proteínas oxidadas e diminuição de glutationa reduzida (GSH), principalmente no fígado e coração (Mogulkog et al, 2006; VENDITTI; DI MEO, 2006). No fígado de roedores, a resposta termogênica a injeções diárias de 0,1 mg/Kg de T_3 é diretamente proporcional ao aumento do consumo de oxigênio, à ativação do fator de transcrição NF-κB e produção das citocinas TNF-α e IL-10, em um primeiro momento pelas células de Kupffer (TAPIA et al., 2003). O envolvimento de citocinas inflamatórias na tireotoxicose ainda é pouco relatado, mas parece contribuir com o estresse oxidativo e vice-versa. Makay e colaboradores (2009) observaram que o estresse oxidativo plasmático em um modelo murino de tireotoxicose induzida por injeções diárias de T_4 é diretamente relacionado com a ativação da expressão das citocinas TNF-α e IL-10, uma vez que o tratamento com o antioxidante melatonina restaura parte das alterações nesses parâmetros.

No músculo esquelético, observa-se aumento de proteínas miofibrilares oxidadas, de marcadores de lipoperoxidação e da atividade das enzimas superóxido dismutase e glutationa peroxidase, com diminuição da GSH (SEYMEN et al., 2004; VENDITTI et al., 1997; VENDITTI et al., 2009; YAMADA et al., 2006). Apesar do dano oxidativo estar presente no músculo esquelético de animais com tireotoxicose, não existem evidências diretas da participação das EROs e ERNs na disfunção muscular causada pelo excesso de hormônios tireoidianos (VENDITTI; DI MEO, 2006).

1.3 Músculo Estriado Esquelético e Mecanismos de Perda de Massa Muscular

A perda de massa muscular é caracterizada por diminuição involuntária do peso corporal (5% a 10%) em um curto período de tempo, devido a aceleração da degradação protéica e redução da síntese de proteínas, representando uma complicação clinicamente significativa de muitas doenças crônicas. Os mecanismos de perda de massa muscular na doença de diferentes processos são mal compreendidos. Independentemente da sua causa, a perda de massa muscular afeta o resultado da doença, levando à fraqueza, incapacidade e qualidade de vida prejudicada. O dano muscular pode ser observado em doenças que levam a acidose metabólica, aumento do catabolismo causado por hormônios (glicocorticóides, hormônios tireoidianos) ou citocinas (IL-1, IL-6, TNF- α), e pela resistência à insulina (CASTANEDA, 2002).

A maquinaria proteolítica das células é altamente seletiva e regulada, uma vez que é capaz de controlar a degradação de proteínas regulatórias críticas, como por exemplo fatores de transcrição, e de proteínas essenciais para o funcionamento celular (LECKER et al., 1999; MITCH; GOLDBERG, 1996). O aumento na degradação ou diminuição da síntese de proteínas leva a uma perda de massa corporal acentuada, sendo a proteólise a primeira causa de perda de peso observada em diversas doenças (MITCH; GOLDBERG, 1996).

As células de mamíferos possuem diferentes vias proteolíticas, com diferentes funções (figura 4). Existem 3 vias proteolíticas de grande importância no músculo esquelético: sistema citosólico cálcio-dependente da calpaína, proteases lisossomais, como por exemplo a catepsina, e o sistema ATP dependente ubquitina- proteassoma (MITCH; GOLDBERG, 1996; LECKER et al., 1999).

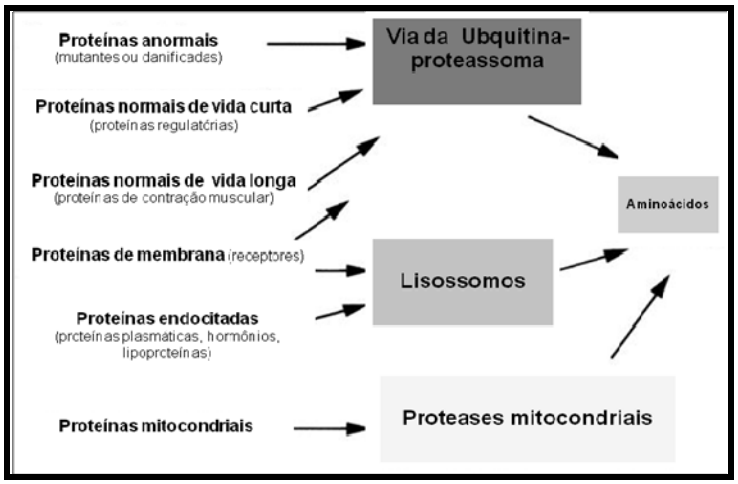

Figura 4 – Papéis dos sistemas de proteólise celular na degradação de várias classes de proteínas. Adaptado de Mitch e Goldenberg (1996).

A ativação da via da calpaína é observada em diversos estados catabólicos como sepse, câncer, distrofias musculares e também na tireotoxicose severa (O'NELL et al., 2009), sendo o cálcio um importante fator para a atividade dessa via (GOLL et al., 2003). Como a via da ubquitina-proteassoma não degrada miofibrilas intactas, é possível que ativação da via da calpaína desempenhe um papel importante na perda de massa muscular por clivagem de proteínas do citoesqueleto, resultando em perturbações na estrutura celular e liberação de miofilamentos, que são posteriormente ubiquitinados e degradados pelo proteassoma (HUANG; FORSBERG, 1998). A proteína lisossomal catepsina é responsável principalmente pela degradação de proteínas de superfície celular, como receptores, ligantes, canais e transportadores (JACKMAN; KANDARIAN, 2004). Estudos mostram seu aumento da expressão de mRNA em modelos experimentais de proteólise, inclusive na tireotoxicose, porém o mecanismo de ação não é conhecido (DEVAL, MORDIER; OBLED, 2001; O'NEAL et al., 2009).

A via da ubquitina-proteassoma atua juntamente com a via da calpaína na degradação de substratos protéicos (JACKMAN; KANDARIAN, 2004). Essa via é altamente específica, degradando apenas proteínas seriamente modificadas, que são reconhecidas e marcadas pela ubquitina. Esse processo é composto de 3 fases: (1) Ativação da carboxila terminal da ubquitina através da conversão em grupamento tiol pela enzima dependente de ATP E1; (2) A ubquitina ativada é transferida pela E1 para um família de proteínas carreadoras chamada E2, e os ésteres de tiol formados nessa reação são os doadores da ubiquitina para a

formação da ligação isopeptídica entre o resíduo de glicina do carbono terminal da ubiquitina e grupos amino de resíduos de lisina das proteínas-substrato; (3) Este passo requer a participação de um terceiro grupo de enzimas, chamado E3, responsáveis pela seleção da proteína substrato através do reconhecimento de um sinal de degradação existente nessa proteína. Enzimas E2 podem contribuir, também, para o reconhecimento da proteína substrato ubiquitinando-a na ausência de E3. Várias moléculas de ubiquitina são adicionadas formando uma cadeia poliubiquitinada, e as proteínas marcadas degradadas pelo proteassoma 26S (ARGILÉS; LÓPEZ-SORIANO, 1996; MITCH; GOLDENBERG, 1996; GLICKMAN; CIECHANOVER, 2002; JACKMAN; KANDARIAN, 2004). O proteassoma 26S é uma protease multimérica dependente de ATP, composta de um complexo regulatório 19S e um complexo catalítico 20S, onde a unidade 19S possui a função de desdobrar as proteínas e encaminhá-las ao complexo 20S para serem degradadas a aminoácidos (MITCH; GOLDENBERG, 1996).

Recentemente, foi mostrado por O'Neal e colaboradores (2009) que ocorre ativação da via ubquitina-proteassoma, com aumento de expressão das ubquitina-ligases E3 atrogina-1 e MURF-1, da via da calpaína e da catepsina em ratos com tireotoxicose severa induzida por injeções intraperitoneais diárias de 100 μg/100g de peso de T_3 durante 3 ou 7 dias. Tawa, Odessey e Goldberg (1997) verificaram que a T_3 aumenta a proteólise no diafragma de ratos, e que o uso de inibidores proteassomais é capaz de inibir 34% da proteólise nesses animais. Sabe-se que o estresse oxidativo está envolvido na ativação das vias proteolíticas da calpaína, caspase-3 e ubquitina-proteassoma (POWERS et al., 2010), porém não há dados na literatura que mostrem uma possível relação entre o estresse oxidativo no músculo esquelético com a ativação da perda de massa muscular na tireotoxicose.

2 OBJETIVO

Avaliar a participação dos radicais livres na lesão muscular esquelética em ratos com tireotoxicose severa experimental.

2.1 OBJETIVOS ESPECÍFICOS

A) Relacionar o status oxidativo do músculo *gastrocnemius* de ratos na tireotoxicose severa experimental com as alterações metabólicas envolvidas.

B) Relacionar a atividade mitocondrial com o estresse oxidativo no músculo esquelético de animais com tireotoxicose severa experimental utilizando-se o inibidor alfa-tocoferol.

ARTIGO[1]

OXIDATIVE SKELETAL MUSCLE INJURY IN RATS WITH EXPERIMENTAL SEVERE THYROTOXICOSIS

Sara Santos Bernardes[2], Flávia Alessandra Guarnier[2], Poliana Camila Marinello[2], André Armani[3], Rubens Cecchini[2], Alessandra Lourenço Cecchini[2*].

Resumo

Miopatia é uma manifestação inicial freqüente na tireotoxicose, caracteriza por diminuição da massa muscular, perda de força e fraqueza. O estado hipermetabólico na tireotoxicose acelera a produção de espécies reativas de oxigênio (EROs) na mitocôndria e induz mudanças na defesa antioxidante de diversos tecidos, inclusive no músculo esquelético. Apesar dessa relação, a participação de EROs na perda de massa muscular na tireotoxicose ainda não é conhecida. O objetivo desse trabalho foi avaliar a participação das EROs nas alterações musculares esqueléticas observadas na tireotoxicose severa experimental. Para isso, foram realizados dois grupos experimentais de ratos Wistar machos tratados com triiodotironina (T3), durante 3 ou 5 dias, e dois grupos experimentais tratados com T3 e alfa-tocoferol, um agente antioxidante de membrana, durante os mesmos períodos. Todos os grupos experimentais foram comparados com seu respectivo par controle. Nosso estudo mostrou que ocorre estresse oxidativo no músculo *gastrocnemius* de ratos com tireotoxicose severa experimental tratados durante 3 ou 5 dias com a T3. As EROs mostraram-se envolvidas na ativação de vias de perda de massa muscular, evidenciado pelo aumento do conteúdo de tirosina intracelular observado 5° dia. O antioxidante alfa-tocoferol melhorou os parâmetros oxidativos no músculo *gastrocnemius,* principalmente no dia 3, onde o estresse oxidativo foi mais evidente, e diminuiu a atividade hipermetabólica mitocondrial e a temperatura interna nos grupos experimentais com tireotoxicose severa. Além disso, a melhora dos

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[1] Este é um trabalho realizado no Laboratório de Fisiopatologia dos Radicais Livres da Universidade Estadual de Londrina, formado pelo artigo científico: **Oxidative skeletal muscle injury in rats with experimental severe thyrotoxicosis.** Bernardes, S. S.; Guarnier, F. A.; Marinello,P. C.; Armani, A.; Cecchini, R.; Cecchini, A. L.
As formatações do artigo seguem as normas da revista *Free Radicals Biology and Medicine* (Anexo A).

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parâmetros oxidativos no 3° dia protegeu parcialmente a perda de massa muscular observada no 5° dia de tratamento com a T3. Os resultados encontrados nesse trabalho sugerem que o alfa-tocoferol retarda a ativação de vias de perda de massa muscular na tireotoxicose, protegendo o músculo esquelético, provavelmente por desviar o catabolismo para a lipólise. Esses achados mostram uma participação ativa das EROs nas alterações musculares observadas na tireotoxicose.

Palavras-Chave: Triiodotironina. Tireotoxicose. Massa muscular. Alfa-tocoferol.

Abstract

Myopathy is an early manifestation of thyrotoxicosis. The hypermetabolic state in thyrotoxicosis accelerates ROS production in the mitochondria and induces changes in the antioxidant defenses in skeletal muscle, but the participation of ROS in muscle wasting loss is still unclear. The aim of the work is to comprehend the participation of ROS in the muscle alterations in rats with experimental severe thyrotoxicosis. For that, alpha-tocopherol (α-T) was administered. The experimental model consisted of two groups of rats treated with hormone triiodotironina (HT) for 3 and 5 consecutive days and two groups of rats that where treated also with α-T. Our study shows that oxidative stress is present in the skeletal muscle on 3 and 5 days hormone treatment. ROS can be involved in the activation of muscle wasting pathways, evidenced by the increase of intracellular tyrosine content by 5 days HT. α-T ameliorate antioxidant parameters, mainly on day 3, decrease mitochondrial respiration and internal temperature in HT α-T groups, protecting partially *gastrocnemius* mass loss observed on day 5. We suggest that, α-T slows down the skeletal muscle wasting pathways in thyrotoxicosis, protecting skeletal muscle mass loss, diverting catabolism to lipolysis. These findings show an active participation of reactive oxygen species on muscular alterations observed in thyrotoxicosis.

Keywords: Triiodothyronine. Thyrotoxicosis. Skeletal muscle. Alpha-tocopherol.

INTRODUCTION

Thyrotoxicosis is the syndrome caused by an excess of free thyroid hormones, by which any or all systems of the body can be affected. The symptoms and signs depend on the increase of the hormones, for how long that it has been elevated,the rate at which the hormone level rose, and individual biological variations [1]. It results in a generalized acceleration of metabolic processes. In most cases, thyrotoxicosis is due to hyperactivity of the thyroid gland, or hyperthyroidism.

Occasionally, thyrotoxicosis may be due to other causes such as excessive ingestion of thyroid hormone, toxic adenoma or subacute thyroiditis [2,3]. Common manifestations include palpitations, nervousness, easy fatigability, hyperkinesia, diarrhea, excessive sweating. There is often marked weight loss without loss of appetite [3].

Muscle is one of the largest tissues in the body, and is susceptible to the metabolic and trophic effects of the endocrine system. Excess thyroid hormone upregulates metabolic activity, resulting in increased energy utilization. The specific pathogenesis of thyrotoxic myopathy is unclear; perhaps the myopathy is caused by a combination of increased energy use with increased catabolism, resulting in an inefficient system that manifests primarily as muscular weakness [4].

Thyrotoxicosis is associated with general muscle weakness, which is part of the initial clinical manifestation of approximately 80% of patients [5]. Mild short-term experimental hyperthyroidism increases whole-body protein turnover and breakdown before any measurable changes in energy expenditure or glucose and fat metabolism, suggesting that amino acid and protein metabolism is an early and primary target for thyroid hormone action in humans [6]. Clément, Viguerie & Diehn [7], showed that T3 induces many genes involved in protein turnover and in energy metabolism in human skeletal muscle. Myopathy affects men with thyrotoxicosis more commonly than women and may overshadow the other manifestations of the syndrome [8]. Patients with thyrotoxicosis are characterized by a decreased muscle mass of around 20% and an impairment of muscle strength around 40%. These abnormalities persist for between 5 and 9 months after initiation of medical treatment [9]. In the progression of disease, the patient is severely weaken and may be unable to rise from a sitting or lying position and may be virtually unable to walk. This disorder may resemble progressive muscular atrophy or polymyositis; however, fasciculation is absent, and little if any inflammatory change is evident on biopsy. Instead, the muscle is atrophic and infiltrated with fat cells and lymphocytes [8].

The hypermetabolic state in thyrotoxicosis accelerates reactive oxygen species (ROS) production in the mitochondria and induces changes in the antioxidant defenses in different tissues, as skeletal muscle [10]. At the cellular level, oxidative stress promotes a wide spectrum of responses, depending on the cell type, the level of ROS achieved, and the duration of the exposure [11]. It is known that ROS interact with cellular signaling pathways that regulate gene expression and,

therefore, contribute to skeletal muscle remodeling [12]. The aim of this is work was to evidence the participation of ROS in muscle wasting loss though the administration of alpha-tocopherol (α-T), a cell membrane antioxidant, in parameters of body weight, skeletal muscle mass, metabolic rate and muscle oxidative status in rats with experimental severe thyrotoxicosis.

MATERIALS AND METHODS

Animals and treatment. Were used 48 male Wistar rats weighing 220-250 g, divided in 8 experimental groups: 1.Control 3 days; 2. Thyrotoxicosis 3 days; 3. Control α-T 3 days; 4. Thyrotoxicosis 3 days treated with α-T; 5. Control 5 days; 6. Thyrotoxicosis 5 days; 7. Control α-T 5 days; 8. Thyrotoxicosis 5 days treated with α-T group. Experimental thyrotoxicosis was induced by daily intraperitoneal injections of 100 ug 3,5,3-triiodothyronine (T3; Sigma, St. Louis, MO) per 100 g body weight during three or five consecutive days, as described previously [13]. Control rats were injected with corresponding volumes (0.2 ml/100 g body weight) of solvent (5mM NaOH/NaCl 0.9%). Four groups were also treated with intraperitoneal 100 mg/Kg of α-T solubilized in corn oil, started 12 hours before first T3 injection, and repeted every day. All injections were performed between 9 and 10 AM. The rats were obtained from the animal house of the Biological Sciences Center at the Universidade Estadual de Londrina, are maintened in light-dark cycle 12:12, and had free access to water. Previously was realized a *per fed* group and the same amount of food was offered for all groups throughout the experiments (Nuvilab CR1; Nuvital Nutrients Ltda., Curitiba, Brazil). Body weight and internal temperature, by digital thermometer rectal insertion, were measured daily. Twenty-four hours after the final T3 injection, rats were anesthetized with ether, the blood samples were obtained from heart and serum was stored at - 20°C for measurement of T3 levels. Then, the rats were killed by decapitation and the *gastrocnemius* muscle and peritoneal fat rapidly excises and weighed. *Gastrocnemius* was stored at -80°C for oxidative analysis. The rats were treated and cared for in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The

experimental protocol was approved by the Institutional Animal Care and Utilization Committee at the Universidade Estadual de Londrina.

Measurament of T3 levels. Total T3 levels in serum were measured by chemiluminscence assay with commercial Kit.

Weight Loss Index. The weight loss index was calculed using the proposed formula by Guarnier et al. [14], where [(initial body mass - final body mass + body mass gain of control) / (initial body mass + body mass gain of control)] x 100%.

Skeletal Muscle Relative Weight (SMRW). This index was calculated by the relationship between the total body weight and the weight of the *gastrocnemius* muscle.

Visceral fat weight. To characterize the fat weight loss, retroperitoneal and perigonadal fat, were rapidly excises and weighed.

Tissue preparation for oxidative analyses. *Gastrocnemius* muscle homogenates were prepared as described by Guarnier et al. [14], with some modifications. Briefly, muscles pieces were placed on ice and homogenized for 50-s in an Ultraturrax homogenizer containing 10 mg/mL or 50 mg/mL of tissue in 10 mM KH_2PO_4/K_2HPO_4 buffer and 120 mM KCl at pH 7.4. Total homogenate was used for the *tert*-butyl hydroperoxide-stimulated chemiluminescence and thiobarbituric acid-reactive substances assays. The supernatant from total homogenate obtained by centrifugation at 11,000*g* for 15 min at 4ºC containing 50 mg of tissue/mL of the same buffer, was used for the total antioxidant capacity, glutathione, catalase and superoxide dismutase assays. For carbonyl protein test, was prepared 10 mg/mL gastrocnemius muscles using 50 mM phosphate buffer, 1 mM EDTA, pH 7.4. The samples was homogenized as described above and centrifuged at 3,000*g* for 10 min at 4ºC.

Measurement of tert-butyl hydroperoxide-initiated chemiluminescence. Reaction mixtures were placed in 2-mL luminescence tubes containing total muscle homogenate from gastrocnemius (10 mg/mL), 30 mM KH_2PO_4/K_2HPO_4 buffer (with 120 mM KCl, pH 7.4), and 3 mM *tert*-butyl hydroperoxide, in a final volume of 1 mL. The tert-butyl hydroperoxide-initiated chemiluminescence (CL) reaction was assessed in a TD/20 20 luminometer (Turner Designs, Sunnyvale, CA), with a response range of 300-650 nm. The tubes were kept in the dark until the moment of assay, which was carried out in a room at 28ºC

[15, 16]. For each animal, an 40-min curve, where each point represented the differential smoothing of 600 readings, was obtained by interpolation. The entire curve and area, extracted by integral calculus of each animal curve, was used to determine the lipid hydroperoxides present in the sample. The results were expressed in Relative Light Unit (RLU) per mg tissue.

Thiobarbituric acid-reactive substances (TBARS) assay. Malondialdehyde (MDA) formed during lipid peroxidation reacts in the TBARS assay to generate a colored product, a $(TBA)_2$-MDA adduct. After extraction with n-butanol, the complex $(TBA)_2$-MDA absorbs light at 532 nm. TBARS levels was measured as described by Oliveira and Cecchini [15], using ε_{535}=156 $mM^{-1} \cdot cm^{-1}$ and expressed in nmols per 10 mg tissue.

Carbonyl protein content. Carbonyl protein content was measured as described by Reznick and Parker [17], with modifications. 0,5 mL of each protein extract was placed in glass tubes. A volume of 2 mL of 2,4- dinitrophenylhydrazine (DNPH) solution prepared in 2.5 N HCl was added to each tube, and the reaction mixtures were incubated for 1 h at RT, with vortexing every 15 min. Next, the samples were washed with 2,5 mL of 20% TCA (w/v) and centrifuged for 10 min to collect the protein precipitates. Another wash was performed using 10% TCA, and protein pellets were dispersed mechanically. Finally, the pellets were washed 3 times with 2 mL of ethanol-ethyl acetate (1:1, v/v) to remove free DNPH and lipid contaminants. The final precipitates were dissolved in 2 mL of 0.6 M guanidine hydrochloride, and any insoluble materials were removed by additional centrifugation. Carbonyl content was calculated by reading the peak absorbance at 355-390 nm of the DNPH-treated samples versus samples treated with only 2.5 M HCl. A formula was used to calculate the concentration of carbonyls: *C = Abs (355-390nm) x 45.45 nmol/mL*, where C is the concentration of DNPH/mL, and 45.45 its absorption coefficient [17]. The procedures were performed in an ice bath until the TCA wash. Carbonyl content was expressed in nmol per mg total protein.

Superoxide dismutase (SOD) activity. SOD activity was determined according to Marklund and Marklund [18], based on the inhibition of pyrogallol autoxidation in an aqueous solution of SOD. This oxidation is accompanied by yellow color formation in the reaction medium, monitored at 420 nm. Aliquots of *gastrocnemius* supernatant (50 mg/ml) was diluted in Tris buffer with 1 N HCl and 5 mM EDTA, pH 8.0, were added to pyrogallol. The reaction was monitored

continuously for 5 min. The autoxidation of pyrogallol alone was used as control. The amount of SOD that is able to inhibit 50% of pyrogallol autoxidation is defined as the enzymatic activity unit (U). Final SOD results were expressed in U per mg total protein.

Catalase activity. *Gastrocnemius* muscle catalase activity was determined using the technique of Aebi [19]. The homogenate was prepared as described above, and 0,2 ml added in a quartz cuvette content 1,5 ml of Tris buffer 1M HCl and 0,2 of deionized water. The reaction began with the addition of 0,1 ml of 200 mM H_2O_2. Absorbance was read at 240 nm for 180 s and the velocity of absorption for catalase was calculated from the absorbance difference between times 0 and 60 seconds. The result was expressed as absorption velocity in one ml of tissue homogenate per mg total protein.

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 in the same manner in the supernatant previously incubated with 4-vinylpyridine for 60 min at room temperature, according to the method described by Tietze [20]. Volumes of supernatant were adjusted for the assay with muscle homogenate, containing 50 mg/mL. The results were expressed in µM per mg total protein. The stress index (SI) was calculed by the equation: SI = GSSG / (GSH-GSSG) x 100%.

Measurement of the total radical-trapping antioxidant parameter (TRAP). Total antioxidant capacity of muscle homogenate prepared as described before was measured by CL, in a reaction medium containing 20 µM 2-azo-bis-(2-amidinopropane) and 200 µM luminol. 70 µL of tissue supernatant or trolox (final concentration of 0.7 µM) were added to the reaction medium. The time of total quenching was compared with trolox quenching, and the results were expressed in µM trolox [21].

Mitochondrial cytochrome c oxidase activity. Cytochrome c oxidase activity in mitochondria was measured as described by Wharton & Tzagoloff [22], with some modifications. Briefly, *gastrocnemius* muscles were rapidly excised and placed in ice-cold isolation medium (IM) containing 200 mM mannitol, 75 mM sucrose, 1mM EDTA, 0.05% fatty acid- free albumin, 1 mM Tris, pH 7.4. Then the muscles were freed of extracellular fat and connective tissue, finely minced, and washed with IM. Tissue fragments were gently homogenized (10% w/v) in IM using a

glass Potter–Elvehjem homogenizer set at a standard velocity (1000 rpm) for 10 seconds, three times. The homogenates were freed from debris by centrifugation at 550g for 10 min at 4°C, and mitochondria were isolated from the supernatant centrifugation at 10,000g. The mitochondrial pellets were washed in MI twice, centrifuged again at 10,000g and then resuspended in 200 μl MI. The suspension was frozen and thawed three times in liquid nitrogen and kept under ice until used to determine enzyme activity and total protein content. Cytochrome c oxidase activity was measured spectrophotometrically by the oxidation of 30 μM ferricytochrome c at 550 nm (**ε=** 20.5 mM^{-1} cm^{-1}), at 38°C. Ferricytochrome c was prepared by reduction of ferrocytochrome c using excess sodium dithionite, followed by chromatographic desalting with Sephadex G-25 [23]. The results were expressed in μM ferrycitochrome c / min per mg total protein.

Intracellular tyrosine content. Intracellular tyrosine content is proportional tyrosine release in skeletal muscle [24]. *Gastrocnemius* was homogenized in Ultraturrax homogenizer containing 100 mg/mL of tissue in deionized water, and tyrosine was measured by spectrophotometry, according Udenfriend and Cooper [25]. Results are expressed in μM of tyrosine per mg tissue, and calculated from standard curve concentration of L-tyrosine at 450 nm.

Protein concentration. Protein was determined by the method of Lowry et al. [26], modified by Miller [27], used bovine serum albumin (BSA) as standard.

Statistical Analysis. Control and experimental groups were compared using the Student unpaired t test. Chemiluminescence curves were compared also using two-way analysis of variance (ANOVA). The results are show as mean±SEM of 6 animals and $P < 0,05$ was considered significant.

RESULTS

Body weight decreased during the course of 5 days (Figure 1A), and alpha-tocopherol treatment did not change the weight loss. Figure 1B show that the

internal temperature increased in HT animals and not in alpha-tocopherol treated animals ($p<0.05$).

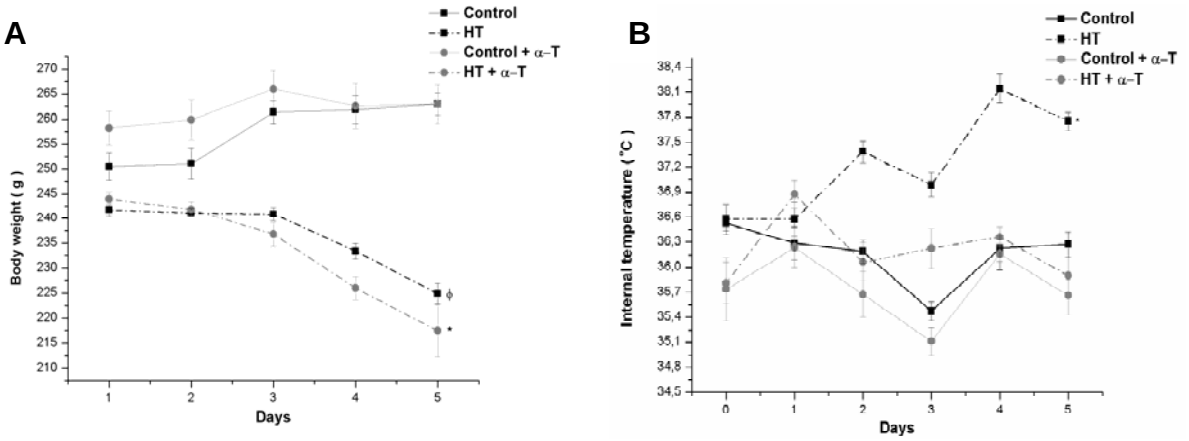

Figure 1 – (A) Body weight and (B) internal temperature curves. Rats were treated for 3 or 5 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Body weight and internal temperature were measured daily. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$). Results are expressed as mean ± SEM. HT, hormone treated group; HT+α-T, alpha-tocopherol treated hormone group.

3 days hormone treatment

Table 1 shows the characterization of serum T3 levels, body, muscle and fat wasting in thyrotoxic animals after 3 days hormone treatment (HT). Alpha-tocopherol treatment do not change the increase in serum T3 levels. The Weight Loss Index and the Skeletal Muscle Relative Weight (SMRW) did not change with alpha-tocopherol treatment, but peritoneal fat decreased 35.49% in HT group treated with the antioxidant ($p < 0.05$), without differ in relation to HT group. Lipid peroxidation levels, measured by tert-butyl hydroperoxide initiated chemiluminescence and TBARS reaction increased with HT, and the treatment with alpha-tocopherol decreased these parameters (Figures 2A, 2B and 3A). Carbonyl proteins did not change in relation to control group, and decreased in alpha-tocopherol treated groups when compared to non-treated groups (Figure 3B).

Table 1 – Serum T3 levels, weight loss index, Peritoneal fat, Skeletal Muscle Relative Weight (SMRW) and *gastrocnemius* weight of rats with severe thyrotoxicosis. Rats were treated for 3 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$). HT, hormone treatment; α-T, alpha-tocopherol treated group.

				Gastrocnemius	
	T3 (ng/mL) (mean± SEM)	Weight loss index (%± SEM)	Peritoneal fat (mg) (mean±SEM)	SMRW (%± SEM)	Weight (mg) (mean±SEM)
Control	0.898±0.016	none	742.8 ± 57.42	0.470±0.005	983.6±12.77
HT	4.758±1.075*	7.808±0.877	626.8 ± 44.97	0.470±0.009	947.9±16.90
Control+α-T	1.015±0.166	none	830.8 ± 37.79	0.472±0.007	1125.0±17.11
HT+α-T	4.723±0.896ϕ	9.303±0.518	536.0 ± 41.94ϕ	0.480±0.006	1140.0±17.32

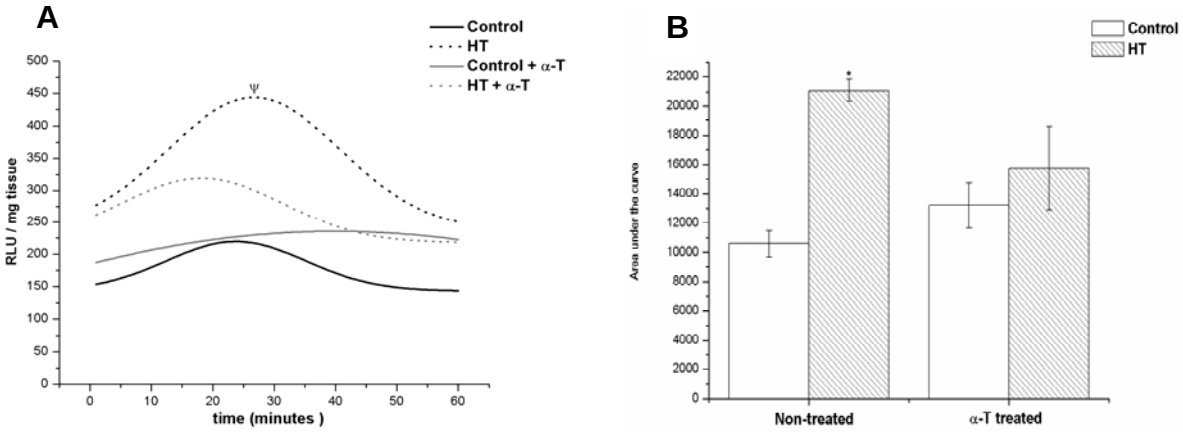

Figure 2 – Lipid hydroperoxide levels in *gastrocnemius* of rats with severe thyrotoxicosis. Rats were treated for 3 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Muscles were studied 24 hours after the last T3 injection. (A) Tert-butyl hidroperoxide–initiated chemiluminescence was monitored continuously for 60 minutes. Each curve represents a Gaussian fit of the mean of 600 readings of emitted light; (B) Area under the chemiluminescence curve. ψ : All curves are different by Two-way ANOVA test ($p < 0.001$). Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$).Results are expressed as mean ± SEM. HT, hormone treated group; α-T, alpha-tocopherol.

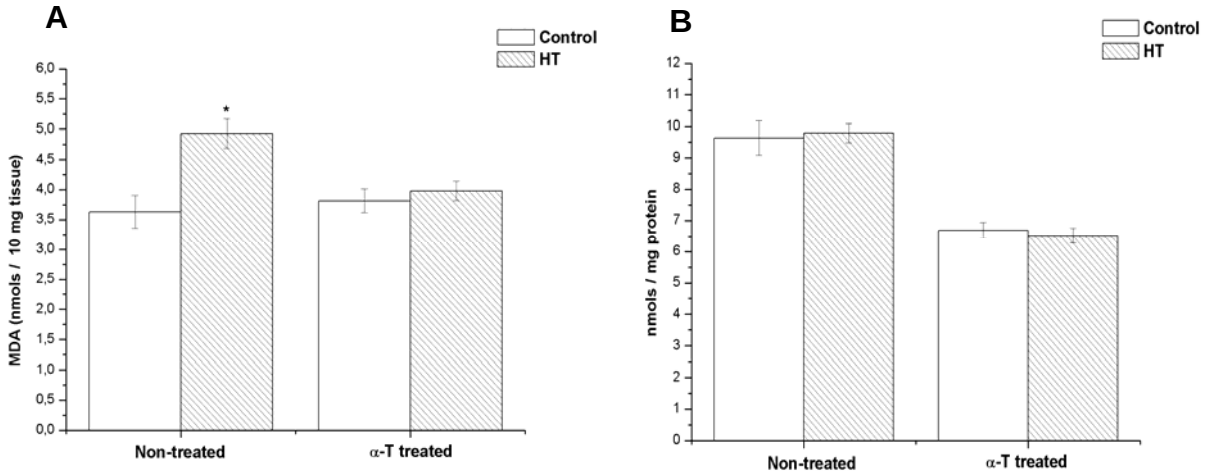

Figure 3 – (A) MDA and (B) Carbonyl protein levels in *gastrocnemius* muscle of rats with severe thyrotoxicosis. Rats were treated for 3 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Muscles were studied 24 hours after the last T3 injection. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$).Results are expressed as mean ± SEM. HT, hormone treated group; α-T, alpha-tocopherol.

GSH and GSSG levels decreased in HT group, and alpha-tocopherol treatment did not change GSH, but increased GSSG levels. Stress index increased in HT group, and antioxidant treatment did not decrease this parameter (Table 2). In non alpha-tocopherol treated group, the activity of SOD was increased and of catalase was decreased, but in the antioxidant treated group there was not found difference in catalase and SOD activity (Figure 4A and 4B), despite catalase activity increased 60,27% when compared to HT non-treated group ($p < 0.05$). The antioxidant treatment also increased total antioxidant capacity in HT group (Figure 5). Mitochondrial cytochrome c oxidase activity, a indicative of cellular respiration and, consequently, metabolic rate, there was increased in HT group, and decreased in both groups treated with alpha-tocopherol (Figure 6).

Table 2 – Reduced (GSH), oxized (GSSG) glutathione and stress index (SI) in *gastrocnemius* muscle of rats with severe thyrotoxicosis. Rats were treated for 3 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Muscles were studied 24 hours after the last T3 injection. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$). HT, hormone treatment; α-T, alpha-tocopherol.

	GSH (μM/ mg protein) (mean± SEM)	GSSG (μM/ mg protein) (mean± SEM)	Stress Index % (GSSG/GSH-GSSG) (mean± SEM)
Control	3.412 ± 0.127	0.129 ± 0.016	3.838 ± 0.530
HT	1.567 ± 0.099*	0.084 ± 0.006*	5.407 ± 0.391*
Control+α-T	4.525 ± 0.813	0.077 ± 0.015	5.283 ± 0.568
HT+α-T	1.860 ± 0.367$^{\phi}$	0.111 ± 0.031	7.327 ± 0.562$^{\phi}$

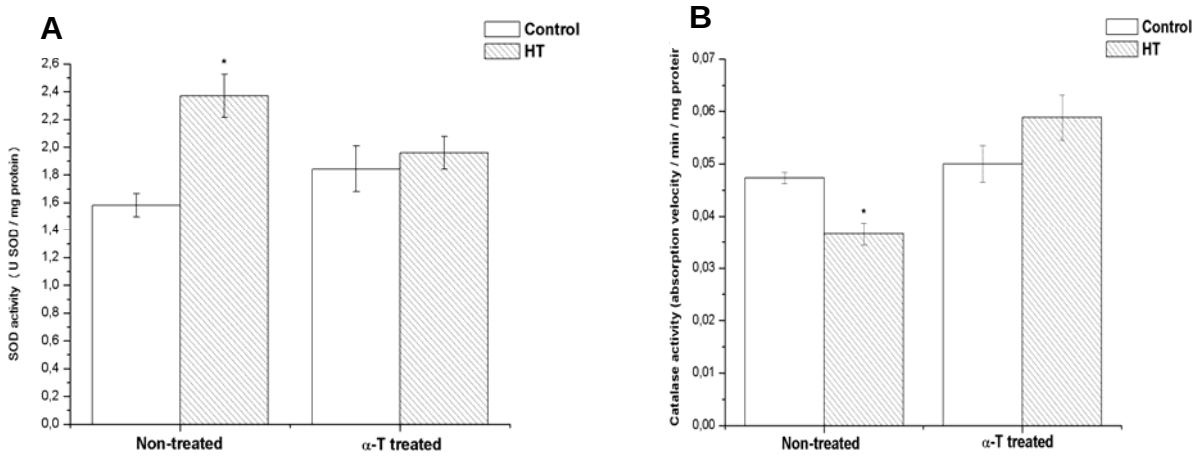

Figure 4 – (A) SOD and (B) Catalase activity in *gastrocnemius* of rats with severe thyrotoxicosis. Rats were treated for 3 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Muscles were studied 24 hours after the last T3 injection. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$).Results are expressed as mean ± SEM. SOD activity was expressed in U / mg protein at 37°C and catalase in Absorption velocity / mg protein at 25°C. HT, hormone treated group; α-T, alpha-tocopherol.

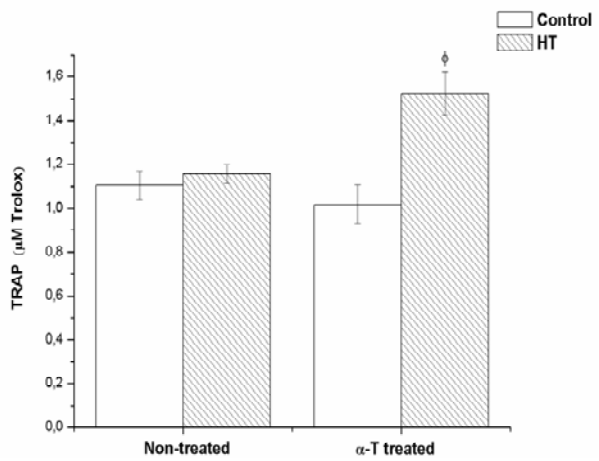

Figure 5 – Effect of thyrotoxicosis on total antioxidant capacity (TRAP) in *gastrocnemius* of rats with severe thyrotoxicosis. Rats were treated for 3 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Muscles were studied 24 hours after the last T3 injection. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$). Results are expressed as mean ± SEM. TRAP are expressed in μM Trolox at 37°C. HT, hormone treated group; α-T, alpha-tocopherol.

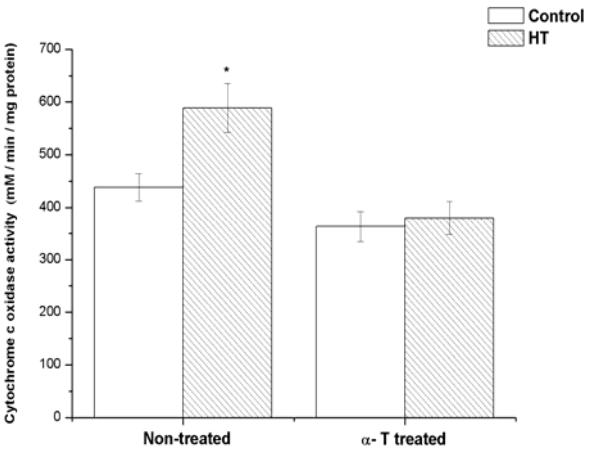

Figure 6 – Effect of thyrotoxicosis on cytochrome c oxidase actitivity in *gastrocnemius* mitochondria of rats with severe thyrotoxicosis. Rats were treated for 3 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Muscles were studied 24 hours after the last T3 injection. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$). Results are expressed as mean ± SEM. Citochrome oxidase activity was expressed in μM ferricytochrome consumed per minute / mg protein at 38°C. HT, hormone treated group; α-T, alpha-tocopherol.

5 days hormone treatment

Table 3 shows the characterization of body, muscle and fat wasting in thyrotoxic animals after 5 days hormone treatment (HT) and serum T3 levels. Alpha-tocopherol treatment do not change the increase in serum T3 levels. Peritoneal fat decreased 41,75% in HT groups treated with antioxidant ($p<0.05$). The Weight Loss Index did not change with alpha-tocopherol treatment. Skeletal Muscle Relative Weight (SMRW) decreased in HT group, indicate mass muscle loss, there was restore to antioxidant treatment. When the HT was treated with alpha-tocopherol, *gastrocnemius* weight decreased 13.72% instead of 23,7% in non-treated group. Lipid peroxidation levels measured by tert-butyl hydroperoxide initiated chemiluminescence increased, and antioxidant treatment did not change this parameter (Figure 8A). MDA levels, showed a tendency to increased ($p=0.0649$) in HT group, and the treatment with alpha-tocopherol did not change MDA levels (Figure 9A) ($p=0.394$). Carbonyl proteins decreased in relation to control group, in non-treated and alpha-tocopherol treated pairs (Figure 9B).

Table 3 – Serum T3 levels, weight loss index, Peritoneal fat, Skeletal Muscle Relative Weight (SMRW) and *gastrocnemius* weight of rats with severe thyrotoxicosis. Rats were treated for 5 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$). HT, hormone treatment; α-T, alpha-tocopherol treated group.

				Gastrocnemius	
	T3 (ng/mL) (mean± SEM)	Weight Loss Index (%± SEM)	Peritoneal fat (mg) (mean±SEM)	SMRW (%± SEM)	Weight (mg) (mean±SEM)
Control	0.908±0.024	none	913.2 ± 37.76	0.506±0.007	1207 ± 28.16
HT	3.630±0.243*	14.300±0.871	870.3 ± 26.29	0.430±0.008*	921.2 ± 26.64*
Control+α-T	0.761±0.077	none	1021 ± 78.44	0.491±0.012	1137 ± 23.87
HT+α-T	4.89±0.777$^{\phi}$	18.850±1.949	594.7 ± 27.78$^{\phi}$	0.470±0.024$^{\phi}$	981.0 ± 47.94$^{\phi}$

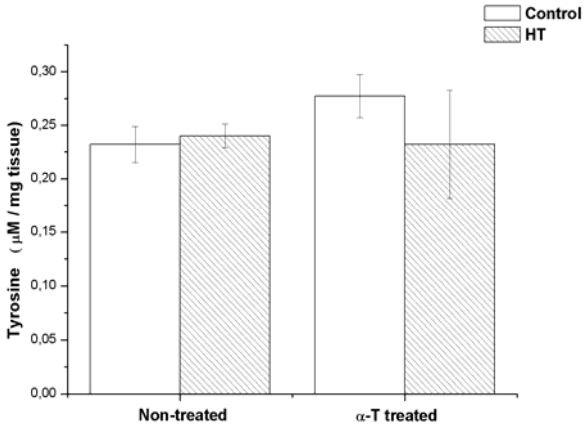

Figure 7 – Intracellular tyrosine levels in *gastrocnemius* muscle of rats with severe thyrotoxicosis. Rats were treated for 3 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Muscles were studied 24 hours after the last T3 injection. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$). Results are expressed as mean ± SEM. HT, hormone treated group; α-T, alpha-tocopherol.

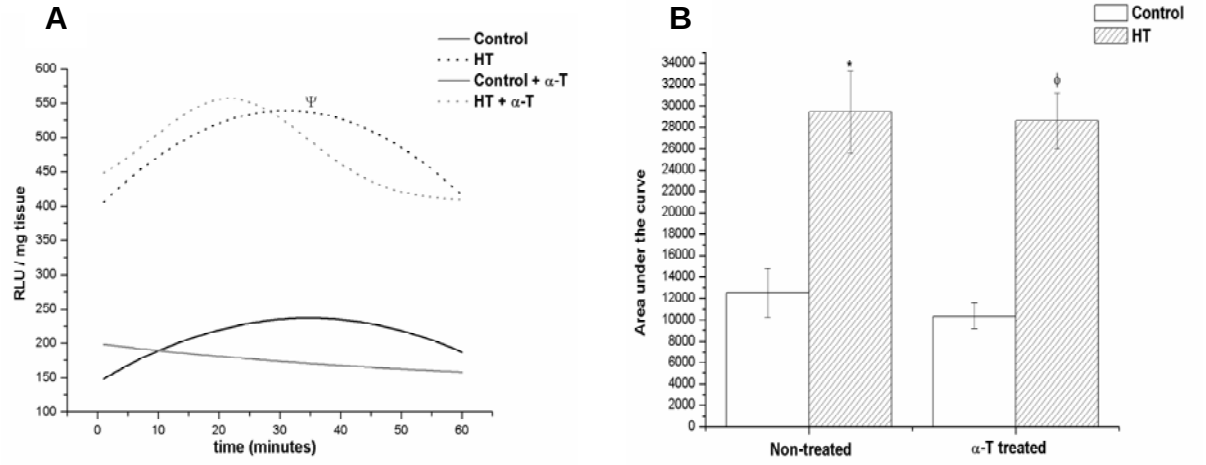

Figure 8 – Lipid hydroperoxide levels in *gastrocnemius* of rats with severe thyrotoxicosis. Rats were treated for 5 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Muscles were studied 24 hours after the last T3 injection. (A) Tert-butyl hidroperoxide–initiated chemiluminescence was monitored continuously for 60 minutes. Each curve represents a Gaussian fit of the mean of 600 readings of emitted light; (B) Area under the chemiluminescence curve. ψ : All curves are different by Two-way ANOVA test ($p < 0.001$). Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$).Results are expressed as mean ± SEM. HT, hormone treated group; α-T, alpha-tocopherol.

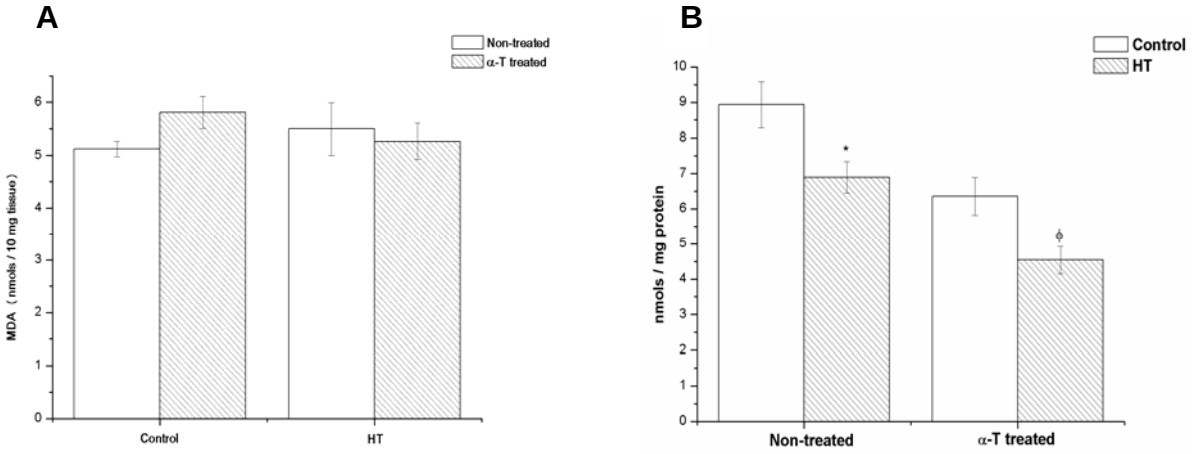

Figure 9 – (A) MDA and (B) Carbonyl protein levels in *gastrocnemius* muscle of rats with severe thyrotoxicosis. Rats were treated for 5 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Muscles were studied 24 hours after the last T3 injection. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$). Results are expressed as mean ± SEM. HT, hormone treated group; α-T, alpha-tocopherol.

GSH and GSSG levels decreased in HT group, and alpha-tocopherol treatment did not change these parameters. Stress index did not change in 5 days thyrotoxic animals (Table 4). In non alpha-tocopherol treated group, the activity of SOD and of catalase did not change, but with α-T treatment increased these antioxidants enzymes activities in relation to respective controls (Figure 10A and 10B). The antioxidant treatment restores total antioxidant capacity in HT group, and also increased total antioxidant capacity in HT group in 125,8% ($p < 0.05$), when compared with alpha-tocopherol non-treated group (Figure 11). Mitochondrial cytochrome c oxidase activity decreased in both groups treated with alpha-tocopherol (Figure 12). Intracellular content of amino acid tyrosine there was increased significantly only in 5 days HT treated group. Alpha-tocopherol treatment reduced 33,0% this amino acid content in *gastrocnemius* muscle ($p <0.05$), indicating decreased in protein breakdown (Figure 13).

Table 4 – Reduced (GSH), oxized (GSSG) glutathione and stress index (SI) in *gastrocnemius* muscle of rats with severe thyrotoxicosis. Rats were treated for 5 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Muscles were studied 24 hours after the last T3 injection. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$). HT, hormone treatment; α-T, alpha-tocopherol.

	GSH (μM/ mg protein) (mean± SEM)	GSSG (μM/ mg protein) (mean± SEM)	Stress Index (GSSG/GSH-GSSG) (mean± SEM)
Control	4.950 ± 0.260	0.2150 ± 0.010	4.845 ± 0.466
HT	1.820 ± 0.100*	0.0910 ± 0.020*	6.320 ± 1.090
Control+α-T	3.838 ± 0.490	0.2100 ± 0.030	6.943 ± 0.684
HT+α-T	1.302 ± 0.070ϕ	0.1146 ± 0.010ϕ	8.427 ± 0.430

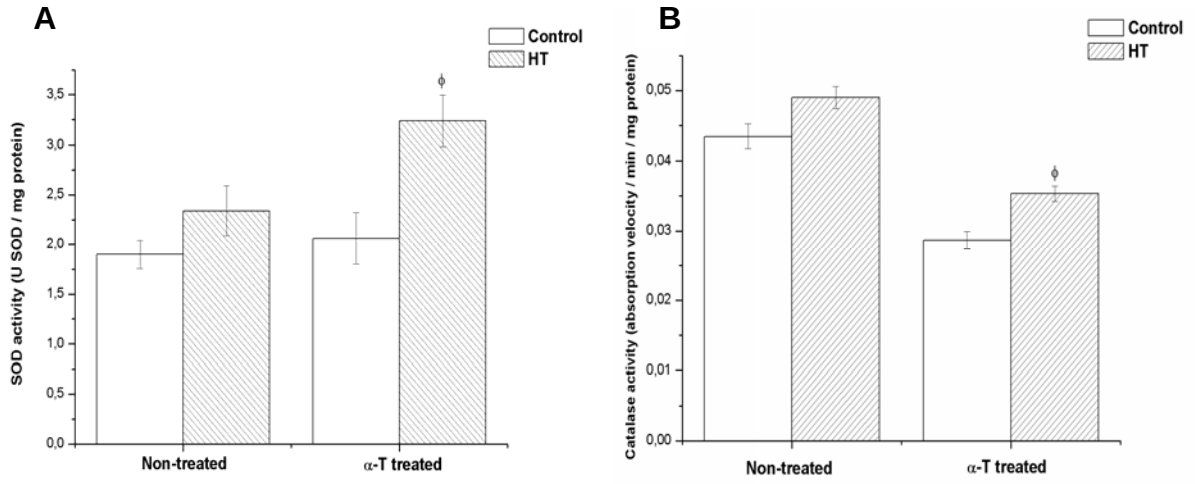

Figure 10 – (A) SOD and (B) Catalase activity in *gastrocnemius* of rats with severe thyrotoxicosis. Rats were treated for 5 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Muscles were studied 24 hours after the last T3 injection. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$). Results are expressed as mean ± SEM. SOD activity was expressed in U / mg protein at 37°C and catalase in Absorption velocity / mg protein at 25°C. HT, hormone treated group; α-T, alpha-tocopherol.

Figure 11 – Effect of thyrotoxicosis on total antioxidant capacity (TRAP) in *gastrocnemius* of rats with severe thyrotoxicosis. Rats were treated for 5 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Muscles were studied 24 hours after the last T3 injection. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$). Results are expressed as mean ± SEM. TRAP are expressed in μM Trolox at 37°C. HT, hormone treated group; α-T, alpha-tocopherol.

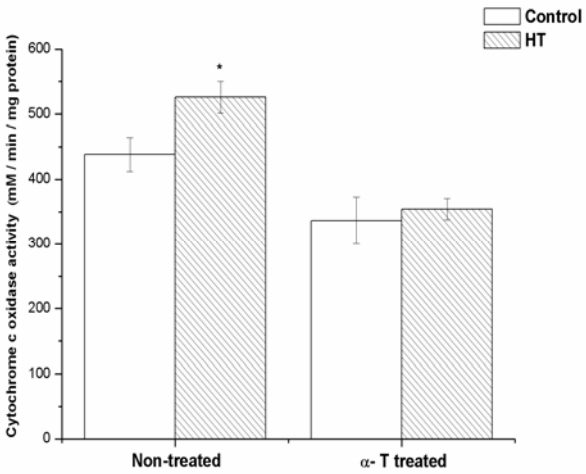

Figure 12 – Effect of thyrotoxicosis on cytochrome oxidase actitivity in *gastrocnemius* mitochondria of rats with severe thyrotoxicosis. Rats were treated for 5 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Muscles were studied 24 hours after the last T3 injection. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$). Results are expressed as mean ± SEM. Citochrome oxidase activity was expressed in μM ferricytochrome consumed per minute / mg protein at 38°C. HT, hormone treated group; α-T, alpha-tocopherol.

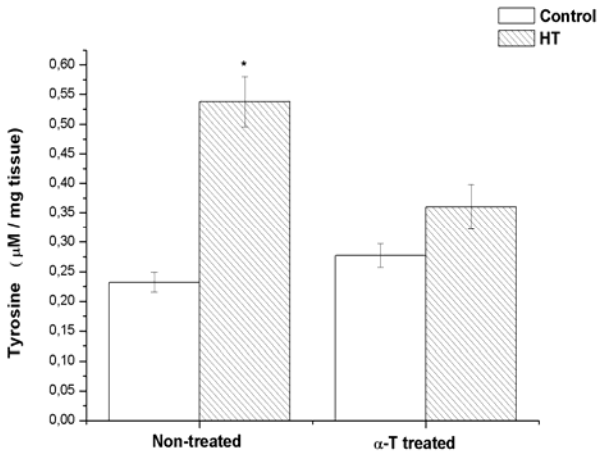

Figure 13 – Intracellular tyrosine levels in *gastrocnemius* muscle of rats with severe thyrotoxicosis. Rats were treated for 5 days with 100 μg/ 100 g body weight of T3 or corresponding volumes of solvent and treated or not with α-T 100 mg/ kg body weight. Muscles were studied 24 hours after the last T3 injection. Control and respective experimental group were compared using the Student unpaired t test. No symbol: No statistical difference; *: Statistical difference in relation control group ($p<0.05$); ϕ : Statistical difference in relation control+α-T group ($p<0.05$).Results are expressed as mean ± SEM. HT, hormone treated group; α-T, alpha-tocopherol.

DISCUSSION

The biochemical basis of the muscle weakness caused by thyroid hormones is uncertain but may be related to the impaired ability to phosphorylate creatine [8]. Previous studies reported that loss of muscle mass in the thyrotoxicosis state is mainly caused by increased protein degradation, in particular degradation of the myofibrillar proteins actin and myosin by proteolytic pathways [28, 13]. Moreover, reduced protein synthesis may also contribute to loss of muscle mass in hyperthyroid patients [29, 6]. It is known that ROS contribute to skeletal muscle remodeling, activating many proteolytic pathways in skeletal muscle [12], although there are not evidences that support the participation of oxidative stress in the muscle loss in thyrotoxicosis. All experimental HT groups exhibited high weight loss index, but only 5 days treatment with T3 decreased *gastrocnemius* muscle mass and SMRW significantly. The treatment with a antioxidant agent decreased muscle loss for 23.69% to 13.72%, and decreased peritoneal fat content in 41.75% compared to

respective control group. Maybe this fat loss contributes to increase in SMRW, since the muscle loss accompanied by fat loss may interfere in this relation.

In aerobic cells O_2 is reduced to water by cytochrome c oxidase complex in mitochondria, organelle present on abundance in skeletal muscle. This reaction occurs without the release of any intermediate in the O_2 reduction. However, despite the efficiency of the mitochondrial electron transport system, the nature of the alternating one-electron oxidation-reduction reactions it catalyses predisposes electron carriers to side reactions, in which an electron is transferred to O_2 directly, instead of to the next electron carrier in the chain, generating $O_2^{\bullet -}$, and this is observed in thyrotoxicosis. This radical is then converted by spontaneous or catalyzed dismutation into hydrogen peroxide (H_2O_2), which can be turned into highly reactive hydroxyl radical ($^{\bullet}OH$) in the presence of transition metals in the Fenton reaction [30]. Thyroid hormones promotes calorigenesis involving increases of ROS production in liver, resulting in transient elevations in the serum levels of tumor necrosis factor-alpha (TNF-alpha) and interleucine-1 (IL-1) through NF-kappa-B-associated mechanism. This event is virtually abolished by pretreatment with a kupffer cell inhibitor (GdCl3), the antioxidants alpha-tocopherol and N-acetylcysteine (NAC), and an antisense oligonucleotide targeting the primary RNA transcript of TNF-alpha, prior to hormone administration [31]. It is known that TNF- alpha and IL-1-alpha are considered endogenous pyrogens due to their direct effects on the hypothalamus, leading to activation of responses that decrease heat loss and increase heat production [32]. Thyroid hormones also induce higher basal proton leak in mitochondria, but the mechanism(s) underlying this event and the manner which the thyroid-hormone induce the leak are still poorly understood. There is increase in the expression of mitochondrial uncoupling protein-2 and 3 (UCP-2 and UCP-3) in skeletal muscle and liver, however it is quite clear that this do not cause the increased of basal proton leak caused by thyrotoxicosis [33]. In our study, the increased in internal temperature corresponds to increased in cytochrome c oxidase activity in HT groups, and the decreased in this parameter obtained through alpha-tocopherol administration was accompanied by a decreased in this enzyme activity. Cytocrome c oxidase is the terminal complex of the mitochondrial respiratory chain, responsible for approximately 90% of oxygen consumption in mammals, and is essential for virtually all energy production in cells. It is located in the mitochondrial inner membrane, and catalyses the oxidation of cytochrome c^{2+} to cytochrome c^{3+}

and the total reduction of oxygen [34]. The effect of alpha-tocopherol and their metabolites in mitochondria are unclear. Gille et al. [35] proposes that alpha-tocopherol reacts with mitochondrial substrates, as ubquinones, generating products that interfere in electron carrier in mitochondria. It is known that the administration of this antioxidant decrease the rates of superoxide radical generation, probably to modulate enzymes and substrates in respiratory chain [36, 35].

The acceleration of lipid peroxidation in skeletal muscle of rats with thyrotoxicosis has constantly been observed [10]. Assayama et al. [37] observed that treatment with alpha-tocopherol reverts partially TBARS levels on skeletal muscle, independently of the changes in oxidative enzymes and antioxidant enzymes, in other words, decrease in lipid peroxidation. In our study,lipid hydroperoxide measured by the chemiluminescence method incresead on 3 and 5 days HT. Tert-butyl hydroperoxide-initiated chemiluminescence was used to evaluate the integral level of nonenzymatic antioxidant defense. A lower level of antioxidant as a consequence of a past or current situation of oxidative stress will correspond to a higher chemiluminescence and has a positive correlation between chemiluminescence and lipid peroxidation [16, 38]. In this study it was evidenced a qualitative difference in all chemiluminescence curves, indicating alteration in cell membrane properties, as antioxidant composition, which modifies the kinetics pattern in α-T treated groups, by exhausting the oxidizable species more rapidly [39]. Treatment with α-T reverts chemiluminescence area in 3 days HT treatment, but not in 5 days HT treatment. Guarnier et al. [14] show that when chemiluminescence curve/area was increased, the mRNA expression of atrogin-1, a muscle-especific ligase in ubiquitylation associated with muscle loss, was also increased, and alpha-tocopherol treatment diminished the expression of this ligase, evidencing the participation of ROS on the modulation of proteolitic pathway through ubiquitin-proteassome proteolysis in cancer cachexia model. MDA levels, an end product of lipid peroxidation, was increased on day 3 in HT group and did not show changes on day 5. Alpha-tocopherol decreased MDA levels in HT group on day 3 and did not change MDA levels on day 5. These results indicate that day 5, MDA formed reacts with others substances, like proteins, oxidatively modifying them, and alpha-tocopherol is not be able to change it.

Yamada et al. [40] showed that high T3 levels increased carbonylation of myosin heavy chain in rat *soleus*, accompanied by a decreased of

GSH levels, and also reduced force production in this muscle. In our study, there was a decreased of protein carbonylation both in control as in HT groups treated with α-T, although there was significant change in HT groups in relation of correspondent control group only on day 5. In some myopathies, excessive oxidative stress may render the proteolytic capacity of this system insufficient, and thereby facilitate the accumulation of abnormal proteins through covalent crosslinking reactions and increased surface hydrophobicity [41], however in thyrotoxicosis it was not evidenced.

HT increased SOD and decreased catalase activities on day 3 and no changed on day 5. In 3 days HT α-T treated animals , the SOD and catalase activity returns to control levels. The catalase activity increased 60.27% when compared to HT non-treated group. Lawler et al. [42] found that hight levels of catalase can ameliorate the oxidative stress and muscle fatigue. Alpha-tocopherol treatment on day 5 increased these enzymes activities in relation to respective control groups, although catalase activity decrease 27% when compared to HT alpha-tocopherol non-treated group. Interestingly, when SOD increased, catalase activity decreased. This can be explained by the fact that superoxide in excess induces the SOD activity and inhibits catalase activity [11].

GSH and GSSG levels decreased in HT groups, with the exception of GSSG on day 3, that did not changed with alpha-tocopherol treatment. The stress index (GSSG/GSH-GSSG) increased in HT and HT plus α-T treatment only on day 3. Unlike what is found in several pathologies that involves oxidative stress where GSSG increase while GSH decrease, Jahngen-Hodge et al. [43] suggests that in tissues with high proteolityc activity, GSSG can form complexes with E1 and E2 ligases, enzymes involved in the ubquitin-proteasome protein breakdown pathway, as negative regulatory mechanism, and the stress index is not considered a good parameter to evaluate the oxidative stress rate in these experimental models. GSH also decreases by high glutathione peroxidase (GPX) activity, in presence of high levels of hydrogen peroxide, for instance. Total antioxidant capacity increased when HT group were treated with α-T on day 3, and antioxidant treatment also restore antioxidant capacity to controls levels on day 5. This occurs probably due directly antioxidant capacity of α-T, and also because this substance improved antioxidant parameters on *gastrocnemius* muscle.

Tyrosine is not synthesized or metabolized in skeletal muscle. Increased tissue levels or release of this amino acid, therefore, is consistent with increased protein breakdown, although reduced protein synthesis and changes in transmembrane transport of the amino acids may give rise to similar results [24, 44]. The oxidative stress and intracellular tyrosine levels results found in our study reinforce that alpha-tocopherol treatment in thyrotoxicosis slows down the skeletal muscle wasting pathways.

O'Neal et al. [13] found in their study that after 3 days administration of triiodothyronine 100 μg/ 100 g body weight there was an activation of proteolytic pathways, and decreased muscle weight and protein content in rat´s *extensor digitorum longus* muscle. Our study shows that oxidative stress in skeletal muscle also occurs in severe thyrotoxicosis, on 3 and 5 days hormone treatment, which can be involved in the activation of skeletal muscle wasting pathways. The membrane antioxidant α-T ameliorate antioxidant parameters, mainly on day 3, decrease mitochondrial respiration and internal temperature in all experimental times. Moreover, on day 3, when oxidative stress is more evident, α-T ameliorates these parameters, protecting *gastrocnemius* mass loss observed on day 5. Probably, alpha-tocopherol slows down the skeletal muscle wasting pathways in thyrotoxicosis, protecting skeletal muscle mass loss, diverting catabolism to lipolysis. These findings suggesting an active participation of reactive oxygen species in skeletal muscle alterations observed in thyrotoxicosis.

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LIST OF ABBREVIATIONS

α-T	Alpha-tocopherol
ANOVA	Analysis of variance
CL	Chemiluminescence
EDTA	Ethylene-diamine tetraacetic acid
HT	Hormone treated
MDA	Malondialdehyde
RLU	Relative Light Unit
ROS	Reative Oxygen Species
SOD	Superoxide dismutase
T3	3,5,3'-*l*-triiodothyronine
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TRAP	Total Radical-Trapping Antioxidant Paramete

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ANEXO

ANEXO A

Guide for Authors

Free Radical Biology & Medicine is an international, interdisciplinary publication encompassing chemical, biochemical, physiological, pathological, pharmacological, toxicological, and medical approaches to research on free radicals and oxidative biology. The journal welcomes original contributions dealing with all aspects of free radical and oxidant research including both in vitro and in vivo studies.

Publications

Original Contributions: Peer-reviewed, high-quality, concise research investigations that represent new and significant contributions to science.

Review Articles: Reviews of major areas or subareas in free radical biology. These articles are peer-reviewed. Letters suggesting topics or authors for reviews or forums or inquiring about the suitability of particular topics should be addressed to Dr. Henry Forman at the address listed above.

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Letters to the Editor: Comments on papers published in the journal and on other matters of interest to free radical researchers.

Book Reviews: As solicited by the editors.

Announcements and Calendar: Notices of forthcoming meetings, courses, and other events relevant to free radical researchers.

The Radical View: Editorials and news of general interest.

Methods in Free Radical Biology & Medicine: By invitation only.

Preparation of papers

Authors should consult a recent issue of the journal to familiarize themselves with the conventions and layout of the articles.

Responsibility for the accuracy of the material in the manuscript, including bibliographic citations, lies entirely with the authors.

Full-length papers

The entire text, including figure and table legends and the reference list, should be double-spaced, leaving a left margin of approximately 3 cm (1 inch). All pages should be numbered consecutively.

Title Page. Page 1 should be concise, descriptive, and informative. It should include (1) the title of the article (80 spaces maximum); (2) the authors' full names (first name, middle initial(s), and surname); (3) affiliations (the name of department (if any), institution, city, and state or country where the work was done), indicating which authors are associated with which affiliations; (4) the name, address, telephone and fax numbers, and e-mail address of the corresponding author.

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References. Type references double-spaced and number them consecutively in the order in which they are first mentioned in the text, not alphabetically. Cite references in the text, tables, and legends in sequential, numerical order, placing the numbers in square brackets. References cited only in tables or figure legends should be numbered in accordance with a sequence established by the first mention in the text

of the particular table or figure. Journal titles are to be abbreviated according to the *List of Journals Indexed in Index Medicus* published by the U.S. Department of Health and Human Services. Examples of reference style are as follows:

Journal:

[1] Smith, M. A.; Casadesus, G.; Joseph, J. A.; Perry, G. Amyloid-β and τ serve antioxidant functions in the aging and Alzheimer brain. *Free Radic. Biol. Med.* **33**:1194-1199; 2002.

Book:

[2] Sen, C. K.; Packer, L.; Hänninen, O., eds. *Handbook of oxidants and antioxidants in exercise.* Amsterdam: Elsevier; 1999.

Chapter in edited book:

[3] Zuo, L.; Clanton, T. L. Detection of reactive oxygen and nitrogen species in tissues using redox-sensitive fluorescent probes. In: Sen, C. K.; Packer, L., eds. *Redox cell biology and genetics, part A. Methods in enzymology, volume 352.* San Diego: Academic Press; 2002: 307-325.

Abstract:

[4] Freeman, B.; Aslan, M. Tissue oxidation and nitration reactions in a mouse model and humans with sickle cell disease (abstract). *Free Radic. Biol. Med.* **33:**S298; 2002.

Manuscripts that have been accepted for publication may be cited as "in press" in the reference list using the estimated year of publication:

[5] Hoshino, N.; Kimura, T.; Yamaji, A.; Ando, T. Damage to the cytoplasmic membranes of Escherichia coli by catechin-copper (II) complexes. *Free Radic. Biol. Med.* In press; 1999.

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Tables. Tables should be used sparingly: they should be used only when the data cannot be presented clearly in the text. Each table and every column should be provided with an explanatory heading, with units of measure clearly indicated. The same data should not be reproduced in both tables and figures. Footnotes to a table should be indicated by superscript, lowercase letters. Tables and illustrations (along with their footnotes or captions) should be completely intelligible without reference to the text.

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Nomenclature and Abbreviations. Stylistic details must be kept constant. For example, electron spin resonance is abbreviated either ESR or EPR (for electron paramagnetic resonance). Either can be used, but both should be given and stated as equivalent at the first mention. (This is the recommendation of the International EPR Society.) Formulas for radicals follow IUPAC recommendations and contain a superscripted (not centered) dot that precedes a charge, if any. Thus, superoxide is represented by $O_2^{\cdot -}$, not $O_2^{\ \cdot -}$, or some other permutation.

Other examples are $HO^{\cdot}$ or $^{\cdot}OH$ (not $OH^{\cdot}$), $RO^{\cdot}$, $ROO^{\cdot}$/$^{\cdot}NO_2$, $^{\cdot}CH_2OH$, etc. In the text, names of radicals are preferred, rather than using formulas in the middle of sentences. For names of radicals, use alkoxyl, peroxyl, and hydroxyl and not alkoxy, peroxy, etc. (correct nomenclature requires the 'l' on the end of radicals, as in methyl, hydroxyl, etc.). Use *tert*, not t-, etc., for abbreviations. For example, CORRECT: *tert*-butoxyl, *sec*-peroxyl; INCORRECT: t-butoxy, s-peroxy.

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Standard three-letter codes for the common amino acids may be used freely and without definition, but the one-letter codes should be restricted to comparisons of long protein sequences. Similar considerations apply to nucleosides and nucleotides. Standard three-letter codes for carbohydrates and for purine and pyrimidine bases may also be used. All other abbreviations should be defined when they first appear in the text. If an extensive list of abbreviations is used, please provide an alphabetical list with definitions followed by the references at the end of the article.

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