FERNANDO HENRIQUE BORGES

RELAÇÃO ENTRE O ESTRESSE OXIDATIVO E A CAQUEXIA CARDÍACA EM RATOS WISTAR PORTADORES DA FORMA SÓLIDA DO TUMOR DE WALKER-256

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

Orientador: Prof. Dr. Rubens Cecchini.

Co-orientadora: Profa. Dra. Flávia Alessandra Guarnier.

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Londrina, 04 de Outubro de 2013.

Dedico este trabalho aos meus pais, Durvalina e José e a meus irmãos, Andréa e Eduardo, pela confiança e pela oportunidade de poder realizar mais um sonho.

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“Não se deixe levar pela distância entre seus sonhos e a realidade. Se você é capaz de sonhá-los, também pode realizá-los.”

William Shakespeare.

BORGES, FH. **Relação entre estresse oxidativo e a caquexia cardíaca em ratos Wistar portadores da forma sólida do tumor de Walker-256**. 2013. 67 f. Dissertação (Mestrado em Ciências da Saúde) – Universidade Estadual de Londrina, Londrina, 2013.

RESUMO

A caquexia do câncer é uma doença caracterizada pela perda progressiva de massa corporal, não envolvendo apenas a musculatura esquelética, mas também a cardíaca. Embora existam relatos de perda de massa muscular cardiaca em ratos portadores de tumor, até o momento existem poucas relações entre estresse oxidativo e caquexia cardiaca. Assim, o presente trabalho objetivou investigar a relação entre estresse oxidativo e ativação de diferentes vias proteolíticas no coração de ratos portadores de tumor de Walker-256. Os animais foram divididos em: animais sem tumor e animais com tumor em 2 diferentes tempos (T5 e T10) - implantados subcutâneamente $8x10^7$ células/mL. Após o sacrifício, foram retirados o coração, divido em lados esquerdo (CE) e direito (CD) e o tumor. O índice de caquexia foi calculado. Os parâmetros de estresse oxidativo (EO) cardíaco foram analisados através das técnicas de quimiluminescência estimulada por terc-butil hidroperóxido (QL), quantificação de substâncias reativas ao ácido tiobarbitúrico (TBARS), e quantificação de proteínas carboniladas (PC). A proteólise foi determinada por meio de kits comerciais. A espessura dos ventrículos foi verificada por morfometria. A partir do 5º dia todos os animais inoculados com tumor apresentaram caquexia (6,85x±0,63% para 5 dias e 17,76x±1,82% para 10 dias). No músculo cardíaco, o CE não apresentou perda de massa, porém, no CD houve perda de 29% em 5 dias e 40% em 10 dias, o que foi reforçado na análise de morfometria. O CD mostrou baixos níveis de lipoperoxidação. As PC apresentaram o mesmo perfil em ambos os lados, tendo um aumento significativo em 5 dias e uma queda em 10 dias. O TBARS não apresentou diferenças significativas em nenhum tempo. A atividade proteolítica apresentou-se aumentada quando somente a calpaina foi avaliada no 5 dia, no CD. Os resultados demonstram que apesar de os animais apresentarem caquexia, não houve alteração de massa no musculo cardíaco do CE, diferentemente do CD. Com base nos resultados, pode-se concluir que, metabolicamente, as duas câmaras se comportam independentemente e que, o EO possui um papel diferente em cada um dos processos. Além disso, a modulação leva a distinta ativação de vias proteolíticas.

Palavras-chave: Caquexia cardíaca. Atrofia cardíaca. Estresse oxidativo. Tumor.

BORGES, FH. **Relationship between oxidative stress and cardiac cachexia in Wistar rats bearers of the solid form of Walker-256 tumor**. 2013. 67 p. Dissertation (Master's Degree in Health Science) – Universidade Estadual de Londrina, Londrina, 2013.

ABSTRACT

Cancer cachexia is a disease characterized by the progressive body mass loss, involving not only skeletal muscle but also cardiac muscle. Although it has been reported cardiac muscle wasting in tumor-bearing rats, there is little evidence linking oxidative stress and cardiac cachexia. Thus, the present study aimed to investigate the relationship between oxidative stress and activation of different proteolytic pathways in the heart of rats with Walker-256 tumor. Animals were divided in: animals without tumor and animals with tumor in 2 different times (T5 and T10) - $8x10^7$ cells/mL, subcutaneously implanted. After sacrifice, heart and tumor were removed and the heart was divided into left (LH) and right (RH). Cachexia index was calculated. The cardiac oxidative stress parameters (OS) were evaluated by the *tert*-butyl hydroperoxide stimulated chemiluminescence (CL), by the quantification of thiobarbituric acid reactive substances (TBARS), and of carbonyl protein (CP). Proteolysis was determined using commercial kits. The ventricles thickness was verified by morphometric analysis. From the 5th day, all tumor-inoculated animals presented cachexia (6.85±0.63% for 5 days and 17.76±1.82% for 10 days). In the cardiac muscle, LH did not presented loss of mass, however, in the RH there was loss of 29% in 5 days and 40% in 10 days, which was reinforced in the morphometric analysis. The RH presented low levels of lipoperoxidation. The CP demonstrated the same profile in both sides, showing increased levels only in 5 days. TBARS did not significantly alter in any experimental time. The analysis of proteolytic activity in both sides showed that only calpain activity of the RH presented alterations, with an increased activity observed only in the 5^{th} day. The results show that even though the animals presented cachexia, there was no changes in cardic mass of the LH, differently of the RH. Based on results, it is concluded that metabolically, the two chambers behave independently and OS has a different role in each case. Furthermore, these modulation leads to activation of proteolytic pathways in different ways.

Keywords: Cardiac cachexia. Cardiac atrophy. Oxidative stress. Tumor.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AIDS	sindrome da imunodeficiência adquirida
AMPK	adenosina monofosfato quinase
ATP	adenosina trifosfato
ATPase	adenosina trifosfatase
BG	banho de gelo
Ca^{2+}	cálcio
Cu-Zn-SOD	cobre-zinco-superóxido dismutase
DNPH	2,4-dinitrofenilhidrazina
EDTA	ácido etilenodiaminotetracético
ERN	espécies reativas do nitrogênio
ERO	espécies reativas do oxigênio
GPx	glutationa peroxidase
H_2O_2	peróxido de hidrogênio
HCl	ácido cloridríco
IFN-γ	interferon gama
IL-1	interleucina-1
IL-6	interleucina-6
K_2HPO_4	fosfato de potássio dibásico
KCl	cloreto de potássio
KH_2PO_4	fosfato de potássio monobásico
MDA	malondialdeido
Mn-SOD	manganês-superóxido dismutase
NaCl	cloreto de sódio
NADPH	nicotinamida adenina difosfonucleotídeo
NFκB	fator de transcrição nuclear kappa B
$O_2^{\bullet}$-	ânion superóxido
$OH^{\bullet}$-	hidroxila
PBS	tampão fosfato-salina
QL	quimiluminescência estimulada por *terc*-butil hidroperóxido
SOD	superóxido dismutase
TBA	ácido tiobarbitúrico
TBARS	substâncias reativas ao ácido tiobarbitúrico

TCA	ácido tricloroacético
TNF	fator de transcrição tumoral
URL	unidade relativa de luz

SUMÁRIO

1 INTRODUÇÃO

1.1 CAQUEXIA NO CÂNCER

A caquexia (do grego, *kakos* - mau e *hexis* – estado), é uma síndrome predominantemente associada à perda de massa muscular e à acelerada degradação de proteínas (LANGSTEN; NORTON, 1991; WALSMITH; ROUBENOFF, 2002). Está presente em muitas doenças crônicas como: câncer, AIDS, insuficiência cardíaca crônica, falência pulmonar crônica, cirrose hepática, insuficiência renal, artrite reumatóide, sepse, entre outras. A síndrome da caquexia está intimamente relacionada com a diminuição da sobrevida dos indivíduos que a portam por ser responsável pela falência multipla de órgãos.

É uma causa importante de morbidade e mortalidade, ocorrendo em até 80% dos pacientes com câncer avançado (pulmão, gastrointestinal e próstata) e 40% em pacientes com câncer de mama, sendo responsável por 20% dos óbitos destes indivíduos (TISDALE, 2002). Diferentes tipos de tumores demonstram diferentes tendências à indução de caquexia. A perda de massa está comumente associada com qualidade e expectativa de vida reduzida, sendo a perda de 30% de massa corporal total incompatível com a vida (TISDALE, 2004).

A caquexia geralmente representa uma consequência clínica de doenças crônicas que possuem resposta inflamatória sistêmica. Frequentemente apresenta síntese hepática elevada de proteínas de fase aguda, resultando em esgotamento de aminoácidos essenciais (WIGMORE, 2000). A depleção de massa corporal magra é a principal característica da caquexia, e envolve não apenas a musculatura esquelética, mas também proteínas cardíacas, resultando em alterações importantes no desempenho do coração (ARGILÉS *et al*., 2005).

A produção de uma variedade de citocinas pró-inflamatórias e a liberação de fatores caquéticos pelo tumor induzem um estado hipermetabólico no hospedeiro, causando um desequilíbrio no balanço energético, que resulta em perda funcional e de massa em diversos órgãos. Desta forma, os sintomas da síndrome caquética, incluindo a anorexia, aparecem em decorrência de estados que variam de alterações no balanço energético, produção de citocinas, alterações na produção de hormônios, variação no nível de atividade, e aumento da expressão de enzimas e proteínas (YOUNES *et al*., 2000; TISDALE, 2000; FEARON; MOSES, 2002;

ARGILÉS *et al*., 2003; ARGILÉS *et al*., 2005).

Alterações no metabolismo energético, ativação inespecífica crônica do sistema imune, e implantação de um quadro inflamatório são situações que levam ao aumento de espécies reativas de oxigênio e nitrogênio (MANTOVANI *et al*., 1998). Além disso, durante os últimos anos, os radicais livres têm sido apontados como importantes moduladores de processos biológicos, principalmente no que diz respeito ao controle de massa muscular, tanto esquelética, como cardíaca (POWERS, 2010). O estresse oxidativo é caracterizado pelo desequilíbrio entre a produção de espécies reativas de oxigênio (ERO) e nitrogênio (ERN) e a neutralização dos mesmos através de um sistema de defesa antioxidante (HALLIWELL; GUTTERIDGE, 2007). Vários mecanismos podem levar ao estresse oxidativo em pacientes com câncer.

1.2 TUMOR DE WALKER-256

O tumor de Walker-256, um carcinosarcoma mamário, tem sido empregado em modelos experimentais para induzir caquexia em ratos. Em curto espaço de tempo após sua implantação, verifica-se redução no peso do animal devido ao aumentado catabolismo de proteínas, lipídeos e carboidratos, além da dimunuição na ingestão de alimento. Aos 14 dias após o implante, a massa tumoral pode representar uma fração considerável do peso do animal e a morte frequentemente ocorre após este período (VICENTINO *et al*., 2002).

1.3 ESTRESSE OXIDATIVO

Radical livre é qualquer espécie capaz de uma existência independente que contenha um ou mais elétrons desemparelhados. Um elétron desemparelhado é aquele que ocupa um orbital atômico ou molecular sozinho (HALLIWELL; GUTTERIDGE, 2007). A presença de um ou mais elétrons desemparelhados muitas vezes torna estas espécies altamente reativas, apesar de esta reatividade ser variável. As fontes de geração intracelular de radicais livres incluem: autoxidação de pequenas moléculas, cadeia de transporte de elétrons, neutrófilos e resposta inflamatória (NADPH oxidase), membrana plasmática, retículo endoplasmático, peroxissomas e fissão homolítica (HALLIWELL; GUTTERIDGE,

2007).

Levando em consideração os conceitos de radicais livres e de defesa antioxidante, chegamos ao termo estresse oxidativo, que é utilizado quando o delicado balanço entre produção de radicais livres e defesa antioxidante é quebrado. Apesar de ser amplamente utilizado na literatura referente a radicais livres, este termo ainda não é muito bem definido. SIES (1991) definiu o termo como o “distúrbio no balanço pró oxidante/antioxidante, a favor do primeiro, levando à potencial lesão”. Tal dano é frequentemente chamado de dano oxidativo. Em princípio, estresse oxidativo pode resultar de: diminuição de antioxidante, ou aumento na produção de espécies reativas.

Muitos estudos indicam que as ERO são importantes mensageiros das vias de sinalização, envolvidas na adaptação celular (ALLEN; TRESINI, 2000). Enquanto altas concentrações de radicais livres podem lesar componentes celulares, níveis baixos a moderados podem desempenhar múltiplas funções regulatórias em vias de sinalização celular, como o controle da expressão gênica, e modulação da função do músculo estriado esquelético. A expressão sinalizada por ERO contribui para a adaptação de fibras musculares em resposta tanto ao aumento da atividade contrátil (como no exercício físico), quanto em períodos prolongados de desuso (como na imobilização). Este paradoxo na sinalização quanto à função das ERO deve-se provavelmente a diferenças tanto na magnitude, quanto no padrão temporal da produção de radicais livres (POWERS *et al*., 2010).

1.4 SISTEMAS ANTIOXIDANTES

Durante a respiração aeróbia e do metabolismo celular, as ERO, tais como o radical ânion superóxido ($O_2^{\cdot}$-), o peróxido de hidrogénio (H_2O_2) e radicais hidroxilas ($OH^{\cdot}$) são gerados como subprodutos. A geração do $O_2^{\cdot}$- torna-se um mediador nas reações em cadeia oxidativa para a produção de H_2O_2 por dismutação do radical $O_2^{\cdot}$- (McCORD; FRIDOVICH, 1969) seguido da redução para a produção de água e oxigênio. O H_2O_2 é estável e pode atravessar a membrana celular. No entanto, na presença de ferro livre no interior da célula, o H_2O_2 e $O_2^{\cdot}$- interagem, ocorrendo a reação de Haber-Weiss, para gerar $OH^{\cdot}$, que é altamente reativo e pode causar dano oxidativo (HALLIWELL; GUTTERIDGE, 2007). A geração das ERO é controlada por processos enzimáticos e não enzimáticos. Defesas enzimáticas

contra $O_2^{\cdot-}$ incluem a superóxido dismutase (SOD), que dismuta o ânion superóxido em peróxido de hidrogênio (FRIDOVICH, 1978). A dismutação do $O_2^{\cdot-}$ é mediada principalmente por Cu-Zn-SOD localizada no citoplasma e Mn-SOD localizada nas mitocôndrias (WEISIGNER; FRIDOVICH, 1973). O H_2O_2 gerado é convertido pela reação da glutationa peroxidase (GPx) (MILLS, 1957) e catalase (CHANCE; SIES; BOVERIS, 1979) para gerar finalmente água e oxigênio.

1.5 CAQUEXIA CARDÍACA

É uma síndrome de ordem metabólica que tem tomado um grande espaço de estudo nos últimos anos, estando diretamente relacionada à insuficiência cardíaca, e geralmente associada a um mau prognóstico (ANKER; SHARMA, 2002). Seu mecanismo ainda continua desconhecido, sendo associado a fatores neuro-hormônais, metabólicos e imunológicos que interagem para provocar um desequilíbrio anabólico/catabólico (MOUGHRABI; EVANGELISTA, 2007).

Pacientes diagnosticados com caquexia cardíaca têm uma taxa de mortalidade de 50% em 18 meses desde o diagnóstico, o que é expressivamente maior do que a maioria dos tipos de câncer (ANKER; COASTS, 1999).

A caquexia cardíaca foi descrita pela primeira vez por Pittman e Cohen em 1964. Em 2005, a Associação de Classificação Funcional de Nova York, classificou a caquexia cardíaca como sendo parte de uma síndrome de desnutrição grave, que ocorre habitualmente em pacientes com insuficiência cardíaca congestiva, em classe funcional III ou IV.

As células do coração possuem muito mais mitocôndrias entre as miofibrilas (cerca de 23%) do que as células do músculo esquelético (cerca de 2%), com isso, acabam gerando uma quantidade maior de peróxido de hidrogênio (H_2O_2) (SCHOEN, 2005). Desta forma podem gerar um aumento na lipoperoxidação e o desequilíbrio da homeostasia do cálcio (RADI; BUSH; FREEMAN, 1993).

Apesar de já ter sido relatada a perda de massa cardiaca em ratos portadores de tumor (BARREIRO *et al*., 2005; MARIN-CORRAL *et al*., 2010), a participação do estresse oxidativo nesta perda não está bem elucidada.

Trabalhos recentes apontam para o coração como um dos principais órgãos comprometidos na síndrome da caquexia. Alguns grupos apontam para o fato de algumas vias de indução serem comuns às vias sinalizadoras encontradas

na atrofia do músculo estriado esquelético, uma vez que ambos os tecidos possuem semelhanças entre si. A razão pela qual o câncer pode afetar a regulação da massa do coração ainda não é bem esclarecida, mas pode estar relacionados com a quantidade ou alguns tipos de fatores humorais liberados pelo tumor (MANNE, 2013). Alguns autores sugerem que a IL-6 está associada com a diminuição da síntese de proteína muscular (VAN HALL; STEENSBERG; FISCHER, 2008) e a ativação da AMPK - proteína quinase ativada por adenosina monofosfato (KELLY; KELLER C; AVILUCEA, 2004). A ativação da AMPK pode atuar como um modulador molecular que ativa as vias de conservação de energia, como por exemplo, a via de síntese de proteínas (CHAN; DYCK JR, 2005).

1.6 PROTEÓLISE MUSCULAR

Muitos sistemas proteolíticos contribuem para a degradação de proteínas musculares. As proteases mais investigadas no músculo estriado esquelético são as proteases lisossomais, as proteases ativadas pelo cálcio (ou calpaínas) e o sistema proteasomal (POWERS *et al.*, 2004).

Apesar de as proteases lisossomais serem ativadas no músculo estriado esquelético, levando à atrofia, a importância destas proteases parece limitada (FURUNO; GOLDBERG, 1986). Evidências apontam que tanto as calpaínas, quanto o sistema proteasomal desempenham papel importante na perda protéica durante a atrofia (IKEMOTO *et al.,* 2001). Estudos revelam que outra protease, a caspase-3, também pode contribuir para formas específicas de atrofia muscular (DU *et al*., 2004).

As proteínas musculares (50-70%) existem em forma de complexos de actina e miosina. O sistema proteasomal pode degradar complexos ou monomêros de actina e miosina, enquanto as proteases lisossomais não degradam complexos intactos de actina-miosina (TIDBALL; SPENCER, 2002). Essa observação sugere que a liberação de miofilamentos seja o limite para ativação da degradação protéica. Alguns trabalhos indicam que tanto as calpaínas quanto a caspase-3 são capazes de produzir dissociação dos complexos de actina-miosina (DU *et al.*, 2004; GOLL *et al.*, 2003). A ativação de uma ou ambas as proteases é necessária para a degradação protéica de miofilamentos durante a perda muscular.

1.7 ESTRESSE OXIDATIVO E PROTEÓLISE MUSCULAR

O estresse oxidativo provoca modificação no potencial redox da célula que conduz a mudanças nos padrões de regulação da sinalização da expressão gênica. O que se sabe é que a sinalização redox pode influenciar uma série de ativadores transcricionais, levando à alterações na expressão gênica e mudanças no fenótipo da célula. Estas mudanças seriam específicas para cada tipo celular ou grupo de células. De maneira geral, o principal mecanismo pela qual a sinalização redox controlaria a expressão gênica seria através do controle das quinases e fosfatases pela fosforilação/desfosforilação de proteínas ou peptídeos responsáveis por sua vez, pelo aumento ou a ativação de fatores de transcrição (CHIARUGI; CIRRI, 2003; TORRES; FORMAN, 2003). Como exemplo, as ERO são conhecidas por ativar o fator de transcrição nuclear κ-B (NF-κB) (KANDARIAN; JACKMAN, 2006), e isso parece ser fundamental no entendimento da adaptação celular do músculo estriado esquelético, tanto em estados fisiológicos, quanto patológicos.

Contudo, a caquexia por si só leva a atrofia cardíaca, pelo aumento da proteólise, promovendo a perda da função do órgão. (MORRINSON; EDWARDS, 1991). Não é relatado ainda qual o verdadeiro fator que desencadeia a caquexia, porém parece haver uma forte influência do TNF, IL-1 e IL-6 (YASUMOTO *et al*., 1995). Acredita-se que esses fatores podem desencadear a ativação do fator de transcrição NFkB, sendo ele o responsável pela degradação muscular (HU *et al.,* 2004).

1.8 MECANISMO DE PROTEÓLISE MUSCULAR

1.8.1 Calpaínas

As calpaínas (I e II) são proteases com um grupo cisteína, dependentes de cálcio, que são ativadas no músculo estriado esquelético durante o desuso ou estados patológicos (GOLL *et al.*, 2003). Apesar de as calpaínas não degradarem diretamente as proteínas contráteis, liberam proteínas sarcoméricas pela clivagem de proteínas do citoesqueleto (como titina e nebulina) responsáveis

pela ancoragem dos elementos contráteis (PURINTRAPIBAN; WANG; FORSBERG, 2003). A atividade das calpaínas é regulada por muitos fatores, incluindo cálcio citosólico e concentração de calpastatina, um inibidor endógeno de calpaína (GOLL *et al*., 2003). Além disso, a atividade da calpaína pode ser aumentada por qualquer fator capaz de elevar concentrações de cálcio citosólico e/ou diminuir níveis de calpastatina. Sabe-se que a inatividade muscular está associada com sobrecarga de cálcio e ativação de calpaína (KOURIE, 1998). Apesar de os mecanismos responsáveis por esta sobrecarga de cálcio sejam desconhecidos, é provável que haja envolvimento de ERO no distúrbio iônico das células (KONDO; NISHINO, ITOKAWA, 1994). Uma explicação para esta hipótese é que a formação de aldeídos provenientes da cadeia de lipoperoxidação, como o 2,4-hidroxinonenal e o malondialdeído, reduzem a atividade da bomba de Ca^{2+}-ATPase (SIEMS *et al*., 2003), retardando a remoção do cálcio do meio intracelular, promovendo seu acúmulo, e consequentemente ativando as vias dependentes de cálcio.

1.8.2 Caspase-3

Numerosas vias de sinalização podem levar à ativação de um único grupo de proteases denominado de “caspases”. Juntas, as caspases são endoproteases que degradam proteínas e, em alguns casos, causam morte celular programada (apoptose). Na célula, as caspases são expressas como precursores inativos chamados de pró-caspases, e a ativação das caspases pode levar à quebra de proteínas e apoptose (POWERS *et al*., 2004). Evidências sugerem que a caspase-3 possa desempenhar um papel importante na perda de massa desenvolvida no diabetes, promovendo degradação dos filamentos de actina e miosina (no complexo). A supressão da atividade da caspase-3 suprime a perda de massa desencadeada pelo diabetes (DU *et al.,* 2004). O controle da caspase-3 é complexo e envolve muitas vias de sinalização. No caso do diabetes, é possível que a caspase-3 seja ativada pela ativação da caspase-12 (via liberação de cálcio), e/ou ativação da caspase-9 (via mitocondrial). A chave da interação entre as vias de ativação da caspase-3 é que ambos os caminhos podem ser ativados por ERO (PRIMEAU; ADHIHETTI; HOOD, 2002). A liberação de cálcio ativa caspase-3 por uma via de sinalização que culmina na ativação da caspase-12. A ativação da calpaína também pode contribuir para a ativação da caspase-3 via liberação de

cálcio. A via mitocondrial de ativação da caspase-3 é complexa e pode ser iniciada por numerosas interações incluindo as ERO e um índice pró-apoptótico alto na mitocôndria (LEEUWENBURGH, 2003). As ERO podem levar à liberação do citocromo c, resultando na ativação da caspase-9 e na subsequente ativação da caspase-3.

1.8.3 Sistema Proteassomal

No sistema proteassomal de proteólise, as proteínas podem ser degradadas pelas partes proteassomais 20S e 26S (GRUNE *et al*., 2003). A porção 26S do proteassoma é composta pela parte central 20S com um complexo regulatório 19S (também chamado de PA700), que possui atividade de ATPase e desempenha um papel importante na degradação ATP-dependente de proteínas ubiquitinadas. No 26S, a ubiquitina se liga covalentemente aos substratos de proteína e os marca para degradação. As proteínas ubiquitinadas são reconhecidas e ligadas pela parte 19S. A energia da hidrólise do ATP remove as cadeias de poliubiquitina, liberando as proteínas que alimentam a porção 20S, que, por sua vez, pode reconhecer proteínas oxidativamente modificadas sem ubiquitinação (GRUNE; DAVIES, 2003). Parece possível que o estresse oxidativo de maneira isolada possa acelerar a quebra protéica via 20S.

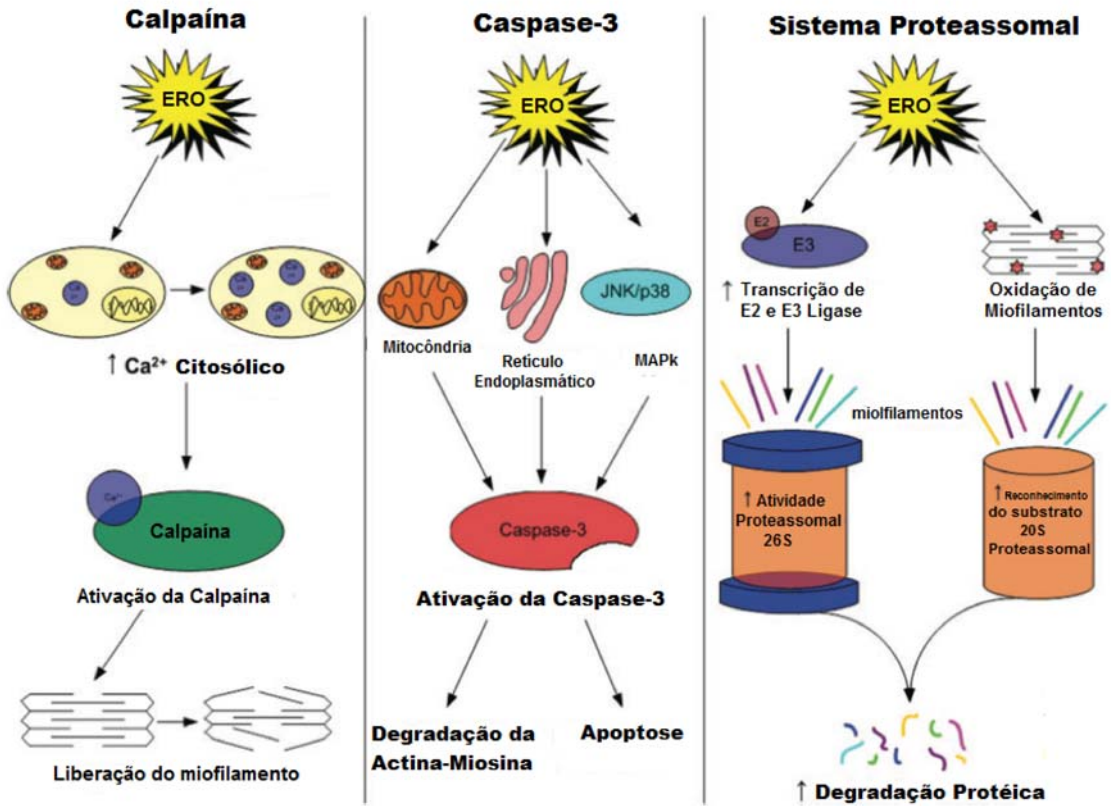
A ligação de ubiquitina a substratos de proteína requer uma enzima ativadora (E1), uma enzima conjugadora (E2), e, em muitos casos, uma enzima ligadora tecido-específica (E3). Duas E3, a atrogina-1 (também chamada de F-box) e *ring finger-1* são específicas do músculo estriado esquelético, utilizadas em pesquisa como importantes marcadores de perda de massa muscular (GOMES *et al.*, 2001).

Tem sido demonstrado que as ERO regulam a expressão gênica destes componentes básicos do proteasoma (LI *et al.*, 2003), assim como todas as outras vias proteolíticas.

Levando em consideração que calpaínas e caspases são ativadas pelo cálcio, um mecanismo que provavelmente liga estresse oxidativo com sobrecarga de cálcio consiste na formação de aldeídos induzida pelas ERO sendo capaz de inativar a Ca^{2+}-ATPase, diminuindo a remoção do cálcio da célula, com conseqüente ativação das vias (KOURIE, 1998). Além disso, a via de Caspase-3 também pode ser ativada diretamente por ERO, ou, indiretamente, ser ativada pela

saída do citocromo c da mitocôndria, por meio da ação de ERO (POWERS; KAVASIS; McCLUNG, 2007).

Figura 1 – Vias proteolíticas: As espécies reativas de oxigênio são consideradas como precursoras de ativação de diversas vias proteolíticas no músculo esquelético, incluindo calpaína, caspase-3 e o sistema proteassomal



Fonte: adaptado do artigo de revisão de Power, 2010.

Uma das grandes limitações sobre relacionar o estresse oxidativo e a perda de massa cardíaca, está na pouca literatura disponível sobre esse assunto.

2 JUSTIFICATIVA

A caquexia é uma síndrome amplamente conhecida em pacientes portadores de câncer. Dados clínicos demonstram que o coração é um órgão extensivamente comprometido durante a síndrome da caquexia pelo câncer, apresentando perda de massa e falência funcional. Evidências recentes demonstram que a perda de massa na caquexia cardíaca possui similaridades com a perda de massa no músculo estriado esquelético (MANNE *et al.*, 2013). Apesar de recentes trabalhos demonstrarem a perda de massa cardíaca relacionada com o estresse oxidativo (BARREIRO *et al.*, 2005; MANNE *et al.,* 2013), pouco se sabe sobre a influência dos radicais livres na ativação de vias proteolíticas. Isso torna este campo um amplo nicho de investigação.

3 OBJETIVOS

Investigar a participação entre o estresse oxidativo e a caquexia cardíaca em ratos portadores de tumor de Walker-256 em diferentes tempos experimentais.

3.1 OBJETIVOS ESPECÍFICOS

- Avaliar a relação entre peroxidação lipídica e atividade proteolítica no modelo em questão;

- Verificar a relação entre oxidação de proteínas e atividade proteolítica no modelo em questão.

4 METODOLOGIA

4.1 ANIMAIS

Foram utilizados ratos Wistar machos pesando entre 200-250g, provenientes do Biotério Central e mantidos no Biotério do Departamento de Ciências Patológicas da Universidade Estadual de Londrina durante a fase de experimentos. Os animais foram acomodados em gaiolas coletivas contendo no máximo 5 animais, com ração (Nuvilab CR-1, Nuvital) e água *ad libitum*. Todos os procedimentos foram realizados mediante aprovação do projeto pelo Comitê de Ética em Experimentação Animal da Universidade Estadual de Londrina sob o número 029/2013. Este projeto seguiu as recomendações do Código Brasileiro para Utilização de Animais de Laboratório.

4.2 MANUTENÇÃO DO TUMOR

O tumor de Walker 256, um carcinosarcoma, é mantido em laboratório através de passagens semanais de células viáveis para a cavidade abdominal de ratos Wistar adultos machos. Para indução do tumor sólido durante a fase experimental, 8,0 x 10^7 células viáveis, determinadas pelo método de contagem em câmara de Neubauer e exclusão pelo azul de Tripan foram retiradas das passagens intraperitonial e inoculadas intramuscular no flanco direito dos animais do grupo experimental.

4.3 DELINEAMENTO EXPERIMENTAL

Os animais foram divididos em 2 grupos: animais sem e com tumor - implantados subcutâneamente com solução de 8,0 x 10^7 células viáveis em PBS. Os animais com tumor foram subdivididos em diferentes tempos experimentais, 5 e 10 dias.

4.4 EUTANÁSIA DOS ANIMAIS E COLETA

No momento da eutanásia, os animais foram submetidos à anestesia com ketamina/xilazina (100/160 mg/kg), sofreram abertura da cavidade abdominal e a ruptura do músculo diafragma, o coração foi imediatamente colhido e divido em direito e esquerdo, sendo pesado, aliquotado e estocado em nitrogênio líquido. O músculo gastrocnêmio esquerdo de todos os grupos de animais também foram retirados e pesados para a realização do cálculo da perda de músculo esquelético. O tumor dos grupos de animais experimentais, tumor 5 e 10 dias foi dissecados cuidadosamente para pesagem e realização do cálculo do índice de caquexia. Após esses procedimentos, o músculo gastrocnêmio e o tumor foram descartados.

4.5 PREPARO DO MÚSCULO CARDÍACO

Para cada determinação, uma parte do miocárdio foi retirado do nitrogênio líquido, pesado e levado à homogeneização em gelo por 30s, em homogeneizador Ultra-Turrax, em tampão KH_2PO_4/K_2HPO_4 em KCl 120mM, pH 7,4. Para a técnicas de quimiluminescência estimulada por *terc*-butil hidroperóxido (QL), quantificação de malondialdeído, o homogenato foi preparado na concentração de 10mg/mL. A quantificação de proteínas, com um padrão de albumina bovina, foi realizada nos homogenatos de 50mg/mL.

4.6 DETERMINAÇÃO DO ESTADO CAQUÉTICO

A determinação da caquexia foi feita de acordo com a seguinte fórmula:

onde :

$$\%\ \text{perda de massa corpórea} = \left(\frac{\text{mi - mf + gmc + massa do tumor}}{\text{mi + gmc}}\right) \times 100$$

mi - massa inicial do animal com tumor;

mf - massa final do animal com tumor;

mt - massa do tumor;

gmc - ganho de massa do animal controle.

Para este cálculo, foi utilizada a média do ganho de massa corpórea obtida através do acompanhamento do ganho de massa corporal apresentado pelos animais do grupo controle durante 5 e 10 dias. Os animais foram considerados caquéticos quando a porcentagem de perda de massa corpórea for maior que 5%. A perda de massa muscular (%) e a perda de massa do miocárdio (%) foram calculadas usando os valores de massa dos músculos gastrocnêmios e do miocárdio respectivamente, em relação à média apresentada dos respectivos músculos dos animais do grupo controle (GUARNIER *et al.,* 2010)

4.7 Técnicas para Determinação de Estresse Oxidativo

Todos os homogenatos preparados a partir dos músculos cardíacos dos grupos de animais foram submetidos às seguintes técnicas para determinação do estresse oxidativo prévio sofrido pelo tecido: quimiluminescência estimulada por *terc*-butil hidroperóxido (FLECHA; LLESUY; BOVERIS, 1991), quantificação de proteínas carboniladas (REZNICK; PARKER, 1994) e quantificação de substâncias reativas ao ácido tiobarbitúrico (OLIVEIRA; CECCHINI, 2000).

4.7.1 Quimiluminescência Estimulada por *terc*-butil Hidroperóxido

A técnica foi realizada conforme descrito por Flecha, Llesuy e Boveris (1991). O composto *terc*-butil hidroperóxido é um potente formador de radicais peroxil que ataca os lipídeos de membrana gerando lipoperóxidos que podem reagir com outros lipídeos, oxidando-os. Assim, o *terc*-butil inicia uma reação de lipoperoxidação que pode ser detectada através da emissão de fótons ocorrida durante a formação dos lipoperóxidos. Para esse teste utilizou-se 10mg/mL do tecido cardíaco homegenizado em tampão fosfato monobásico a 10mM pH 7,4 NaCl 0,9%. Em 875µL do homogenato foi acrescentado 105µL do mesmo tampão utilizado para preparar o homogenato e posto em incubação por 5 minutos em banho 37ºC, após esse período foi acrescentado 20µL da solução de *terc*-butil hidroperóxido (38,6µL de *terc*-butil em 1mL de água), deixando que o luminômetro TD 20/20 marca Promega® faça a leitura utilizando uma sensibilidade de 68,5%. Os resultados foram expressos em unidade relativa de luz por miligrama de tecido (URL/mg tecido).

4.7.2 Quantificação de Proteínas Carboniladas

As proteínas carboniladas foram quantificadas como descrito por Parker e Reznick (1994), com modificações. Cerca de 50mg do músculo cardíaco foram colocados em tubos contendo 1mL de tampão de homogenização [tampão fosfato 50mM, 1mM de ácido etilenodiamino tetra-acético (EDTA), pH 7,4]. As amostras de tecido foram homogenizadas, e centrifugadas a 3000g durante 10min a 4°C. Foi distribuído em 3 tubos, um volume de 250µL da cada amostra, os tubos permaneceram em banho de gelo (BG) durante todo o tempo. Foi adicionada em dois tubos de cada amostra 1mL de solução de 2,4-dinitrofenil-hidrazina (DNPH) e no terceiro tubo 1mL de 2,5N de ácido clorídrico (HCl), incubando-os durante 1 hora em BG agitando em vórtex a cada 15min, a seguir as amostras foram centrifugadas a 3000g durante 10min a 4°C. Em seguida, todas as amostras foram lavadas com 1250mL de 20% (p/v) de ácido tricloroacético (TCA) e incubadas durante mais 20 minutos em BG. Outra lavagem foi realizada com TCA a 10% e incubadas por mais 20 min em BG. Após este período as amostras foram centrifugadas a 3000g durante 10min a 4°C. Finalmente, os péletes foram lavados três vezes com 1mL de etanol (1:1, v/v) para remover os contaminantes de lípideos livres e DNPH. No final os precipitados foram dissolvidos em 1mL de 0,6M de cloridrato de guanidina. A quantificação foi calculada através da leitura da absorvância a 355-390nm, pico das amostras tratadas com DNPH e comparadas com as amostras tratadas apenas com 2,5N de HCl. Foi utilizada a seguinte fórmula para o calculo: C = (Abs 390 - Abs 355) x 45,45 nmol/mL, sendo que, C é a concentração de DNPH/mL e 45,45 coeficiente de absorção. Os valores finais foram expressos em nanogramas por mililitro de proteína total.

4.7.3 Quantificação de Substâncias Reativas ao Ácido Tiobarbitúrico

Realizado conforme descrito por Oliveria e Cecchini (2000). A peroxidação lipídica do tecido cardíaco de todos os grupos foi determinada pela reação de TBARS. O malondialdeído (MDA) formado durante a peroxidação lipídica reage com o ácido tiobarbitúrico (TBA), para produzir um produto colorido, o aduto (TBA)-MDA. Em solução ácida, este aduto absorve a luz em 532nm e é facilmente extraído em solventes orgânicos, tais como n-butanol. Foram medidos os níveis de

MDA e os resultados expressos em nmol/g de tecido, tal como descrito anteriormente.

4.8 Comportamento da Modulação da Massa Cardíaca

O nível de atividade proteolítica total foi determinado por meio de kits comerciais (Proteasome-Glo™ Chymotrypsin-Like Cell-basead Assay para atividade da quimotripsina, Proteasome-Glo™ Caspase-Like Cell-basead Assay, para a atividade da caspase e Calpain-Glo™ Protease Assay para análise da atividade da calpaína) que aponta, ou não, desequilíbrio no catabolismo protéico do músculo cardíaco.

4.9 Quantificação de Proteínas

A quantificação de proteínas dos homogenatos foi determinada, utilizando albumina bovina como padrão (LOWRY *et al.*, 1951; MILLER, 1959), para normalização dos resultados.

4.10 Morfometria dos Ventrículos

Os ventrículos foram retirados e separados dos átrios por corte transversal, sendo esses últimos descartados. Os cortes transversais dos ventrículos foram colocados em frascos contento formalina tamponada 10% e deixados por 24 horas. Após esse período, os músculos foram lavados em água corrente por 24 horas e depois armazenados em álcool 70% até o momento da inclusão. As peças foram incluídas em parafina e cortes semi-seriados de 5µm de espessura foram realizados (5 cortes/lâmina). Após a confecção das lâminas, as peças foram coradas em solução de hematoxilina e eosina, fotografados em fotomicroscópio no aumento de 5x (Ziess Discovery V8 com câmera AxioCam ERC5s) e a análise morfométrica para quantificação das espessuras dos ventrículos foram realizadas por meio do programa Image-Pro Plus® 4.5 (Media Cybernetics, Rockville, EUA).

4.11 ANÁLISE ESTATÍSTICA

Os resultados foram expressos por meio da média e erro padrão da média dos grupos. A análise de significância foi realizada por *two-way* ANOVA para os testes de quimiluminescência estimulada por *terc*-butil hidroperóxido e *one way* ANOVA seguido do teste do Bonferroni como pós teste, para as demais análises. Os resultados foram considerados significativos quando $p<0,05$. O programa Origin 8.6, GraphPad Prisma 5 e Microsoft Excel 2011 for Mac foram utilizados para as análises estatísticas. O número de animais (n=8) foi determinado baseado em estudos prévios do nosso laboratório (GUARNIER *et al*., 2010) e de outros estudos (AGTEN *et al,* 2011), possibilitando, inclusive, a exclusão de resultados que foram discrepantes dentro de um mesmo grupo (determinação de *outlier* pelo teste de Grubbs: http://www.graphpad.com/quickcalcs/Grubbs1.cfm), assumindo uma distribuição normal dos resultados (testes paramétricos).

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ANEXOS

ANEXO A

Artigo

Differences between right and left heart in aspects related with oxidative and proteolytic profile on a model of cancer-induced cardiac cachexia.

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Abstract

AIMS: Measure the relationship between heart mass loss in cachexia, oxidative stress and proteolytic inhibition/activation.
METHODS AND RESULTS: In the present study, we evaluated right (RH) and left (LH) heart of rats bearing Walker-256 solid tumor for 5 and 10 days and compared to healthy animals. Determination of cachexia was calculated on 5th (6.85%) and 10th (17.76%) days after tumor implantation. Cardiac mass (CM) did not change for the LH and decreased in the RH (29% at 5th and 40% at 10th days). When *t*-butyl stimulated chemiluminescence was evaluated, increasing in LH and decreased in the RH was found. TBARS test remained the same for both sides all times, and carbonyl proteins increased ($p<0.05$) at 5th and decreased at 10th day after tumor implantation for both sides studied. When proteolytic activities were evaluated, calpain-like increased at 5th day only in the RH. CM was progressively decreased on RH with tumor progression, and oxidative stress was inversely proportional to the increase of calpain-like activity. On the LH, oxidative stress and calpain showed different behaviors.
CONCLUSION: Oxidative stress appears to be differently involved in mass modulation on both sides. Their behaviours are different and can influence responses presented on total heart assays.

Keywords: Oxidative stress. Cardiac cachexia. Cardiac atrophy.

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Introduction

Cancer cachexia is a complex disease characterized by progressive loss of weight, in association with anorexia, asthenia, anemia and alterations on immune function [1]. Depletion of lean body mass is the main characteristic of cachexia [2], but recent evidences have demonstrated involvement of cardiac proteins besides skeletal muscle depletion, what results in important changes on heart performance [3].

In recent years, free radicals have been implicated as important modulators of biological processes, particularly in aspects regarded to the control of muscle mass, both skeletal and cardiac [4,5,6,7]. Recent evidences point to the fact that on muscle tissue, activation of proteolytic pathways appear to be related to increased levels of reactive oxygen species (ROS) [8]. One of the mechanisms purposed is that high ROS production leads to highly reactive aldehydes, causing dysfunction in the Ca^{2+}-ATPase pump, delaying the removal of cytosolic calcium, and thus, the activation of calcium-dependent pathways [9].

Cadiac cachexia is a syndrome of metabolic order that has taken great study space in recent years, being directly related to heart failure, and generally associated with a bad prognosis [10] Patients diagnosed with cardiac cachexia has a mortality rate of 50% at 18 months since diagnosis, which is significantly greater than most cancers [11]. Although loss of heart muscle mass has been reported on rats bearing tumor with presence of oxidative stress [14] and that modulation of cardiac mass seems to be similar to the modulation presented on skeletal muscle [12,13], until the present moment there is no evidence about the relationship between oxidative stress, cardiac cachexia and proteolytic inhibition/activation on cardiac tissue.

For this purpose, the presence of oxidative stress and the activation of main proteolytic pathways on myocardium mass modulation were investigated in rats with solid form of Walker-256 tumor, that is characterized by rapid growth and marked metabolic changes in the host.

Methods

Animals and tumor implantation

Adult male Wistar rats (200-250g) were placed in collective cages containing not more than 5 animals, with commercial food (CR Nuvilab-1®, Nuvital®) and water *ad libitum.* Rats were divided into two groups, designated as controls and tumor hosts. The former group received 500uL of phosphate-buffered saline (PBS) administered intramuscularly (n=8). The other group received a Walker-256 tumor cell suspension ($8.0x10^7$ cells in 500uL of PBS) injected intramuscularly into the right hindlimb flank (n=16). Tumor cells were maintained in our laboratory as an ascitic intraperitoneal tumor ($2.0x10^6$ cells/500uL of PBS). At the moment of implantation, tumor cells were removed from the peritoneal cavity (with 8μL/mL of 5000 IU/mL heparin) and centrifuged at 1000xg. Intermediate phase was collected and the percentage of viable cells was determined by trypan blue dye method of exclusion (nonviable cells stained blue), using Neubauer chamber.

After 5 and 10 days, animals implanted with tumor cells were anesthetized with ketamine and xylazine (100/160mg/kg i.m.) and euthanized by rupture of diaphragm muscle. The heart was immediately collected, weighed, divided in right (RH) and left (LH) portions, and stored at -80°C until use. The left gastrocnemius muscle of all groups was also removed and weighed for loss of skeletal muscle calculation. On tumor bearing groups, tumors were carefully dissected and weighed for calculation of cachexia index [14]. Since a pilot study demonstrated that there were no differences between controls when 5^{th} and 10^{th} days were analyzed, control group was collected 5 days after PBS implantation and considered in all comparisons. All procedures were approved by the Ethics Committee on Animal Experimentation in our Institution (ref.029/2013).

Morphometry of the ventricles

One portion of the ventricles were removed and fixed in formalin 10% for 24 hours. After this time, the muscles were washed in current water for 24 hours and then stored in alcohol 70% until inclusion. Heart pieces were embedded in paraffin and semi-serial cuts of 5μm um were performed (5 cuts/slide). After this

procedure, cuts were stained with hematoxylin and eosin. Thickness of ventricles walls was then measured 5 times/cut and thickness of right and left ventricles were determined on Image-Pro Plus® 4.5 (Media Cybernetics, Rockville, USA) software.

Tissue preparation

One part of each side of the heart myocardium was weighed and homogenized into ice for 30 seconds on an Ultra-Turrax homogenizer, in KH_2PO_4/K_2HPO_4 buffer 120mM KCl, pH 7.4, and submitted to the following techniques: chemiluminescence induced by *tert*-butyl hydroperoxide (CL) [15] and quantification of thiobarbituric acid reactive substances (TBARS) [16], with homogenate prepared at a concentration of 10mg/mL. The quantification of carbonyl proteins (CP) was performed at homogenate 50mg/ml [17].

Oxidative stress parameters

Chemiluminescence induced by tert-butyl hydroperoxide. Reaction mixtures were placed in 1ml luminescence tubes containing the following: total cardiac muscle homogenate from control and tumor-bearing rats (10mg/mL); 30mM KH_2PO_4/K_2HPO_4 buffer (with 120mM KCl, pH 7.4); and 3mM *tert*-butyl hydroperoxide, in a final volume of 1mL. The *tert*-butyl hydroperoxide–initiated chemiluminescence (CL) reaction was assessed by luminometer (TD/20 20; Turner Designs) with a response range of 300–650nm. The tubes were kept in the dark until the moment of assay, which was carried out at a room temperature of 32ºC. This technique was described by Gonzalez-Flecha *et al* (1990) [15], using heart as one of the tissues studied. For each animal, in 40min curve, where each point represented the differential smoothing of 3600 readings, was obtained by interpolation. The results were expressed in relative light units per gram of tissue (RLU/mg tissue), and after the final calculation a final curve was determined by Gaussian fit. The entire curve and area, extracted by integral calculus of each animal curve, were used to determine the lipid hydroperoxides present in the sample.

Quantification of thiobarbituric acid reactive substances. Lipid peroxidation of heart homogenates of all groups was determined by reaction of TBARS. Malondialdehyde (MDA) formed during lipid peroxidation reacts with thiobarbituric acid (TBA) to produce a colored product, the adduct with protein (TBA)-MDA. In acid solution, this adduct absorbs light at 532 nm and is easily extractable in organic solvents, such as n-butanol. MDA levels were measured and results expressed in nmol/g tissue as described by Oliveira and Cecchini, 2000 [16].

Quantification of carbonyls protein. Carbonyl protein content was measured as described by Reznick and Parker,1994 [17], with modifications. Briefly, about 50mg of cardiac muscles from control and tumor-bearing animals were placed in glass homogenization tubes containing 1mL of homogenizing buffer [50mM phosphate buffer, 1mM ethylenediamine tetraacetic acid (EDTA), pH 7.4]. Tissue samples were homogenized and incubated for 15min in an ice bath. The samples were centrifuged at 3000g for 10 min at room temperature (RT), and 1mL of each protein extract was placed in glass tubes. A volume of 1mL of 2,4-dinitrophenyl-hydrazine (DNPH) solution prepared in 2.5N HCl was added to each tube, and the reaction mixtures were incubated for 1h at RT, with vortexing every 15min. Next, the samples were washed with 2,5mL of 20% (w/v) trichloroacetic acid (TCA) and centrifuged for 10min to collect the protein precipitates. Another wash was performed using 10% TCA, and protein pellets were dispersed mechanically. Finally, the pellets were washed three times with 1mL of ethanol (1:1, v/v) to remove free DNPH and lipid contaminants. The final precipitates were dissolved in 1mL of 6M 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 and compared with samples treated with only 2.5M HCl. The following formulae was used to calculate the concentration of carbonyls: C=Abs (355–390nm)x45.45nmol/mL, where C is the concentration of DNPH/mL, and 45.45 its absorption coefficient. The procedures were performed in an ice bath until the TCA wash. Carbonyl content was expressed in nM/mg total protein.

*Chymotrypsin-like proteolytic activity***.** To evaluate the proteolytic activity of the heart muscle, was made a luminescent assay, using Kit Proteasome-Glo™ Chymotrypsin-Like Cell-basead Assay (Promega™, Madison, USA), to

measure the activity of chymotrypsin-related portion catalytic proteasomal. The homogenates were prepared at a concentration of 25mg/mL of PBS. The samples were incubated with in the same volume containing the luciferase substrate. Cleavage of the labeled substrate allowed by the barrel proteasomal release aminoluciferinas, that generate light, that was captured in luminometer. The light emission is considered proportional for proteolytic activity of the heart muscle. The results were expressed as RLU/mg of tissue.

Calcium-dependent proteolytic activity. The activity of calcium-dependent proteases was measured by chemiluminescent assay using the kit Calpain-Glo™ Protease Assay (Promega™, Madison, USA) and the kit Caspase-3™ Cell-basead Assay. Homogenates were prepared at a concentration of 6.25mg/mL 10mM KH2PO4 buffer, pH 7.4 in 0.9% NaCl. Luminescent signal is proportional to the calcium-dependent proteolytic activity in samples, also based on the specific reaction of labeled substrate luciferin by the enzyme luciferase. The results were expressed as RLU/mg tissue.

Total protein concentration. When necessary, protein concentrations were determined by the method described by Lowry *et al*. [18], modified by Miller [19] in order to correct concentrations. Bovine serum albumin was used as standard.

Statistical analysis. Results are presented as mean ± SEM of 8 animals. CL curves were compared using two-way analisys of variance (ANOVA) tests. Carbonyl proteins, TBARS, proteolytic activity and morphometry of the ventricles were compared by one-way ANOVA, followed by Bonferroni's multiple comparison test. $p<0,05$ was considered significant.

Results

Characterization of general, skeletal, and cardiac muscle loss. Walker-256 tumor led to a progressive body mass loss. It could be observed through the cachexia index [14] (6.85±0.63% in 5^{th} and 17.76±1.82% in 10^{th} day after tumor implantation when control was used as reference). Gastrocnemius muscle weight was used to evaluate loss of lean mass, and presented, as cachexia index,

progressive loss after tumor implantation (16.9% in 5th and 23.7% in 10th days after tumor implantation when compared with control). Tumor mass also increased during the course of 10 days: from 5.89±2.03g at 5th day after tumor implantation to 12.94±1.65g at 10th day. The evaluation of cardiac mass showed a small decrease in the total weight, but no significant when compared to the control. All absolute parameters are presented on table 1. In addition, left heart (LH) showed increased values at both experimental times (0.670±0.22g at 5th day, and 0.682±0.24g at 10th day), although no statistical difference has been observed when compared with control (0.652±0.13). Paradoxically, Right Heart (RH) demonstrated progressive mass loss (44±0.09mg in 5th and 60±0.08mg in 10th day, when compared with control). These data are represented on table 2.

Morphometric analysis. In accordance with results found on weight, morphometric analysis of right ventricle showed progressive and significant reduction on thickness (38.16% at 5th and 54.04% at 10th days after tumor implantation, when compared with control, $p<0.05$). In addition, as also demonstrated on weight, left ventricle morphometry did not present statistical differences when compared with control group. Quantitative data are exposed on table 3 and a representative panel is presented on figure 1.

Oxidative stress parameters: Tert-butyl hydroperoxide-initiated chemiluminescence (CL) was used to analyze the integrity of non-enzymatic antioxidant defenses and the levels of lipid hydroperoxides in muscle homogenates of animals inoculated with tumor cells. This assay indicates that the increase in CL is closely related to the oxidative stress previously suffered by the tissue. It induces the consumption of antoxidants and augments the formation of lipid peroxides. Figure 2 shows the CL curves for both left (2A) and right (2B) heart sides. The left heart demonstrated increased curves at both experimental times. The peak was achieved at 5th day after tumor implantation (mean peak of 1092.37 RLU/g tissue, against 760.032 RLU/g tissue on control), decreasing at 10th day (mean peak of 857.0001 RLU/g tissue). On the contrary, analysis of the RH showed to be significant and progressively decreased at both times after tumor implantation ($p<0.0001$). The 5th day demonstrated mean CL curve peak of 1236.84 RLU/g tissue and 10th day showed 949.759 RLU/g tissue as mean peak. No significant alterations could be

observed at any of the times evaluated when thiobarbituric acid reactive substances results from RH and LH were compared with their respective controls (2C, 2D). In addition, carbonylated proteins assayed on LH showed significant increasing on 5th day (from 12.85 in controls to 27.42nM carbonyl/mg total proteins).The amounts returned to control at 10th day after tumor implantation. The same profile was observed on RH, with significantly increased values on 5th day (from 6.28 in controls to 13.85nM/mg total proteins), returning to control levels at 10th day (Figure 3A, 3B).

Proteolytic profile activity. The analysis of proteolytic profile on the left heart demonstrated to be downregulated at all pathways and experimental times. Specifically, the activities chymotrypsin-like at 5th day (-57.5148%), and caspase-like at 5th (-58.885%) and 10th day (-54.5191%) were significantly decreased when compared with control group. (Figure 3C) Although 10th day on chymotrypsin-like activity and calpain like at both experimental times did not present statistical differences, downregulation could be observed when data were normalized by control group. On the other hand, right heart showed positive and significant activity of calpain-like at 5th day after tumor implantation (120.884%) besides significant downregulation (-69.4748%) of chymotrypsin-like proteolytic (CTL) activity at 10th day. CTL at 5th day, caspase-like activity, or calpain-like activity at 10th day after tumor implantation did not presented differences when compared with control group (Figure 3D).

Discussion and conclusion

The loss of cardiac mass in chronic diseases, such as heart failure and obstructive pulmonary disease, has been extensivelly reported [20,21,22,23,24,25], but the mechanisms responsible for this process is only partially elucidated [26]. In rats implanted with Walker-256 tumor – a carcinosarcoma of mammary glands of pregnant albino rats [27] – tumor growth occurs quickly, causing progressive weight loss, leading to the cachexia syndrome already in 5 days. The profile of body weight loss in this study was consistent with earlier results from the same experimental model [14] and analysis of skeletal muscle loss. In the present study, the reduction of total cardiac mass was not observed. However, it seems that the signaling to mass loss or gain begins to happen before, since differences could

be observed when left and right heart sides were analyzed separately. The left (LH) and right (RH) side of the heart were separately analyzed to verify different responses front differentiated metabolic demands of the LH and RH. Barreiro *et al*. (2005) [12] and Marin-Corral *et al*. (2010) [13], showed reduction of 23% and 18%, respectively, on total cardiac mass in rats implanted with ascitic hepatocarcinome cells (Yoshida AH-130). None of the studies performed their assays considering different heart sides. The mass loss found in right side was not observed on the left side. The morphometric analysis of the right ventricle showed gradual decrease of its thickness, while the LH thickness reinforced the data observed on weight. Few studies establish relationships or discuss the mechanism of modulation of cardiac mass in cancer. With respect specifically to the change in oxidative balance in the heart, Marin-Corral *et al*. (2010) [13] found mass loss without alteration in carbonyl groups, but with increased levels of adducts of malondialdehyde and hydroxynonenal in the total heart 7 days after tumor implantation. In the present study, both RH and LH presented significant peaks of ($p<0,05$) carbonyls proteins on day 5, decreasing at 10^{th} day after tumor implantation. However, no significant differences were observed in any sides of the heart when tissues were evaluated by TBARS. Evidence that the quantification of TBARS could, with adequate control, be employed as useful measure of oxidative stress suffered by a tissue, arise from use of specific inhibitors of several stages of lipoperoxidation with proportional reduction of TBARS basal levels. Furthermore, comparison with other methods provides a good concordance in terms of interpretation of the phenomenon. The comparison of TBARS method with more specific methods of measurement of MDA, like gas chromatography-mass spectrometry, has shown significant correlation results, led the authors to suggest that quantification of TBARS assay is useful because of its simplicity and sensitivity, since that there is clear understanding of its unespecificity and bials on tissue evaluation [28]. To better evaluate membrane peroxidation and the significance of the change in membrane permeability in tumor induced rats, the chemiluminescence stimulated by *tert*-butyl hydroperoxide was performed.

Chemiluminescence is a very sensitive method, which takes into account the kinetic analysis of the ascending part of the curve, in addition to their peak height. The technique parts from the precept that the higher the levels of preexisting membrane lipoperoxides, the higher variation in both parameters described. Thus, when tissue is challenged with *tert*-butyl hydroperoxide, the presented CL curves

reflects both the attack suffered by the membrane, as tissue antioxidant consumption, allowing this attack. Thus, the change in membrane permeability due to oxidative unbalance could be observed [15]. Therefore, the LH showed increased in CL in all experimental periods, with the peak at the 5th day after tumor implantation. This fact was coincident with the result obtained in carbonyls proteins evaluation. Paradoxically, the right side small qualitative difference in the CL curve when the 5th day after tumor implantation was compared with control. In addition, subsequent day showed significant reduction in CL curve in the same comparison. At this point, the summary of the evaluated parameters of mass and oxidative stress demonstrated that, in the LH, there was no loss of mass with increased protein carbonylation, coincident with the peak of the CL curves, in 5th day. Previous studies from our laboratory [14] showed that, at the same experimental time and model of tumor induced cachexia, peak of protein carbonylation and CL curves in skeletal muscle also occured on the 5th day. This same study observed modulation and activation of ubiquitination-dependent proteolysis, evaluated by the activity of atrogin-1, an enzyme involved in the final step of identifying proteins that will undergo degradation, until then considered specific for the skeletal muscle. The RH also exhibited reduced mass, with the peak of protein carbonylation on the 5th day without important changes in respect to increased membrane alterations verified by the CL curves. We also found significant decrease ($p < 0.05$) in subsequent experimental time. The knowledge that elevated metabolic rates increased O_2 consumption by the mitochondria in muscle tissues is known since the beginning of the 80´s and, consequently, the generation of reactive oxygen species by mitochondria from the superoxide anion. In the same way, adaptive mechanisms appear to decrease oxidative stress by producing increased production and/or mobilization of antioxidants, reduced production of oxidants and reducing radicals leakage during oxidative phosphorylation [29]. Thus, it seems reasonable to infer that the differences between the sides of the heart, especially in respect to increased CL curves obtained in the LH on 5th day, and decreased CL curve in the right side in the 10th after tumor implantation, may be due to different responses from metabolic activity presented, showing that the two sides of heart work independently.

The heart muscle and skeletal muscle have much in common. Cellular components and structures do not have great differences, and their behavior when trophic aspects are considered have the same profile. Recent studies from our group [14,30] and other recent studies [31,32,33] have shown the involvement of

oxidative stress - or their end products – in skeletal muscle atrophy. Powers *et al*. (2010) [8] presented in their review the influence of reactive oxygen and nitrogen species on the induction of three main proteolytic pathways: calpains, caspases and ubiquitin-proteasome. Evidences have shown that the reactive species contribute to skeletal muscle dysfunction through different mechanisms. Brotto and Nosek (1996) [34] demonstrated the decrease in calcium release from the sarcoplasmic reticulum, and Andrade *et al*. (1998) [35] demonstrated reduced sensitivity to calcium in muscles exposed to H_2O_2. In addition, moderate oxidative stress has been reported on induction of protein oxidation, with consequent increasing on intracellular proteolysis [36,37,38]. Extensively oxidized proteins are modified and degraded by the proteasomal system [39,40], particularly those modified by aldehydes and peroxides. Furthermore, Usui *et al.* (2011) [41] observed that the downregulation of atrogin-1 synthesis positively influenced cardiac atrophy, partially by stabilizing portion IκB-α and NFκB inactivation, important signaling factors in favor of proteolysis. In this study, we evaluated the behavior of the 3 main proteolytic pathways on both sides of the heart and the two sides had different behaviors again.

When the right heart proteolytic pathways were analyzed, caspase-like activity was reduced or indifferent compared to their respective control at all times. A small, but significant, decrease was showed at 10^{th} on chymotrypsin-like proteolytic activity. However, calpain-like activity was coincident with the peak of protein carbonylation. It seems reasonable to assume then that since lipid peroxidation was not observed in this tissue at the same time, the increase of the calcium concentration has been released from the sarcoplasmic reticulum front an initial demand that adapts, desensitizes and reduces on subsequent days. When the left side was analyzed, all proteolytic activities were reduced, showing downregulation of all pathways. It seems that lipid peroxidation and protein carbonylation levels (approximately 1.8 times of increase) were not sufficient for upregulation of pathways across the increased metabolic demand. When the right side becomes insufficient, it is not able to support the blood peripheral demand of organs. The left ventricle performs mechanical compensation by modifying the left ventricular chamber, becoming rounded, and allowing reduction in pulmonary arterial hypertension caused by right ventricular failure [42]. This mechanism justifies the fact that a gradual small increase in the thickness of the left ventricle was observed. Similar results were found in an experimental model of pulmonary emphysema induced by papain [30].

Downregulation in chymotrypsin-like pathways when compared with control was observed, with increased weight and wall thickness of the right ventricle (*cor pulmonale*), with lipidperoxidation by CL, levels of malondialdehyde and protein carbonylation unchanged when compared to control.

Based on the results presented, it can be concluded that cancer leads to loss cardiac mass, regulated by proteolytic pathways. The behavior of both sides of the heart is different due to their different metabolic demands to the disease. Oxidative stress appears to be differently involved in mass modulation on both sides. In addition, heart must be separated in future analysis, since the behaviours are different and can influence responses presented on total heart assays.

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FIGURE LEGENDS

Figure 1 - Morphometric analysis of the ventricles showed reduced thickness - indicated by the arrow.

Figure 2 - Chemiluminescence induced by tert-butil hydroperoxide (2A and 2B) on cardiac muscle of rats bearing Walker-256 tumors at different time points. The total curve was used for statistical analysis by two-way ANOVA using the Bonferroni test as *post-hoc*. Each curve represents the mean of 8 animals. $p<0.05$ was considered significant. Figures 2C and 2D represent quantification of thiobarbituric acid reactive substances on cardiac muscle of rats bearing the Walker-256 tumors at different time points. Results are expressed as mean ± standard error of 8 animals. Control and experimental groups were compared by one-way ANOVA using Bonferroni test as *post-hoc*, $p<0.05$ was considered significant. (A) $p<0.05$ when compared to control group and (B) $p<0.05$ when compared with 5 days.

Figure 3 - Quantification of carbonyl proteins (3A and 3B) on cardiac muscle of rats bearing the Walker-256 tumors at different time points. Results are expressed as mean ± standard error of 8 animals. Figures 3C and 3D represent quantification of proteolytic capacity in cardiac muscle of rats bearing the Walker-256 tumors at different time points. Results are expressed as percentage normalized by control levels. Control and experimental groups were compared by one-way ANOVA using Bonferroni test as *post-hoc*, $p<0.05$ was considered significant. (A) $p<0.05$ when compared to control group and (B) $p<0.05$ when compared with 5 days. CTL: chymotrypsin-like proteolytic activity.

TABLES

Table 1 – Characterization of tumor weight, and muscle loss on rats implanted with solid form of Walker-256 tumor.

Groups	Tumor (g)	Cachexia Index (%)	Gastrocnemius muscle (g)
Control	----	----	1.278 ± 0.23
Tumor 5 days	5.89 ± 2.03	6.85 ± 0.63	1.062 ± 0.27
Tumor 10 days	12.94 ± 1.65	17.75 ± 0.29	0.975 ± 0.29

Groups represent number of days after subcutaneous injection of $8.0x10^7$ tumor cells. Each group consisted of 8 animals. Values are expressed as mean ± standard error. Cachexia index (CI) was calculated by the formula: CI = ((inicial body weight – final body weight + body mass gain of control group) / (initial body weight – body mass gain of control group) x 100 (Guarnier *et al*., 2010 – [21]).

Table 2 – Parameters related to heart weight.

Groups	Heart (g)	Heart weigh left side (g)	Heart weight right side (g)
Control	0.806 ± 0.15	0.652 ± 0.13	0.153 ± 0.06
Tumor 5 days	0.776 ± 0.27	0.670 ± 0.22	0.109 ± 0.09 (A)
Tumor 10 days	0.775 ± 0.25	0.682 ± 0.24	0.093 ± 0.08 (AB)

Groups represent number of days after subcutaneous injection of $8.0x10^7$ tumor cells. Each group consisted of 8 animals. Values are expressed as mean ± standard error. (A) $p<0.05$ when compared to control group and (B) $p<0.05$ when compared to tumor 5 days by one-way ANOVA and Bonferroni´s test as *post hoc*.

Table 3 - Morphometric analysis

Groups	Left Heart (µm)	Right Heart (µm)
Control	1220 ± 30	607 ± 21
Tumor 5 days	1104 ± 20	420 ±16 (A)
Tumor 10 days	1048 ± 12	279 ± 08 (AB)

The values show the thickeness of the right and left ventricles. Values are expressed as mean ± standard error of 8 animals. (A) $p<0.05$ when compared to control group and (B) $p<0.05$ when compared to tumor 5 days by one-way ANOVA and Bonferroni´s test as *post hoc*.

Figure 1

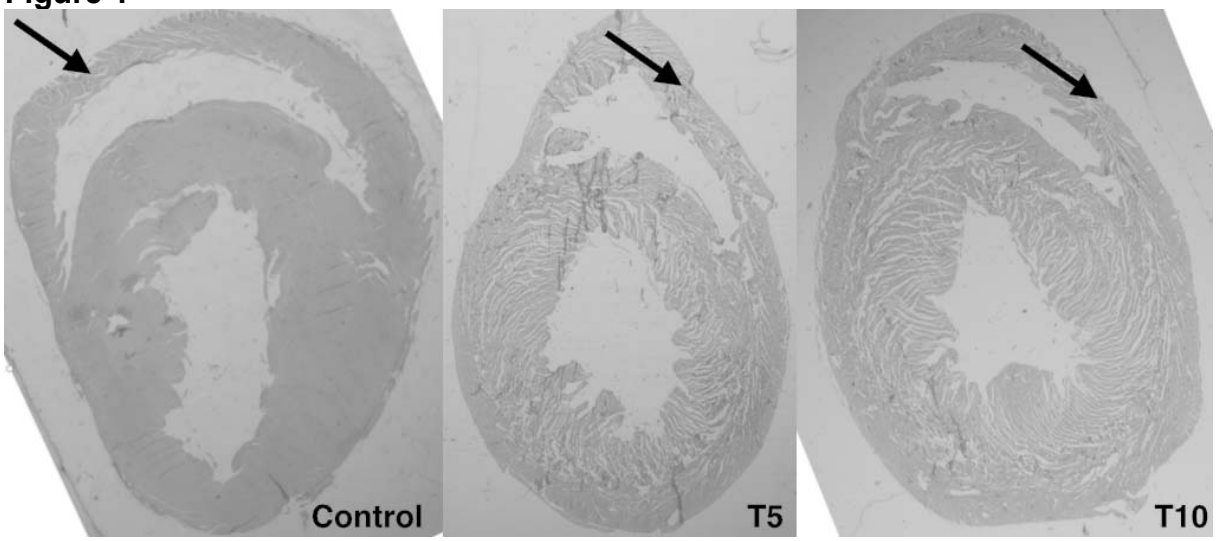


Figure 2

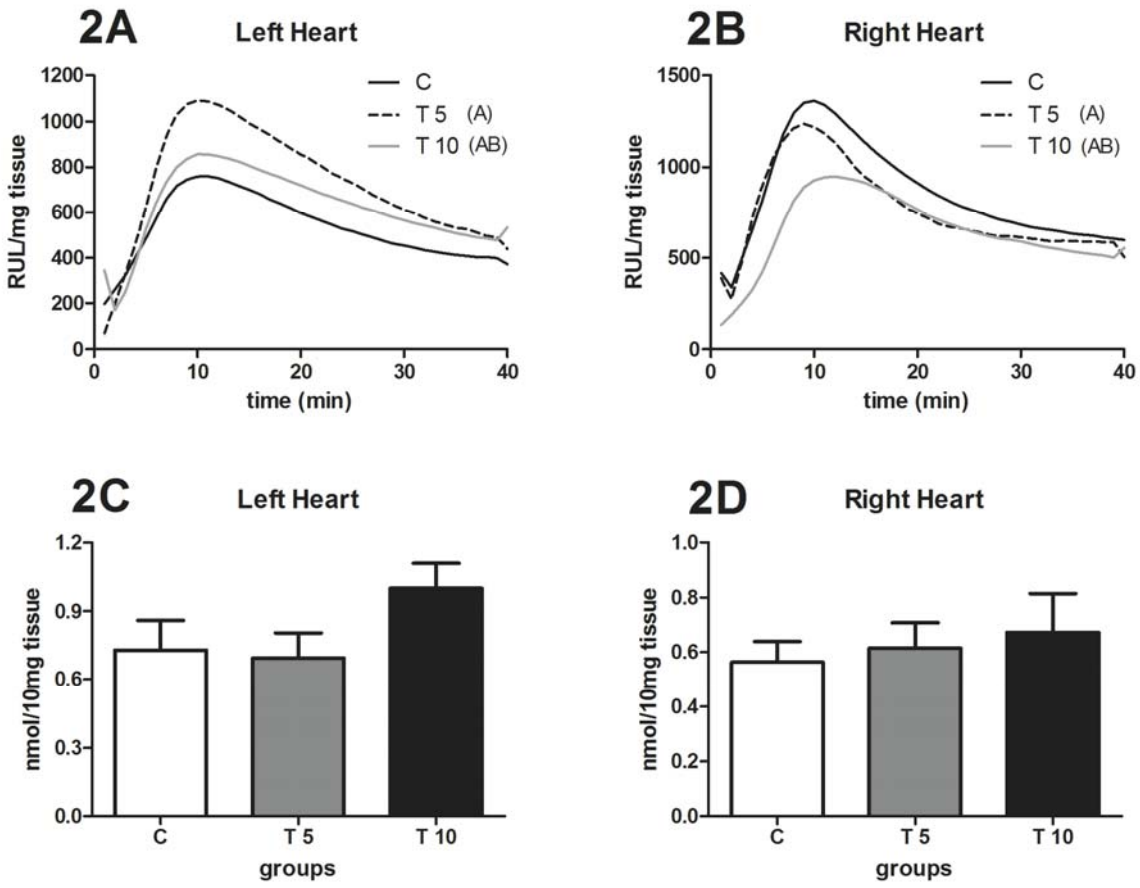
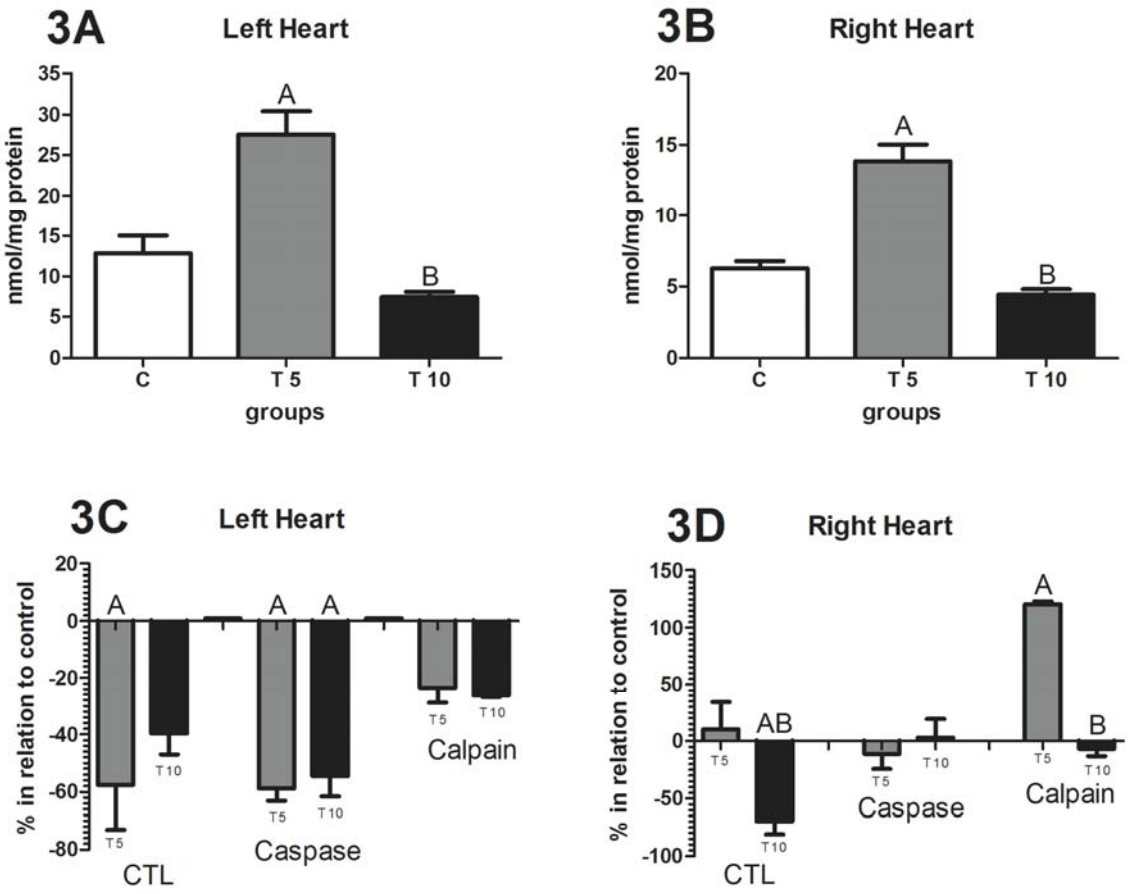


Figure 3


3A
Left Heart
nmol/mg protein
35
30
25
20
15
10
5
0
A
B
C
T 5
T 10
groups
3B
Right Heart
nmol/mg protein
20
15
10
5
0
A
B
C
T 5
T 10
groups
3C
Left Heart
% in relation to control
20
0
-20
-40
-60
-80
A
A
A
T5
T10
T5
T10
T5
T10
CTL
Caspase
Calpain
3D
Right Heart
% in relation to control
150
100
50
0
-50
-100
AB
A
B
T5
T10
T5
T10
T5
T10
CTL
Caspase
Calpain

ANEXO B

Normas da Revista European Journal of Heart Failure

Scope of the Journal
The *European Journal of Heart Failure* is the International Journal of the European Society of Cardiology dedicated to the advancement of knowledge in the field of heart failure. The Journal publishes reviews and editorials in order to improve the understanding, prevention, investigation and treatment of heart failure. Molecular and cellular biology, pathology, physiology, electrophysiology, pharmacology, as well as the clinical, social and population sciences all form part of the discipline that is heart failure. Accordingly, submission of manuscripts on basic, clinical and population sciences is invited. Original contributions on nursing, care of the elderly, primary care, health economics and other specialist fields related to heart failure are also welcome.

HEART Network
The European Journal of Heart Failure participates in the HEART network which is a network of Editors from most cardiovascular journals. Information is exchanged between editors on a regular basis. The network has recently approved a common ethics standard. □□

Its purpose is to ensure transparency and honesty in the scientific process that promotes ethical conduct in performance and publication of research.

The following will be considered as parts of this process: □□

a. Disclosure of potential conflicts of interest for all involved in the performance of research and in the evaluation and publication process of a manuscript. Relevant relationships with commercial interests should be disclosed according to the guidelines of the journal's sponsoring society, or, when no such guidelines exist, according to those of the AHA, ACC, or ESC. □□

b. establish thorough review processes particularly alert to discovering scientific fraud and data falsification, redundant or duplicate publication, and plagiarism, and to adopt a uniform standard of dealing with authors guilty of fraudulent practices. □□

c. to maintain confidentiality and embargos where appropriate.

d. to create uniform criteria to establish authorship. To qualify for authorship, individuals must have made substantial contributions to the intellectual content of the paper in at least one of the following areas: conceived and designed the research, acquired the data, analyzed and interpreted the data, performed statistical analysis, handled funding and supervision, drafted the manuscript, or made critical revision of the manuscript for important intellectual content. Authors must give final approval of the version to be submitted and any revised version to be published. For multi-centre trials, individuals who accept direct responsibility for the manuscript should fully meet the criteria for authorship defined above and contributors not meeting these criteria should be acknowledged. □□

e. avoidance of false claims of ownership, priority, by attention to previous publications. □□

f. avoidance of excessive claims of benefits of a product/technique, in the publication as well as with news media.

g. noting compliance with institutional review board requirements and, when appropriate, approved laboratory procedures for animal research, and that the research conforms to the ethical standards of the Declaration of Helsinki, the Geneva Declaration, the Belmont Report, and Good Clinical Practices from the FDA, and the submission conforms to the International Committee of Medical Journal Editors (ICMJE): Uniform Requirements for Manuscripts Submitted to Biomedical Journals: writing and editing for biomedical publication (Haematologica 89:264, 2005).

Article categories
The *European Journal of Heart Failure* accepts the following categories of articles:

Full Length Articles These should not exceed 3500 words (excluding references, tables and figures) and may include up to a maximum of 6 figures and/or tables and up to 30 references. Full length articles should be divided into the following sections: (1) Title page, (2) Abstract and up to six Keywords, (3) Introduction, (4) Methods, (5) Results, (6) Discussion, (7) Acknowledgements, (8) Funding, (9) Conflict of Interest, (10) References, (11) Figure legends, (12) Appendices, (13) Tables, (14) Figures. The Abstract should be divided into the following sections 'Aims', 'Methods and results' and 'Conclusion', it should not exceed 250 words.

Reviews The *European Journal of Heart Failure* publishes a limited number of scholarly, comprehensive review papers. Reviews should not exceed 3500 words. They should summarize and critically evaluate research in the subject area, and should discuss implications for the future. Reviews have unstructured abstracts with no headings, which should not exceed 250 words and may include up to 45-50 references. Please see below for systematic reviews.

Systematic Reviews: These reviews should follow the format of full length articles, please see above for the required format. These should be submitted as a full length article during the submission process.

Editorials All editorials should be limited to 1500 words (excluding references), with a maximum of 15 references. They do not require an abstract.

Short Reports These reports should not exceed 1500 words and should comprise a background section (~100 words), aims (~50 words), methods (~300 words), results (300 words) and conclusion (250 words). The editorial team reserves the right to decide which of the tables/figures submitted are necessary. A structured abstract not exceeding 250 words is also required for Internet purposes.

Letters to the Editor Relevant correspondence will be considered. This should not exceed 400 words in length excluding references.

Case Reports These reports should not exceed 1200 words. Case Reports should include an unstructured Abstract with no subheadings (not exceeding 100 words), and Introduction, a description of the case(s) under the heading “Case Report” and a discussion of the findings in the context of current practice.

Study Design Papers These should not exceed 3500 words (excluding references, tables, and figures) and may include up to a maximum of 6 figures and/or tables and

up to 30 references. Study design papers should be divided into the following sections: (1) Title page, (2) Abstract and up to six Keywords, (3) Introduction, (4) Study Design, (5) Discussion, (6) Acknowledgements, (7) Funding, (8) Conflict of Interest, (9) References, (10) Figure legends, (11) Appendices, (12) Tables, (13) Figures. The Abstract should be divided into the following sections 'Aims', 'Methods', and 'Conclusion', it should not exceed 250 words.

Submission of manuscripts
The *European Journal of Heart Failure* uses a web-based submission and review system at www.editorialmanager.com/eurjhf/. Online submission facilitates the submission of manuscripts from authors and streamlines the reviewing and publication process.

Authors may send queries concerning the submission process to ejhf.editorialoffice@wiley.com. For enquiries about the review process and journal procedures, the editorial office can be contacted at +44 1482 461778. As a matter of policy, the status of documents will not be discussed by telephone.

Once you have prepared your manuscript according to the instructions below, please go to the online submission system by clicking here. First-time users must click "Register" on the navigation menu at the top of the screen. The system will send an automatic e-mail with your user name and password. Detailed guidelines for authors and reviewers are available at the submission site.

Covering letter The covering letter should include the following:

i) a declaration that "the manuscript, or part of it, has neither been published (except in the form of abstract or thesis) nor is currently under consideration for publication by any other journal";

ii) an explanation as to why your paper would be of particular interest to the readers of the European Journal of Heart Failure;

iii) a statement declaring that all named authors have seen and approved the final version of the manuscript.

Short title: Published papers include a running header (max. 80 characters), which is a shortened version of the article title. Please insert a suggested short title in the ‘Short title’ field on the submission screen.

Review of manuscripts
All manuscripts correctly submitted to the *European Journal of Heart Failure* will first be reviewed by the Editors. Some manuscripts will be returned to authors at this stage if the paper is deemed inappropriate for publication in the *European Journal of Heart Failure*, if the paper does not meet submission requirements, or if the paper is not deemed to have a sufficiently high priority. All papers considered suitable by the Editors to progress further in the review process will undergo appropriate peer review and all papers provisionally accepted for publication may undergo a detailed statistical review.

Manuscripts will usually be evaluated by at least two reviewers from an international panel. Editors will make every effort to reach a decision within 6 to 8 weeks of receipt of the manuscript but on some occasions, due to reasons beyond our control, this

may take longer. □□

Authors may supply the names and addresses of three referees to whom the manuscript might be sent for review.

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Style and spelling: Oxford English spelling should be used. Authors whose first language is not English are requested to have their manuscripts checked carefully before submission. This will help expedite the review process and avoid confusion. □

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Number each page. Please avoid footnotes; use instead, and as sparingly as possible, notes within brackets. Enter text in the style and order of the journal. Type references in the correct order and style of the journal. Type unjustified, without hyphenation, except for compound words (where two words are joined to form a new word e.g. end-systolic, non-infarcted). Type headings in the style of the journal. Use the TAB key once for paragraph indents. Where possible use Times New Roman for the text font and Symbol for Greek and special characters. Use the word processing formatting features to indicate Bold, Italic, Greek, Maths, Superscript and Subscript characters. Clearly identify unusual symbols and Greek letters. Differentiate between the letter O and zero, and the letters I and l and the number 1.

Check the final copy of your paper carefully, as any spelling mistakes and errors may be translated into the typeset version. □□

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DNA Sequences and GenBank Accession Number: For each and every gene accession number cited in an article, authors should type the accession number in bold, underlined text. Letters in the accession number should always be capitalised. Example: (GenBank accession nos. A**I631510**, **AI631511**, **AI632198** and BF223228), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. **BE675048**), and a T-cell lymphoma (GenBank accession no. **AA361117**).

Title Page: The title page should include the following: (1) the title, (2) the name(s) of authors, (3) the institution(s) where work was performed, (4) the position, institution, and location of all authors, (5) the telephone number, fax number and e-mail address of the corresponding author (6) the institutional affiliations of the authors (including corporate appointments) should be acknowledged in a footnote. □□

Abstract: All abstracts may not contain more than 250 words and should also be submitted as a separate file. The abstract should be formatted with the following heading: (1) Aims, (2) Methods and Results, (3) Conclusion. □□

Keywords: A maximum of six keywords may be submitted.

Introduction: This section should provide a rationale for conducting the study within the context of previous work by other authors. □□

Methods: This section should be sufficiently detailed to enable repetition of the study by other investigators. If pertinent, the section may be divided into headed subsections. For animal studies, this section should contain a statement that "The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985)". Human studies should contain a statement that "The investigation conforms with the principles outlined in the Declaration of Helsinki" (Br Med J 1964;ii:177).In addition details of the ethics committee approval procedures and a statement that all subjects gave written informed consent to participate in the study should be included.

Results: If pertinent, the section may be divided into headed subsections. For presentation of data, figures are preferred to tables. Data should not be duplicated in both figures and tables. Extensive numerical data should be presented in legends to the figures rather than in the main body of text. SI units should be used throughout. □□

Discussion: Four manuscript pages should in general be enough to compare and interpret the findings of the study with regard to previous work by (other) authors. This section should also contain 1-4 paragraphs dealing with topics that are beyond the scope of the study. Limitations to the study should also be discussed.

Figures: The review process will not begin until all figures are received. Figures should be limited to the number necessary for clarity and must not duplicate data given in tables or in the text. They must be suitable for high quality reproduction and should be submitted in the desired final printed size so that reduction can be avoided. Figures should be no larger than 125 (height) x 180 (width) mm (5 x 7 inches) and should be submitted in a separate file from that of the manuscript. □□

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Ensure that the size of the lettering is in proportion with the overall dimensions of the drawing. Ideally, the drawings should be submitted in the desired final printed size to avoid reduction. If submitting line drawings which require reduction, please check that the lettering will be clearly legible after the drawing has been reduced to the size at which it will be printed. After reduction, letters should not be smaller than 2 mm in height.

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Acknowledgements: Substantive contributions of individuals, should be noted in the Acknowledgements, positioned before the conflict of interest statement. □□

Conflict of interest: All authors must make a formal statement indicating any potential conflict of interest that might constitute an embarrassment to any of the authors if it were not to be declared and were to emerge after publication. Such conflicts might include, but are not limited to, shareholding in or receipt of a grant or consultancy fee from a company whose product features in the submitted manuscript or which manufactures a competing product. The statement should be positioned before the list of references. If there are no conflicts of interest, please insert the wording 'Conflict of Interest: none declared'.

Reference format
References should be identified in the text by Arabic numerals and numbered in the order cited. All references should be compiled at the end of the article in the Vancouver style, except that ALL authors should be listed. □□

Complete information should be given for each Reference including the title of the article, abbreviated journal title and page numbers. □□

Personal communications, manuscripts in preparation and other unpublished data should not be cited in the reference list but may be mentioned in parentheses in the

text. Authors should get permission from the source to cite unpublished data. Titles of journals should be abbreviated in accordance with Index Medicus (see list printed annually in the January issue of Index Medicus). If a journal is not listed in Index Medicus then its name should be written out in full.

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2. Asger A, Møller JM, Daugaard PC, Kjær SU, Erik S. Effects of phosphodiesterase-5 inhibition by sildenafil in the pressure overloaded right heart. *Eur J Heart Fail*;doi:10.1016/j.eheart.2008.09.016. Published online ahead of print 12 March 2008. □□*Chapter citation example:* □□

3. Nichols WW, O'Rourke MF. Aging, high blood pressure and disease in humans. In: Arnold E, ed. *McDonald's Blood Flow in Arteries: Theoretical, Experimental and Clinical Principles. 3rd ed*. London/Melbourne/Auckland: Lea and Febiger; 1990. p. 398-420. □□*Webpage citation example:* □□

4. Panteghini M. Recommendations on use of biochemical markers in acute coronary syndrome: IFCC proposals. eJIFCC 14.
h**ttp://www.ifcc.org/ejifcc/vol14no2/1402062003014n.htm (28 May 2004)** □□Where the date in parenthesis refers to the access date.

Supplementary data
Supporting material that is not essential for inclusion in the full text of the manuscript, but would nevertheless benefit the reader, can be made available by the publisher as online-only content, linked to the online manuscript. The material should not be essential to understanding the conclusions of the paper, but should contain data that is additional or complementary and directly relevant to the article content. Such information might include more detailed methods, extended data sets/data analysis, list of Investigators, or additional figures. □□

All text and figures must be provided in suitable electronic formats (instructions for the preparation of Supplementary data can be viewed here). All material to be considered as Supplementary data must be submitted at the same time as the main manuscript for peer review. It cannot be altered or replaced after the paper has been accepted for publication. Please indicate clearly the material intended as Supplementary data upon submission. Also ensure that the Supplementary data is referred to in the main manuscript where necessary.

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All manuscripts selected for publication will be reviewed for the appropriateness and accuracy of the statistical methods used and the interpretation of statistical results. All papers submitted should provide in their Methods section a subsection detailing the statistical methods, including the specific method used to summarize the data, the methods used to test their hypothesis testing and (if any) the level of significance

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Details of all funding sources for the work in question should be given in a separate section entitled 'Funding'. This should appear before the 'Acknowledgements' section. □□The following rules should be followed:

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The full official funding agency name should be given, i.e. ‘the National Cancer Institute at the National Institutes of Health’ or simply 'National Institutes of Health' not ‘NCI' (one of the 27 subinstitutions) or 'NCI at NIH’ (full RIN-approved list of UK funding agencies)

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Where individuals need to be specified for certain sources of funding the following text should be added after the relevant agency or grant number 'to [author initials]'.

An example is given here: ‘This work was supported by the National Institutes of Health [P50 CA098252 and CA118790 to R.B.S.R.] and the Alcohol & Education Research Council [HFY GR667789].

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