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Uso do Ensaio Cometa em Diferentes Modelos Experimentais de Treinamento de Força, Suplementação Nutricional e de Insuficiência Cardíaca em Ratos.

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LISTA DE ABREVIATURAS

$^1O_2$: Oxigênio singlete

1RM: Teste de Uma Repetição Máxima

AST: Área de Secção Transversa

ATP: Adenosina Trifosfato

BH_4: Tetrahidrobiopterina

Ca^{2+}: Cátion Bivalente de Cálcio

DNA: Ácido Desóxirribonucléico

ERN: Espécie Reativa de Nitrogênio

ERO: Espécie Reativa de Oxigênio

FAD: Flavina Adenina Dinucleotídeo

FMN: Flavina Mononucleotídeo

GMPc: Guanosina-3,5-monofosfato

H_2O_2: Peróxido de hidrogênio

IAM: Infarto Agudo do Miocárdio

IC: Insuficiência Cardíaca

LAC: Ligação da Artéria Coronariana

NaCl: Cloreto de Sódio

NADPH: Nicotinamida Adenina Dinucleotídeo Fosfato

NO: Óxido Nítrico

NOS: Óxido Nítrico Sintase

$O_2^{-\bullet}$: Ânion radical superóxido

$OH^{\bullet}$: Radical Hidroxil

$ONOO^-$: Peróxinitrito

PDFVE: Pressão Diastólica Final do Ventrículo Esquerdo

PEVK: Proteínas Cinases Prolina Glutamato Valina Lisina)

pH: Potencial de Hidrogênio

RNA: Ácido Ribonucléico

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ABSTRACT

Introduction: The Comet Assay has been used as a very sensitive tool to detect damage to nuclear DNA. Although its use is almost strictly to the *in vitro* and *in vivo* toxicology studies, its application is poorly explored in sports nutrition and in cardiology. Resistance training (RT) is able to induce physiological and biochemical adaptations. It has been observed that some ergogenic supplements could increase their observed effect on biochemical parameters. The supplementation of L-arginine (Arg) is underexplored of genotoxic tests. As well as in experimental models of sports nutrition, experimental studies in cardiology, especially regarding chronic heart failure (CHF), have not been investigated on the point of view of DNA damage. The lingering damage to DNA can induce significantly harmful outcomes for affected cells and thus, increasing the risk of developing organic dysfunctions or even participate directly and indirectly in the pathogenesis of diseases. **Aims**: Using the technique of the Comet Assay in different experimental situations of RT associated with Arg supplementation, and the CHF induced by myocardial infarction (MI) in rats. **Methods**: A total of 35 male Wistar rats was used. Of these, 23 animals were allocated to the study 1 and 2, involving the association of RT with Arg on biochemical parameters. Finally, 12 animals were allocated to the study 3, which were submitted to MI surgery for coronary artery ligation (CAL) to assess DNA damage and its relationship with hemodynamic variables. For study 1 and 2, the protocols lengthened eight weeks of RT and Arg supplementation which succeeded in hemodynamic, biochemical analysis and DNA damage in lymphocytes. For the study 3, the CHF animals underwent MI by CAL and Sham rats had not the artery ligation. After six weeks, it was performed the analysis of hemodynamic function, area of infarction, cardiac hypertrophy, hepatic and pulmonary congestion and DNA damage in different cells (left ventricle, lungs, diaphragm, gastrocnemius and soleus). **Results**: Regarding the study 1, with preliminary results, the RT showed protection of DNA damage in lymphocytes and the association with Arg protected further genotoxic damage. Regarding the study 2, it was further observed that after statistical analysis, Arg supplementation significantly influenced the protection against DNA damage in lymphocytes, a larger extent than RT alone. The Arg influenced to a greater concentration of total testosterone, extracellular HSP72 and lower uric acid concentration, as well as influenced cardiac hypertrophy in the sedentary supplemented group. In relation to the study 3, the CHF group showed greater DNA damage in all different tissues and it was positively correlated with left ventricular dysfunction and with pulmonary congestion. **Conclusion**: The protection and results in DNA damage both in lymphocytes and in different cells reflect that by appropriate experimental designs, the Comet Assay can be used as a sensitive and enriching tool for scientific investigation, in order to render more robust results.

Key-words: DNA damage. Resistance training. L-arginine. Heart failure. Hemodynamic function.

RESUMO

Introdução: O Ensaio Cometa tem sido utilizado como uma ferramenta muito sensível ao detectar dano ao DNA nuclear. Apesar de seu uso ser quase estritamente à ensaios *in vitro* e *in vivo* toxicológicos, sua aplicação é pouco explorada em estudos de nutrição esportiva e de cardiologia. O treinamento de força (TF) é capaz de induzir adaptações fisiológicas e bioquímicas. Tem se observado que algumas suplementações ergogênicas poderiam aumentar o seu efeito observado sobre parâmetros bioquímicos. A suplementação de L-arginina (Arg) é pouco explorada do ponto de vista genotóxico. Assim como modelos experimentais de nutrição esportiva,trabalhos em cardiologia experimental, principalmente em relação à estudos com insuficiência cardíaca (IC), ainda não foram investigados sobre o ponto de vista de dano em DNA. O dano persistente em DNA pode trazer desfechos significativamente prejudiciais para células afetadas e, portanto, aumentando o risco de gerar disfunções ou até mesmo participar direta e indiretamente na patogênese de doenças. **Objetivos**:Utilizar a técnica do ensaio cometa em diferentes situações experimentais de TF associado a suplementação de Arg, bem como à IC induzida pelo infarto agudo do miocárdio (IAM) em ratos. **Métodos**: Foi utilizado um total de 35 ratos machos Wistar. Destes, 23 animais foram alocados ao estudo 1 e 2, envolvendo a associação do TF com Arg sobre parâmetros bioquímicos. Por fim, 12 animais foram alocados ao estudo 3, os quais foram submetidos à cirurgia de IAM pela ligadura da artéria coronariana (LAC) para avaliar dano em DNA e sua relação com variáveis hemodinâmicas. Para o estudo 1 e 2 foram realizados protocolos de oito semanas de TF e suplementação de Arg a que se sucedeu em análise hemodinâmica, análise bioquímica e dano em DNA em linfócitos. Para o estudo 3, os animais IC sofreram IAM pela LAC e os animais Sham não sofreram oclusão da artéria coronariana. Após seis semanas, foi realizado a análise da função hemodinâmica, área de infarto, hipertrofia cardíaca, congestão hepática e pulmonar e dano em DNA em diferentes células (ventrículo esquerdo, pulmão, diafragma, gastrocnêmio e sóleo). **Resultados**: Quanto ao estudo 1, com resultados preliminares, o TF demonstrou proteger o dano em DNA em linfócitos e a associação com Arg protegeu ainda mais o dano genotóxico. Em relação ao estudo 2, foi observado que após análise estatística mais aprofundada, a suplementação de Arg influenciou significativamente a proteção contra o dano em DNA em linfócitos, em maior intensidade que o TF. A Arg influenciou em maior concentração de testosterona total,HSP72 extracelular e menor concentração de ácido úrico, bem como influenciou na hipertrofia cardíaca no grupo sedentário suplementado. Em relação ao estudo 3, a IC demonstrou maior dano em DNA em todas as células de diferentes tecidos e foi positivamente correlacionada com disfunção ventricular esquerda e congestão pulmonar. **Conclusão**: Os resultados de proteção e dano em DNA tanto em linfócitos e em diferentes células refletem que através de desenhos experimentais apropriados é possível utilizar o Ensaio Cometa como uma ferramenta sensível e enriquecedora da investigação científica a fim de tornar o resultado observado mais robusto.

Palavras-chave: Dano em DNA. Treinamento de força. L-arginina. Insuficiência cardíaca. Função hemodinâmica.

CAPÍTULO I - REVISÃO DE LITERATURA

1.1 Introdução

A molécula de DNA é uma estrutura complexa que desempenha papeis primordiais na manutenção da vida. O DNA é formado por um grupo fosfato, uma pentose e uma base nitrogenada (púrica ou pirimídica) e é com esta combinação que confere à esta estrutura notáveis funções. O DNA é responsável por armazenar informações genéticas de expressão de peptídeos e proteínas-chave envolvidos em processos fisiológicos (1, 2).

Apesar de desempenhar funções essenciais, o DNA está suscetível a uma série de fatores que podem colocar a sua integridade em risco, como os agentes oxidantes. Os agentes oxidantes são formados endogenamente e o excesso de sua produção pode levar a danos à estrutura de DNA. Algumas situações podem aumentar a formação de agentes oxidantes, como doenças degenerativas e o estresse físico excessivo, como por exemplo, o exercício físico agudo (3).

O exercício físico tem demonstrado ser uma excelente ferramenta para promoção de saúde, de um modo geral (4). Por desempenhar efeitos positivos sobre diferentes órgãos e sistemas, o treinamento crônico tem sido demonstrado aumentar defesas imunológicas, reduzir o risco do desenvolvimento de doenças crônicas não-transmissíveis, bem como aumentar a qualidade de vida em diferentes faixas etárias (5-7). No entanto, ao abordar exercício físico, deve-se segregar o exercício de característica aeróbia de exercício de força, uma vez que seus efeitos fisiológicos são muito distintos (8, 9).

Apesar de diversos estudos já demonstrarem benefícios do exercício aeróbio sobre defesas antioxidantes, bem como proteção ao dano oxidativo (10), um número significativamente menor de investigações científicas abordam os efeitos do treinamento de força.

Estratégias coadjuvantes que envolvem a suplementação de nutrientes em associação com o treinamento de força tem sido alvo de investigações científicas nos últimos anos. Os efeitos da suplementação de aminoácidos tem sido amplamente estudados, pois estes apresentam efeitos peculiarmente interessantes sobre o desempenho atlético e estado redox (11). A L-arginina é uma aminoácido com notáveis funções fisiológicas que poderia desempenhar efeitos diferenciados ao associar com o treinamento de força. Uma de suas principais funções é a formação de óxido nítrico e creatina, além de ser intermediário de síntese de proteínas celulares (12).

O óxido nítrico desempenha um papel importante na regulação a curto prazo da pressão arterial, mas seu efeito perpassa modulações fisiológicas, podendo atuar como agente oxidante ou até mesmo antioxidante (13). O paradoxo do óxido nítrico é uma discussão extensa que parece tanto ser um promotor de saúde, quanto pode ser um agente causador de danos celulares. No entanto, em algumas investigações, a suplementação de L-arginina demonstrou ser antioxidante (14) e até mesmo proteger o dano ao DNA (15). A sua função antioxidante ainda não é muito clara, uma vez que poderia atuar como varredor de radicais livres tanto no meio intra, quanto no meio extracelular.

Doenças degenerativas possuem um cenário muito mais prejudicial que em situações de ausência das mesmas, especificamente sobre a biologia do oxigênio. As vias de formação de espécies reativas são ativadas de forma exacerbada, tornando o microambiente celular vulnerável ao dano de biomoléculas essenciais para a manutenção da função celular (16). A insuficiência cardíaca é uma síndrome que acomete não apenas o coração, mas o organismo como um todo. Ela se caracteriza pela inadequada capacidade do coração em manter níveis adequados de suprimento sanguíneo para os tecidos (17).

Nesta condição clínica de insuficiência cardíaca, os efeitos indiretos do prejuízo da função cardíaca desempenham efeitos deletérios sobre outros órgãos, mais especificamente sobre o balanço redox celular (18).

Tendo em vista os diferentes efeitos de situações distintas, como o sedentarismo, o treinamento de força, bem como síndromes degenerativas (insuficiência cardíaca) o mapeamento do dano à estrutura de DNA se torna um importante tema a ser explorado. Por isso, o objetivo da presente revisão de literatura é abordar os pontos-chave de estresse oxidativo, possíveis danos sobre a estrutura do DNA, bem como abordar de forma aprofundada o seu papel e aplicabilidade em situações de treinamento de força, associado ou não à suplementação de L-arginina, bem como em situações de insuficiência cardíaca.

1.2 Estresse Oxidativo

A definição de estresse oxidativo se baseia no desequilíbrio entre a produção de agentes oxidantes com a produção de agentes antioxidantes (19). Os agentes oxidantes

podem ser divididos em dois grandes grupos: as Espécies Reativas de Oxigênio (ERO) e as Espécies Reativas de Nitrogênio (ERN). Da mesma forma, os agentes antioxidantes podem ser divididos em dois grupos: agentes antioxidantes enzimáticos e agentes antioxidantes não-enzimáticos.

As espécies reativas são necessárias e essenciais para a sinalização para a síntese de defesas antioxidantes celulares em processos fisiológicos (20). Por serem formadas em processos fisiológicos, as EROs e ERNs estão presentes na modulação de uma série de situações, como na sua participação em erradicar antígenos via explosão respiratória, no vazamento de elétrons da mitocôndria e também processos de isquemia-reperfusão (21). Na tabela 1 pode ser observado alguns exemplos de EROs e ERNs.

Tabela 1 - Espécies Reativas de Oxigênio e Nitrogênio encontradas em processos biológicos.

Espécies Reativas de Oxigênio	
Oxigênio Singlete	$^1O_2$
Ânion Radical Superóxido	$O_2^{-\bullet}$
Peróxido de Hidrogênio	H_2O_2
Radical Hidroxil	$OH^\bullet$
Radical Perhidroxil	$HO_2^\bullet$
Radical Alcoxil	$RO^\bullet$
Radical Peroxil	$ROO^\bullet$
Radical Hidroperoxil	$ROOH^\bullet$
Ácido Hipocloroso	HOCl
Ozônio	O_3
Espécies Reativas de Nitrogênio	
Óxido Nítrico	NO
Dióxido nítrico	NO_2
Peróxinitrito	$ONOO^-$

Fonte: Bloomer RJ. Effect of exercise on oxidative stress biomarkers. *Adv in Clin Chem*, 2008; 46:1-50.

Por definição, todo radical livre é uma espécie reativa, no entanto, nem toda espécie reativa é um radical. Radical livre é toda molécula que possui um ou mais

elétrons desemparelhados na última camada de valência e é capaz de existir de forma independente, tornando-o altamente instável e com uma afinidade extremamente alta a moléculas adjacentes ao seu local de formação (23).

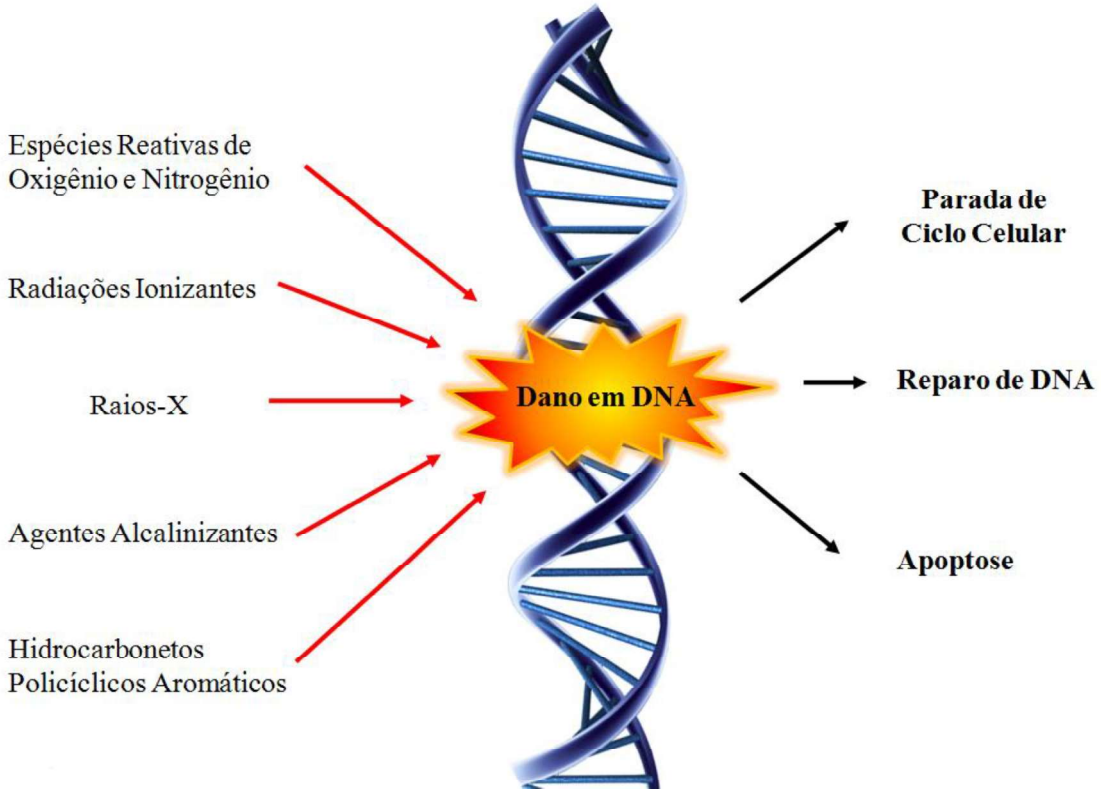
O excesso da produção destas espécies reativas pode levar a prejuízos biológicos. Por haver uma formação generalizada de diferentes espécies reativas em diferentes células, o dano oxidativo pode ocorrer a diferentes biomoléculas (20). As espécies reativas podem causar danos oxidativos a lipídeos (lipoperoxidação ou peroxidação lipídica), proteínas (carbonilação de proteínas) e em DNA (quebras em fitas ou oxidação de bases) (24, 25).

1.3 Dano à Estrutura de DNA

O ácido desoxirribonucléico (DNA) é um composto químico essencial para processos biológicos. A sua atuação não se restringe apenas no armazenamento de códigos genéticos, mas também atua diretamente sobre processos básicos da transcrição de sequências genéticas em compostos de ácidos ribonucléicos (RNA), os quais traduzem este sequenciamento em peptídeos e proteínas essenciais para todos os processos biológicos (1).

Este DNA é formado por grupamentos de fosfatos, bem como por açúcares e bases nitrogenadas (púricas e pirimídicas). Esta conformação estável pode ser alvo de agentes danosos. Dos mais diversos tipos de agentes exógenos e endógenos podem lesionar o DNA, como a radiação ionizante (26, 27), raios-X (28), hidrocarbonetos policíclicos aromáticos (29), agentes alcalinizantes (3) e espécies reativas de oxigênio e nitrogênio (30) (Figura 1). O tipo de dano mais frequente é o dano oxidativo causado por EROs e ERNs (24).

Figura 1 - Diferentes agentes genotóxicos e a suas respectivas respostas celulares ao dano em DNA.



As implicações do dano ao DNA e o seu não-reparo são altamente impactantes no metabolismo celular. O DNA possui o sequenciamente necessário para a sua transcrição em RNA mensageiros e posterior tradução à proteínas com funções biológicas. Uma vez oxidado o esqueleto do DNA, seja em oxidação de base púrica ou pirimídica, ou até mesmo a indução de ligações intermoleculares excessivas entre fitas de DNA, a sua lesão pode ter repercussões devastadoras na homeostase celular (20).

O dano em DNA desempenha um papel importante na mutagênese, carcinogênese e envelhecimento (24). O DNA oxidado está presente em elevada frequência em diversos tecidos, especialmente em células tumorais (31-33). Dentre as ERO que mais danificam o DNA, podem ser destacadas o $O_2^{-}\bullet$, H_2O_2, $^1O_2$ e $OH\bullet$. O $ONOO^-$ é a ERN que possui maior capacidade genotóxica por se difundir facilmente pela célula, podendo reagir com a estrutura de DNA (34).

As ERO e ERN podem causar lesões no DNA de diferentes formas, dentre elas a oxidação de bases púricas/pirimídicas, quebras de fitas simples e fitas duplas (35), O radical hidroxil é considerada a ERO mais perigosa, pois não existe defesas

antioxidantes enzimáticas para neutralizar o seu efeito oxidativo (36). Para reagir com o DNA, este radical livre necessita ser formado próximo ao núcleo celular. O ânion radical superóxido é uma das ERO mais presentes no dano endógeno ao DNA. Entretanto, há defesas antioxidantes enzimáticas próximas ao núcleo, como a superóxido dismutase, que são capazes de dismutar o radical livre no momento que é formado, impedindo seu dano oxidativo (37).

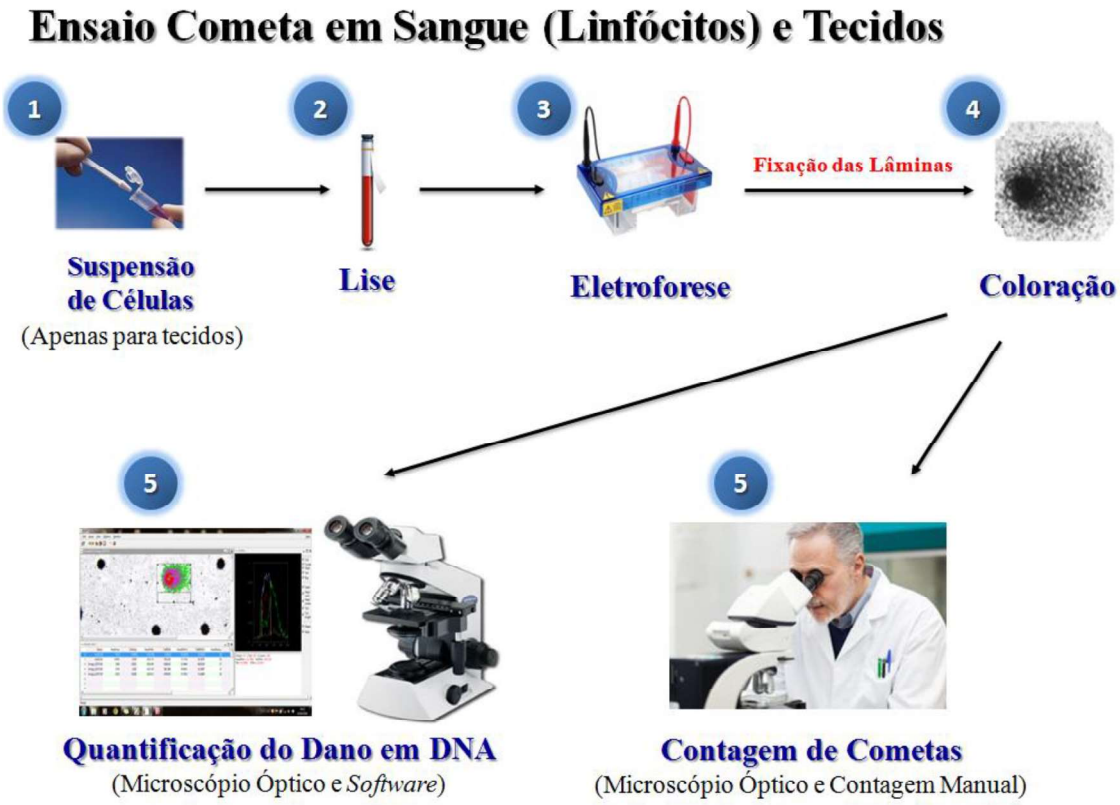
Em situações onde a formação de radical livre é muito superior à defesa antioxidante, ocorre o dano à estrutura de DNA. Dependendo do tipo de lesão, a célula é capaz de identificar a natureza do dano e reparar por diferentes vias (3). Se as lesões genotóxicas persistirem em uma velocidade muito superior ao seu reparo, aumenta o risco de iniciar processos mutagênicos no genoma (33). O acúmulo de bases oxidadas no DNA por radicais livres e EROs tem se demonstrado como um risco para o desenvolvimento de doenças, como o câncer, doenças cardiovasculares e envelhecimento (35, 38).

1.4 Ensaio Cometa

A técnica foi desenvolvida inicialmente com o nome de *Single Cell Gel Eletrophoresis* na década de 70 por Cook e colaboradores (39). Através de uma série de experimentos, foi possível pela primeira vez anexar a estrutura de DNA à uma matriz. Com uma solução detergente de NaCl, o rompimento de membranas plasmáticas permitia que estruturas similares à nucleóides fossem anexadas à uma matriz de agarose. Enquanto o material genético nuclear fosse extraído de proteínas, como histonas, e estivesse na sua forma relaxada, seria possível que fragmentos da sua estrutura, oriundos de quebras simples e duplas de DNA, pudessem se deslocar do centro do nucleóide.

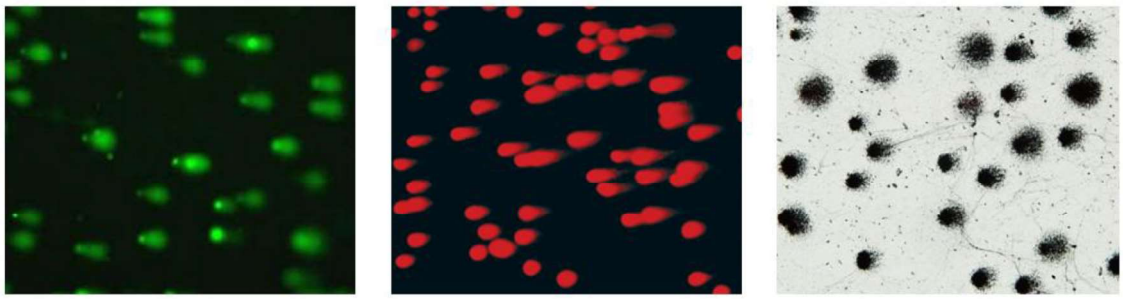
O nome da técnica também é denominado de ensaio cometa, pois a estrutura formada se assemelha à um cometa. Nesta metodologia, os nucleóides embebidos em uma solução de agarose são submetidos à uma corrente eletroforética. Os principais passos da metodologia estão demonstrados na Figura 2. Os fragmentos de DNA são atraídos pelo ânodo, formando a "cauda do cometa". Diferentes densidades de fragmentos de DNA possuem mobilidades diferentes, portanto, fragmentos menores são capazes de se deslocar mais que fragmentos maiores, podendo influenciar diretamente no comprimento da cauda de cometa (40).

Figura 2 - Representação das etapas do Ensaio Cometa (*Single Cell Gel Eletrophoresis*) em sangue (linfócitos) e em tecidos resumido em cinco diferentes etapas.



Após a etapa de eletroforese, é realizado a fixação das lâminas e posterior coloração. A coloração é uma etapa que pode haver diferenças entre protocolos. As colorações disponíveis variam desde reagentes fluoróforos, até reagentes com prata. A escolha do método de coloração depende mais da disponibilidade do laboratório em possuir um microscópio de fluorescência, o que eleva o custo da técnica, ou até mesmo o tipo de *software* de quantificação de dano em DNA. A grande maioria de *softwares* que mensuram o dano em DNA possui apenas versões para lâminas coradas com fluoróforos. Muito frequentemente as condições laboratoriais não permitem a aquisição da metodologia com fluorescência, por isso é amplamente utilizado a coloração de nitrato de prata (41). A coloração de nitrato de prata é capaz de reproduzir os mesmos padrões de dano que reagentes fluorescentes (42). A Figura 3 demonstra as diferenças de coloração do ensaio cometa.

Figura 3 - Tipos de coloração de lâminas (fluorescentes e não-fluorescentes) utilizadas no Ensaio Cometa.



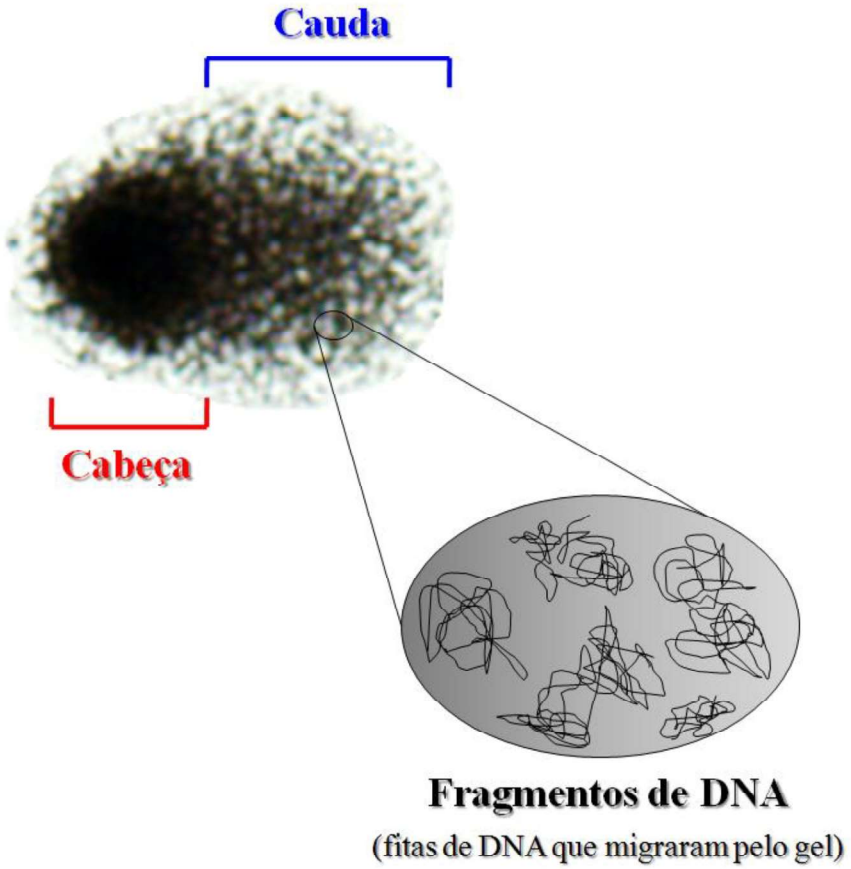
Coloração com Verde SYBR | **Coloração com Brometo de Etídio** | **Coloração com Nitrato de Prata**

Após a coloração de lâminas, é necessário realizar a análise e posterior apresentação dos resultados obtidos no experimento. Há duas grandes possibilidades em apresentar os resultados: contagem de cometas e quantificação direta do dano em DNA. A primeira linha de resultados apresentados com investigações de compostos genotóxicos foi mediante com a utilização de contagem de cometas. Neste processo, há um avaliador que irá distribuir as células em cinco diferentes classes: 0, sem dano; 1 a 4, presença de cauda (indica que há dano em DNA). Nesta classificação o dano é classificado de forma crescente, da classe 1 a 4. Para o final da contagem, é necessário contar 100 células e a partir do número de células com dano de 0 a 4 são gerados resultados como a frequência e índice de dano, sendo o primeiro apresentado em percentual e o último em um escore de 0 a 400 (43). Este método apresenta uma série de limitações, embora seja amplamente utilizado e reflita resultados verdadeiros. Uma das principais limitações é o fato da avaliação ser majoritariamente subjetiva e avaliador-dependente. A fim de reduzir o viés de avaliação, é possível recrutar de dois a três avaliadores, cegados, e analisarem as mesmas lâminas (44). Este tipo de método para mensuração do dano em DNA é recomendado quando não há a possibilidade de realizar a quantificação por *software* das lâminas de interesse.

Por fim, o método mais utilizado e mais confiável é a quantificação direta do dano em DNA. A principal diferença entre a estimativa visual do dano para a quantificação é a isenção da subjetividade do avaliador. Neste caso, *softwares* de análise de imagens medidas de dimensões, comprimentos, diâmetros e raios, bem como densidade e luminosidade por áreas de cada cometa (45). A grande maioria dos

programas utilizados para a quantificação de dano em DNA é realizado um cometa por vez e poucos quantificam com coloração de nitrato de prata, sendo quase uma exclusividade de laboratórios que trabalham com microscópio de fluorescência. Através da análise de cometas por programas específicos é possível obter algumas variáveis, como: percentual de DNA na cauda (% DNA na cauda), comprimento da cauda, momento da cauda e momento da cauda de Olive (42, 43, 46). A Figura 4 representa as diferentes estruturas identificáveis de um cometa com presença de dano.

Figura 4 - Estruturas de um cometa. Nesta figura é possível observar a presença de cabeça, estrutura com DNA íntegro sem dano, e da cauda, estrutura com fragmentos de DNA que são compostos por quebras simples e duplas da fita de DNA.



O cometa possui uma série de protocolos adaptados para diferentes aplicações. Esta técnica pode ser utilizada desde experimentos com cultura de células, como linfócitos (sangue total), até mesmo em células de tecidos. É importante ressaltar que o Ensaio Cometa pode ser utilizado para avaliar quebras simples e/ou duplas. A primeira versão do protocolo foi criada com um pH neutro (pH = 7,0) na etapa de eletroforese.

Esta versão do Ensaio Cometa era capaz de fornecer dano em quebras duplas de fitas de DNA. Mais tarde foi criada uma adaptação por Singh e colaboradores (1988), cuja investigação demonstrou que utilizar pH altamente alcalino (pH > 13,0) sítios apurínicos/apirimídicos em fitas de DNA eram revelados e suas quebras eram observadas de forma mais expressivas após a coloração (48). A partir deste momento, destacou-se ainda mais a importância de realizar o Ensaio Cometa na sua versão alcalina, pois esta adaptação permitia observar todo o dano em DNA, tanto de quebras simples, quanto de quebras duplas (42).

1.5 Aplicabilidade do Ensaio Cometa

O Ensaio Cometa tem sido extensivamente utilizado em investigações toxicológicas das suas mais diversas ramificações. A sua aplicação tem sido investigada além do mapeamento transversal de dano em DNA. O ensaio cometa tem sido utilizado como uma ferramenta a fim de observar proteção de dano genotóxico por irradiação (49), proteção por adequação nutricional (50, 51), proteção de extratos de ervas sobre o fígado (52, 53), proteção hormética entre poluição atmosférica e gravidez (54), bem como em estudos de biomonitoramento ambiental (55).

Recentemente tem sido criticada a utilização do ensaio cometa em estudos que utilizassem nanopartículas, uma vez que resultados falsamente positivos/negativos podem ser encontrados. Por serem muito pequenas, as moléculas de nanopartículas poderiam se ligar ao DNA nuclear, criando um complexo DNA-nanopartícula criando uma instabilidade genômica que seria mascarada pelo aumento da densidade da cabeça do cometa (56). No entanto, mesmo com esta limitação metodológica, o ensaio cometa demonstrou ser confiável no monitoramento do dano em DNA. Em uma recente revisão de Karlsson e colaboradores (2014), demonstram a robustez do ensaio cometa acompanhado com o teste do micronúcleo em células tratadas com nanopartículas de dióxido de titânio. Apesar destes dois testes avaliarem diferentes tipos de lesões, os dois testes demonstraram serem altamente sensíveis e específicos para avaliar o dano em DNA, mesmo com um possível fator confundidor (57).

1.5.1 Aplicação da Técnica de Ensaio Cometa em Modelo Experimental de Treinamento de Força e Nutrição

O Ensaio Cometa tem sido amplamente utilizado em estudos de nutrição (42). São poucos os trabalhos que fizeram uso dessa técnica com o objetivo de investigar os efeitos do treinamento de força sobre o dano em DNA. Já foi demonstrado que o *overtraining* é capaz de danificar o DNA de linfócitos e de músculo esquelético (58). Por esta razão, surgiu o interesse em investigar o efeito crônico do treinamento de força sobre o dano em DNA. Recentemente, alguns trabalhos em humanos têm procurado mostrar o efeito do treinamento de força em idosos sobre a estabilidade genômica (59, 60).

Entretanto, o número de investigações associando suplementos nutricionais com treinamento de força é ainda menor. Por isto, surge um novo campo para investigação cuja aplicação pode se basear em efeitos protetores, sinérgicos ou não com o treinamento de força, ou até mesmo efeito tóxico de substâncias utilizadas em suplementos nutricionais.

1.5.1.1 Adaptações Fisiológicas ao Treinamento de Força

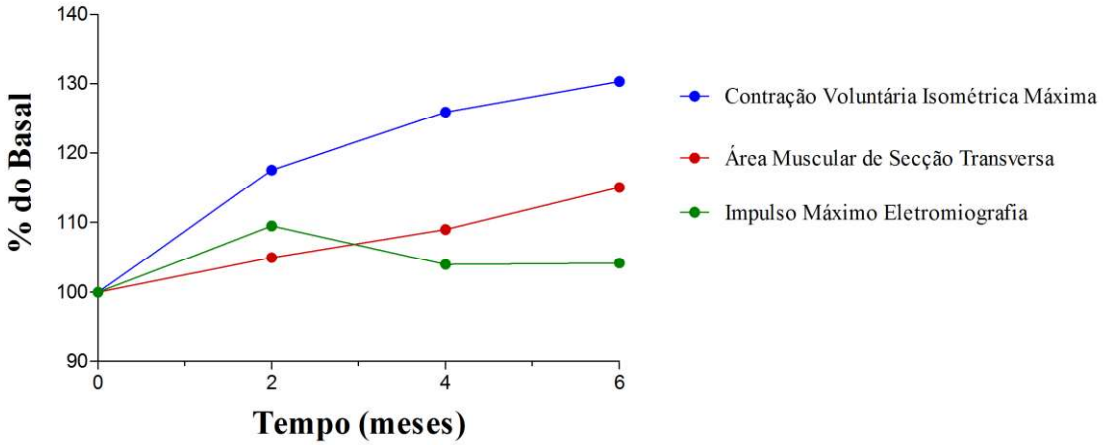
O treinamento de força tem sido utilizado de forma ampla pela população em geral como um método muito eficaz na promoção de ganho de força muscular, aumento de desempenho em esportes de potência, prevenção de lesões musculares e melhora da qualidade de vida (61).

O treinamento de força é conhecido por causar adaptações notavelmente divergentes do treinamento aeróbico. A realização do treinamento de força traz adaptações fisiológicas características do estímulo de força. Dentre elas, pode-se citar o aumento de força máxima, hipertrofia muscular, mudanças na composição de miosina de cadeia pesada e adaptações neurais (62).

O treinamento de força é caracterizado pela imposição de sobrecarga tensional de moderada a alta intensidade, gerando respostas adaptativas diferentes, de acordo com o tipo de fibra muscular, portanto, algumas fibras musculares demonstram maior responsividade à este estímulo do que outras. As fibras musculares do tipo II cujas características metabólicas glicolíticas anaeróbias são mais predominantes, são mais responsivas ao treinamento de força, comparado às fibras musculares do tipo I, as quais possuem metabolismo energético predominantemente oxidativo (8).

Esta seletividade mais expressiva de estímulo por fibras musculares glicolíticas demonstra hipertrofia muscular como principal consequência decorrente do treinamento de força (63-65). Hipertrofia muscular é o processo onde uma célula muscular esquelética aumenta o seu volume e dimensões. Apesar deste último processo ser o efeito mais discutido na literatura científica em relação ao treinamento de força, o seu efeito é observado após meses de treinamento regular (66, 67). O processo inicial do treinamento de força é o aumento de força máxima. Entretanto, nas primeiras semanas de treinamento, o aumento de força é predominantemente influenciado por adaptações neurais (62, 68, 69). A figura 5 demonstra alguns efeitos decorrentes do treinamento de força ao longo de um período de seis meses.

Figura 5 - Efeitos adaptativos morfológicos e fisiológicos de quadríceps no treinamento de força de seis meses. Adaptado de Narici e colaboradores (1996).



Compreende-se por adaptações neurais ao treinamento de força processos estimulatórios que aumentam o recrutamento de unidades motoras musculares esqueléticas (71). Este aprimoramento no recrutamento é afetado por fatores intrinsicamente relacionados ao aumento de força, como a frequência de disparo de despolarização de motoneurônios, potencial de membrana e resistências e capacitância da membrana plasmática de células musculares (72, 73).

Em torno de oito a doze semanas já é possível observar o aumento de área de secção transversa (AST) de fibra muscular, a qual é um marcador de hipertrofia muscular (66, 74). O aumento de AST se correlaciona positivamente com o aumento de volume muscular (75).

A força muscular pode ser mensurada por diferentes metodologias (Tabela 2). O método mais aceito para realização de protocolos de treinamento de força são percentuais calculados a partir do resultado do teste de uma repetição máxima (1RM) (76, 77). O 1RM é considerado o valor máximo de força para determinado exercício, portanto a fim de estabelecer as cargas de treinamento é relativizado em percentual do valor obtido no teste de 1RM.

Tabela 2 - Métodos para calcular a intensidade de exercício de força. RM: Repetição Máxima.

Intensidade Relativa (% de RM)	Intensidade de Uma Sessão Única de Exercício
Carga de RM	Intensidade de Treinamento
Intensidade Média de Exercício	Percepção de Esforço (percepção subjectiva)
Intensidade Absoluta	

Fonte: Fry AC. Resistance exercise intensity and muscle fibre adaptations. *Sports Med*, 2004; 34 (10):663-679.

A intensidade escolhida para o treinamento determina os efeitos observados, como a hipertrofia muscular. O comportamento de intensidade segue uma curva de dose-resposta sobre adaptações musculares (71). Embora o padrão de estímulo hipertrófico seja de dose-resposta, existe um ponto ótimo para a sua intensidade. Tem se observado que tanto em humanos, quanto na grande maioria de animais o maior estímulo para hipertrofia muscular varia entre 80 e 95% de 1RM (68, 78, 79). Protocolos de treinamento de força superiores à 95% de 1RM teoricamente não forneceriam benefícios superiores, em relação à hipertrofia muscular (80).

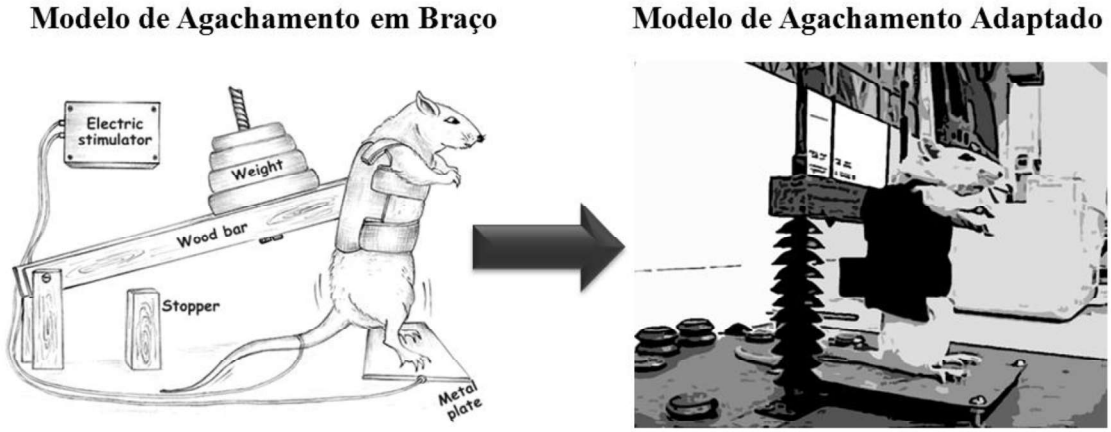
1.5.1.2 Modelos Experimentais de Treinamento de Força

Desde o início da década dos anos 90, o treinamento de força tem sido investigado por meio de modelos experimentais. O objetivo de realizar experimentos com o exercício e treinamento de força com animais era de poder gerar mais conhecimento sobre adaptações fisiológicas, bioquímicas, genéticas, bem como adaptações moleculares.

A primeira tentativa foi realizada por Tamaki e colaboradores (1992), cujo objetivo era criar um modelo de agachamento para ratos. Com este modelo foi possível observar dados reprodutíveis sobre parâmetros de hipertrofia muscular de membros inferiores em ratos. A inovação foi utilizar um estímulo elétrico padronizado para realizar extensão e flexão de membros inferiores. O estímulo elétrico desenvolvido neste trabalho mostrou não lesionar os animais (81). Neste modelo de agachamento, os animais eram posicionados com um colete de neoprene e sua cauda era acoplada a um eletrodo. Com o contato com uma placa metálica (condutor de eletricidade), os animais recebiam o estímulo elétrico para realizar as contrações musculares.

Uma limitação deste modelo era o equipamento de agachamento que era em modelo de braço, tornando-o instável na amplitude máxima durante as repetições. Por isso, em 2004 um grupo de pesquisadores da Inglaterra desenvolveu um equipamento de agachamento com barras guiadas (82). Neste trabalho, Krisan e colaboradores conseguiram trazer o modelo mais próximo da realidade de agachamento com humanos. É possível notas as diferenças entre os dois modelos de agachamento para ratos, apontando principalmente pela barra guiada (Figura 6).

Figura 6 - Ilustração da evolução de modelos experimentais de agachamento para ratos. À esquerda é representado o modelo desenvolvido por Tamaki e colaboradores (1992). À direita é representado o modelo de Krisan e colaboradores (2004) e desenvolvido por Alves e colabores (2014).



Com o passar do tempo, novos modelos foram sendo desenvolvidos. Podem ser citados desde modelos de escaladas com carga acoplada à cauda (83), saltos verticais

em cilindro com água (84) e treinamento de força de membros inferiores em roda suspensa (85). No entanto, o modelo mais representativo e que demonstra maior reprodutibilidade, bem como menor grau de estresse aos animais é o agachamento adaptado. A sua execução com estímulo elétrico garante o mesmo estímulo para contração da musculatura esquelética, não havendo fatores interferentes ao efeito hipertrófico do treinamento.

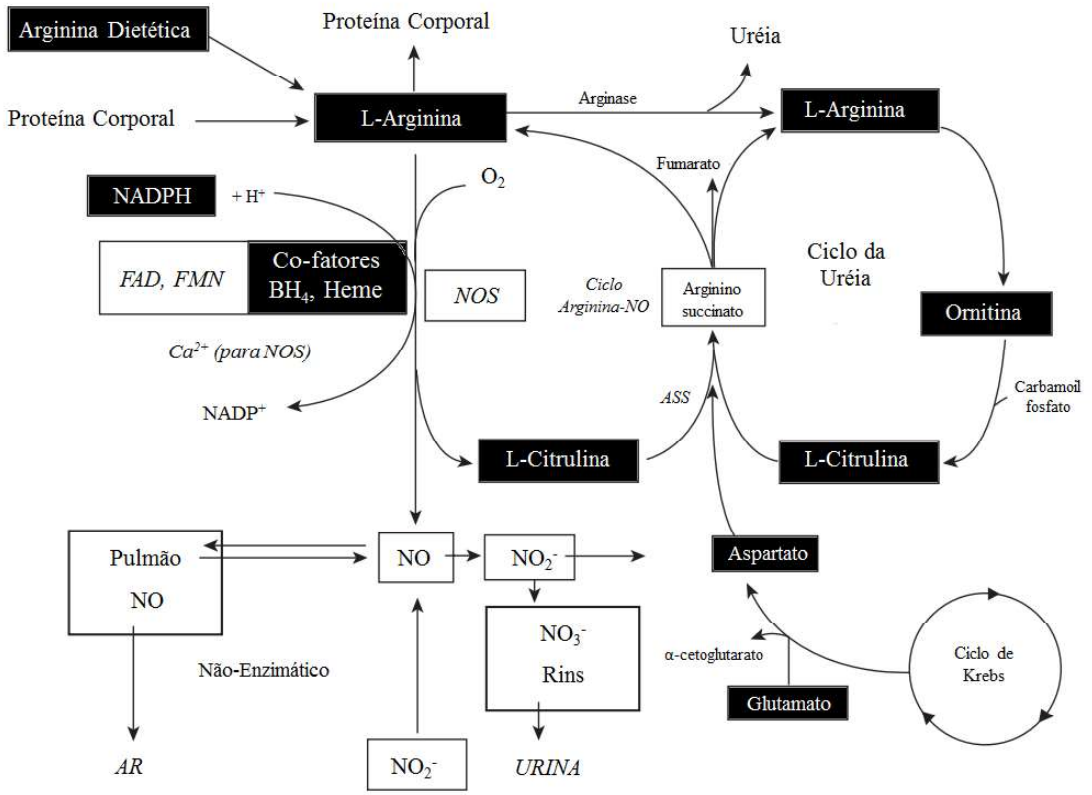
1.5.1.3 Metabolismo da L-Arginina

A L-arginina é um aminoácido proteogênico e condicionalmente essencial, se tornando essencial ou não-essencial dependendo do estágio de desenvolvimento do indivíduo (86). Aminoácidos essenciais não são produzidos pelo organismo humano, mas são necessários para a sua homeostase. Diferentemente, os aminoácidos não-essenciais são produzidos de forma endógena. A arginina é derivada principalmente da citrulina – um produto intermediário do metabolismo intestinal e hepático da glutamina. Uma vez que a citrulina é liberada à circulação sanguínea, os rins a recaptam para sua conversão em arginina (87). Aproximadamente metade da arginina ingerida é rapidamente convertida em ornitina pela arginase, sendo que esta pode ser convertida em outras substâncias ou degradada pela via de poliaminas (88).

A arginina é precursora de diversas substâncias com notáveis funções fisiológicas, como o óxido nítrico (NO), creatina ou proteínas de função diversificadas (89). Quando a arginina não é metabolizada pela arginase e convertida em ornitina, ela é processada por uma destas três possíveis enzimas: oxido nítrico sintase (NOS) - cuja função é sintetizar NO -, arginina-glicina amidinotransferase (sintetização de creatina) ou arginil-RNAt sintetase (sintetização de arginil-RNAt, que é um precursor de essencial à síntese de proteínas) (90).

A fim de sintetizar NO, a NOS necessita de alguns co-fatores para que a reação ocorra, como nucleotídeos doadores de elétrons (FAD, FMN e NADPH) e BH_4 (tetradihidrobiopterina) (91, 92). Ainda é possível sustentar de forma indireta a síntese de NO a partir da citrulina. Intermediários sintetizados no ciclo de Krebs são degradados até formarem aspartato, o qual é metabolizado a arginino succinato, é possível convertê-lo em arginina. A partir da síntese de arginina, o aminoácido serve como substrato para a NOS, sintetizando NO. A Figura 7 demonstra a interrelação de diferentes vias metabólicas da L-arginina no organismo humano.

Figura 7 - Metabolismo da L-Arginina e suas diferentes vias de síntese de intermediários e subprodutos. ASS: Arginino succinato sintase, BH_4: tetrahidrobiopterina, FAD: flavina adenina dinucleotídeo, FMN: flavina mononucleotídeo, NOS: óxido nítrico sintase. Adaptado de Luiking e colaboradores (2010).



O NO é um gás lipossolúvel sintetizado não apenas dependente de NOS, também pode ser formado a partir de nitratos e nitritos (94). O NO possui diversas funções, como vasodilatador no endotélio vascular, mediador na de neurotransmissores noradrenérgicos e não-colinérgicos, plasticidade neuronal e neuroproteção (95, 96). Seu efeito mais notável sobre o controle a curto prazo de pressão arterial é sua difusão no endotélio vascular, onde por ativação da guanilato ciclase, a guanosina trifosfato é degradada à guanosina-3,5-monofosfato (GMPc). Este subproduto atua como segundo mensageiro em uma cascata de sinalização no processo de relaxamento da musculatura lisa de vasos sanguíneos, reduzindo assim a pressão arterial (97).

Além de funções cardiovasculares, o NO é uma espécie reativa de oxigênio e nitrogênio com alta reatividade e taxa de difusão (98). A formação na sua forma reativa pode causar danos oxidativos em biomoléculas, incluindo o DNA (13). Apesar de

investigações demonstrarem efeitos antioxidantes e pró-oxidantes do NO, ainda não está bem estabelecido o seu comportamento no balanço redox celular (99).

1.5.1.3 Suplementação de L-Arginina

O NO desempenha uma série de papeis na regulação da função de diversos órgãos, dependendo do tipo de célula, tecido, bem como a isoforma de NOS responsável (13). Depois de demonstrar notável importância do NO sobre a fisiologia humana, hipóteses foram surgindo de substâncias precursoras deste gás poderia desempenhar efeito ergogênico em situações de exercício físico (100). Através de sua ação vasodilatadora, mediada na reação da conversão da arginina em citrulina, produzindo NO pela catalisação da NOS. A produção de NO teoricamente aumentaria a perfusão sanguínea local, capacitando o músculo esquelético em ter maior acesso à nutrientes essenciais à contração muscular, aumentando assim o desempenho durante o exercício físico.

Outro possível efeito da suplementação de arginina seria o aumento de massa muscular pela modulação positiva na síntese e liberação de hormônio do crescimento (100). Embora esta hipótese pareça muito vaga, nenhum estudo buscou avaliar o efeito crônico da sua suplementação associada ao treinamento de força sobre a massa muscular.

Apenas uma investigação demonstrou efeito ergogênico em exercício isocinético, mesmo com um tamanho amostral pequeno (101). Mais recentemente, em um estudo similar a administração oral de arginina demonstrou aumentar o volume sanguíneo durante o exercício isocinético, entretanto não demonstrou efeitos sobre o desempenho (102). Das poucas investigações que demonstraram efeitos positivos da sua suplementação (14, 103), a grande maioria falhou em demonstrar aumento de força máxima ou desempenho (104-106). A abordagem da suplementação de arginina como um possível antioxidante em situações do exercício de força é escassa. O seu mecanismo ergogênico observados em alguns estudos ainda não está bem estabelecido.

Ainda são necessários mais estudos para avaliar se os efeitos evidenciados são replicáveis no meio científico, para que em um futuro mais distante, seja possível planejar a suplementação de L-arginina como um suplemento eficaz para atletas e praticantes de exercício físico.

1.5.2 Aplicação da Técnica em Modelo Experimental de Insuficiência Cardíaca

1.5.2.1 Epidemiologia

A Insuficiência Cardíaca (IC) é uma síndrome degenerativa com progressão agressiva e de difícil controle (107). Segundo a *American Heart Association*, em uma recente publicação demonstra o impacto de doenças cardiovasculares em desfechos fatais comparado a outras causas de morte. As doenças cardiovasculares representam mais de 30% das causas de morte, tanto em homens, quanto em mulheres, permanecendo em primeiro lugar, seguido por neoplasias (aproximadamente 24%) (17).

No Brasil, o quadro é muito similar ao reportado pelo *American Heart Association*. De acordo com a Sociedade Brasileira de Cardiologia, no Brasil em torno de 30% das mortes são causadas por doenças cardiovasculares (108). Apesar da severidade que a síndrome da IC apresenta, com o passar dos anos, o número de mortes a cada 1.000 pessoas apresenta-se em declínio desde 2000 (17). Através de novas estratégias terapêuticas, foi possível aumentar a expectativa de vida de portadores de IC, bem como aumentar a sua qualidade vida.

Alguns fatores externos desempenham importantes papéis na progressão de doenças cardiovasculares, como o tabagismo, maus hábitos alimentares e inatividade física (17). Por ser uma síndrome com progressão contínua de seus sintomas, a IC representa em torno de 70% dos custos, tanto diretos, quanto indiretos gerados por pacientes portadores de IC (109). A progressão da síndrome por si só não é a única dificuldade à ser enfrentada, um fator que atinge diretamente os custos gerados pela IC é a baixa adesão ao tratamento farmacológico, e de forma mais expressiva ao tratamento não-farmacológico (109).

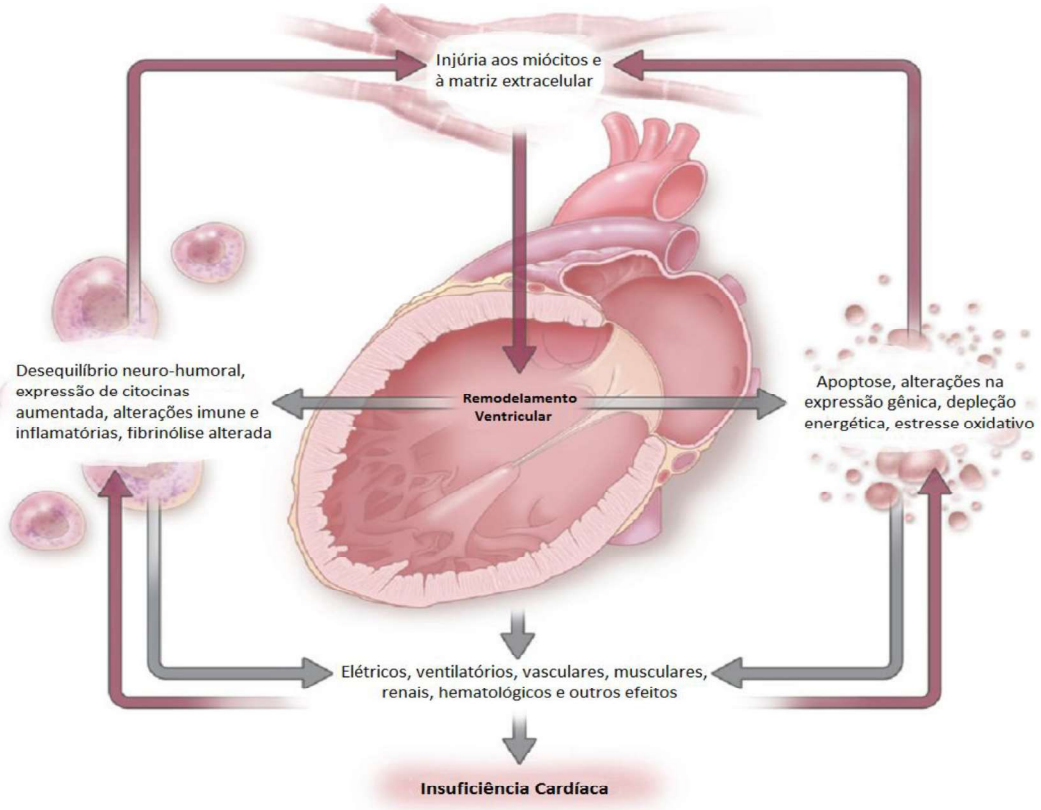
1.5.2.2 Fisiopatologia da Insuficiência Cardíaca

A insuficiência cardíaca (IC) é caracterizada por distúrbios estruturais e funcionais do coração (17). Estas alterações levam à redução do fluxo sanguíneo necessário às atividades metabólicas do organismo, o que pode induzir a sintomas como dispneia, fadiga, congestão pulmonar e sinais, como edema periférico (110, 111). Além disso, muitos destes fatores limitantes estão intimamente relacionados com a perda de

massa magra, bem como redução da capacidade respiratória (112), o que demonstra um cenário característico encontrado em pacientes com IC (113).

Apesar dos grandes progressos em relação às terapias farmacológicas, pacientes com IC possuem uma reduzida capacidade funcional e uma baixa qualidade de vida (114). Este cenário, característico de pacientes com IC, está, em parte, relacionado às alterações tanto estruturais quanto funcionais do sistema cardiovascular e do músculo esquelético, prejudicando a função hemodinâmica e a atividade muscular (115). Como é demonstrado na Figura 8, a fisiopatologia da IC é dependente de alguns fatores-chave para o seu surgimento e progressão.

Figura 8 - Fisiopatologia da Insuficiência Cardíaca e fatores-chave no remodelamento ventricular e progressão da síndrome. Adaptado de McMurray, 2010.



Os pacientes com IC apresentam um severo desequilíbrio do estado redox e alguns marcadores de estresse oxidativo têm sido correlacionados com a disfunção miocárdica e severidade global da IC (117). Um dos mecanismos evidentes pelo qual o estresse oxidativo do miocárdio pode prejudicar a função cardíaca é através de dano oxidativo a proteínas e a membranas celulares, induzindo assim disfunção de organelas celulares, como a mitocôndria, ou morte por apoptose e necrose (118).

O tecido cardíaco é notavelmente suscetível ao estímulo adrenérgico exacerbado. A modulação neuro-humoral pelo aumento na atividade simpática com concomitante redução da atividade parassimpática, eleva a secreção de catecolaminas em pacientes com IC (116). A hiperativiação do componente simpático tem como principal efeito sobre o cardiomiócito aumentar a força de contratilidade, o qual é diretamente modulado pela abertura de canais de Ca^{2+}. A IC tem como característica apresentar uma alteração do metabolismo intracelular de Ca^{2+}. O excesso de liberação de Ca^{2+}, bem como a disfunção da mitocôndria em gerar ATP no cardiomiócito está intimamente relacionado à abertura de poros de permeabilidade transitória mitocondriais, os quais acabam permitindo a saída de espécies reativas de oxigênio e nitrogênio formados na microestrutura mitocondrial (119).

O coração é um órgão com capacidade regenerativa e reparadora limitada. Agudamente após o infarto, o miocárdio sofre uma série de lesões de características hemodinâmicas (120), necróticas e apoptóticas (121), inflamatórias (122), oxidativas e nitrosativas (16), bem como autofágicas (123). Cronicamente, o coração demonstra um panorama diferente. Ao se adaptar com disfunção ventricular esquerda, o coração demonstra uma taxa apoptótica e necrótica muito reduzida, quando comparada ao evento de infarto agudo do miocárdio (124), no entanto é em torno de 10 a 100 vezes superior à um coração saudável (125-127). Entretanto, o cenário de produção de radicais livres e mediadores pró-inflamatórios se torna um fator de notável importância na progressão da IC (128).

1.5.2.3 Remodelamento Cardíaco

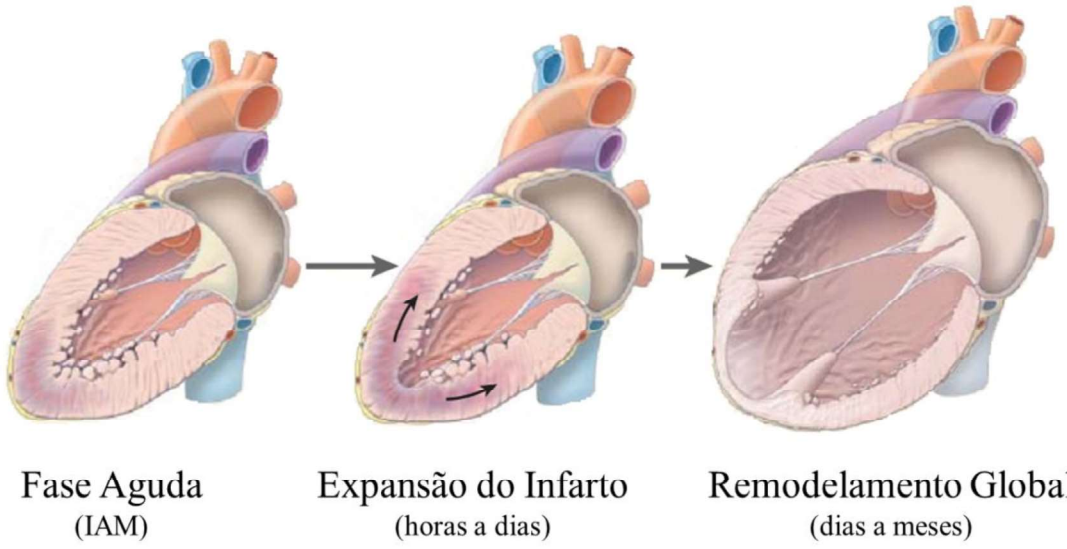
Remodelamento cardíaco é um processo decorrente de elevada demanda cardíaca. A hipertrofia cardíaca é um tipo de remodelamento cardíaco. A hipertrofia cardíaca pode ser subdividida em dois grandes grupos: fisiológica e patológica. A hipertrofia fisiológica é observada em indivíduos fisicamente ativos e mulheres grávidas e não está associado à alterações de funcionamento cardíaco (129). Já a hipertrofia cardíaca patológica é resultante da sobrecarga de volume, pressão ou até mesmo eventos cardiovasculares, como o infarto agudo do miocárdio (130).

A hipertrofia muscular do ventrículo esquerdo na IC é construída a partir de um conjunto de alterações estruturais decorrentes de apoptose e necrose das células endoteliais e miocárdicas, progressiva disfunção da contratilidade do miócito, alteração

na função do fibroblasto cardíaco e extenso remodelamento da matriz extracelular (131). Neste processo, o estresse oxidativo causa alterações morfológicas na mitocôndria, induzindo inchaço da matriz e aumento da espessura do espaço intermembrana mitocondrial. Estas alterações resultam na produção desenfreada de ânion radical superóxido, redução na eficiência da produção de ATP e alterações transcricionais dos genes associados com a IC em relação à contratilidade cardíaca (132). Este processo na IC é denominado de disfunção mitocondrial (118) e está intimamente relacionada em todos os estágios do remodelamento cardíaco.

O remodelamento cardíaco pode apresentar características distintas ao longo do tempo após o infarto (Figura 9). A fase aguda refere-se ao infarto do ventrículo esquerdo o qual causa morte de cardiomiócitos que foram afetados diretamente à isquemia (133). Após algumas horas e até mesmo dias, a área necrótica antes atingida é expandida atingindo o tecido cardíaco adjacente. Por fim, ao longo de semanas e meses, o coração já não possui mais uma área necrótica, mas uma área cicatricial e a alteração da sua forma geométrica original para um formato globóide (134). O coração infartado demonstra elevado acúmulo de tecido fibroso, o qual altera o seu comportamento elétrico, bem como a função ventricular esquerda (135). A cicatriz fibrosa cardíaca reduz a contratilidade e dilatação do ventrículo e esta é dependente da área afetada pelo infarto agudo do miocárdio (136).

Figura 9 - Representação da progressão do remodelamento ventricular esquerdo após o infarto agudo do miocárdio. IAM: Infarto Agudo do Miocárdio. Adaptado de Jessup & Brozena, 2003.



O remodelamento cardíaco resulta no aumento da massa ventricular (hipertrofia muscular cardíaca), no entanto, sem ocorrer um aumento no volume do ventrículo esquerdo (dilatação). Essa alteração é definida como hipertrofia concêntrica patológica, que tem como característica a organização dos sarcômeros em paralelo, consequência de uma sobrecarga por pressão, induzindo aumento do diâmetro celular do cardiomiócito (137).

Com a progressão do remodelamento ventricular, é iniciada a fase de dilatação do ventrículo esquerdo relativo ao aumento da massa ventricular. Este processo é definido como hipertrofia excêntrica e está diretamente ligado à sobrecarga de volume, resultando em adição de sarcômeros em série, que aumenta a célula no seu comprimento (138, 139).

O acúmulo de tecido fibroso no coração infartado altera o comportamento elétrico e também as funções sistólica e diastólica do ciclo cardíaco. A fibrose intersticial em excesso indica um importante aspecto negativo da hipertrofia ventricular esquerda crônica e da IC, além disso, o estresse oxidativo desempenha um importante papel no processo pró-fibrótico em vários órgãos, incluindo coração (140).

1.5.2.4 Alterações Hemodinâmicas

Além de repercussões deletérias sobre a estrutura do miocárdio, a IC leva uma série de alterações hemodinâmicas que afetam indiretamente diversos órgãos e sistemas (141). Após o infarto agudo do miocárdio, é comum que aconteça uma queda do volume ejeção do ventrículo esquerdo, o qual é consequência da perda de tecido muscular cardíaco, aumentando desta forma, a tensão diastólica (pré-carga) e o volume do ventrículo esquerdo e restabelecendo, inicialmente, sua função normal (111, 139, 142).

A disfunção sistólica e diastólica do ventrículo esquerdo permite um ciclo vicioso de prejuízo na distribuição sanguínea à todos órgãos e estruturas periféricas , levando à progressão e severidade da IC (143). A rigidez miocárdica usualmente é o maior determinante sobre a rigidez de câmaras ventriculares cardíacas, sendo mais proeminente que estruturas adjacentes do coração, como o pericárdio e a pressão intratorácica (144, 145). Desta forma, torna-se importante buscar opções farmacológicas e não-farmacológicas que possam atenuar a rigidez do miocárdio que é apresentada em pacientes portadores de IC.

Na IC, comumente é observado disfunção diastólica, na qual se diferencia as alterações hemodinâmicas da IC sistólica no comprometimento da função hemodinâmica (146). Através do processo fibrótico após o infarto agudo do miocárdio, é observado um aumento na rigidez do ventrículo esquerdo (135). Um dos fatores moleculares principais que contribuem para o aumento da rigidez passiva do ventrículo esquerdo na diástole, é a hiperfosforilação de resíduos da estrutura da titina. Nesta proteína, dois resíduos de serina de proteínas cinases prolina-glutamato-valina-lisina (PEVK) são particularmente sensíveis à esta modulação, como a região 26 (S26) e região 170 (S170). A PEVK S26 demonstra ser ainda mais suscetível à hiperfosforilação, o que levaria ao encurtamento de sarcômeros de miofibrilas cardíacas a qual aumentaria, por sua vez, a rigidez passiva na diástole (147).

Outro fator importante que participa na função hemodinâmica do coração é a formação de radicais livres, como o ânion radical superóxido. Este radical livre é formado, além do vazamento de elétrons na mitocôndria, pela xantina oxidase durante o ciclo de contração-relaxamento da musculatura cardíaca (148). Recentemente, tem sido observado que inibidores da xantina oxidase são capazes de atenuar alterações hemodinâmicas sistólicas e, principalmente, diastólicas após sobrecarga de volume (149).

Existem parâmetros que refletem a disfunção ventricular esquerda em pacientes com IC. Um dos parâmetros utilizados que demonstram disfunção no ventrículo esquerdo é a pressão diastólica final do ventrículo esquerdo (PDFVE) (150, 151). Esta pressão indica um aumento de pressão dentro da câmara ventricular esquerda, a qual não deveria estar aumentada em relação à indivíduos sem IC diastólica (152). Alguns fatores contribuem para o seu aumento, como a conteúdo de colágeno na área cicatricial de infarto do miocárdio, o aumento na rigidez passiva do ventrículo esquerdo durante a diástole, bem como alterações no remodelamento do ventrículo esquerdo (153). Recentemente tem sido recomendado a estratificação de valores obtidos de PDFVE de pacientes a fim de estabelecer risco de desfechos cardiovasculares, bem como o seu tratamento (154).

1.5.2.5 Modelos Experimentais de Insuficiência Cardíaca

Modelos experimentais de infarto agudo do miocárdio tem sido extensivamente utilizados em estudos de mecanismos fisiopatológicos, bem como em efeitos de

tratamentos farmacológicos e não-farmacológicos. O infarto agudo do miocárdio é uma das principais causas para o desenvolvimento de IC em humanos (116), por isso torna-se relevante desenvolver modelos experimentais a fim de testar novas alternativas no tratamento da IC.

O modelo mais utilizado para indução do infarto agudo do miocárdio em ratos é a ligação da artéria coronariana (LAC) esquerda descendente (155-157). Em função do seu baixo custo e da sua praticidade, a LAC leva à áreas extensas de infarto, remodelamento cardíaco, bem como disfunção do ventrículo esquerdo (158, 159). Cirurgias de indução de infarto do miocárdio, como a LAC, demonstram taxas de mortalidade variando entre 30 e 50%, dependendo da experiência do cirurgião.

Todo modelo experimental necessita de parâmetros ou um conjunto deles a fim de estabelecer o diagnóstico de IC. Diferentemente de humanos, onde o diagnóstico se baseia em sinais, sintomas e análises ecocardiográficas, modelos experimentais de IC utilizam a PDFVE como um dos principais parâmetros no diagnóstico da IC em ratos (160). De fato, apenas a elevação da PDFVE não é suficiente para estabelecer a presença de IC em ratos. Pelo menos outros critérios devem ser preenchidos, como congestão pulmonar e hipertrofia do ventrículo esquerdo (161).

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CAPÍTULO II - Artigo 1 em Inglês

L-Arginine Supplementation and Resistance Training Promotes Increase of Maximum Strength and Protection from DNA Damage in Rats

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L-Arginine Supplementation and Resistance Training Promotes Increase of Maximum Strength and Protection from DNA Damage in Rats

Abstract

L-arginine (Arg) is a precursor of several substances with remarkable physiological functions, such as nitric oxide (NO) and the amino acid creatine. Arg supplementation has been associated with increased strength in high-intensity exercise. However, there is no clear evidence of association of supplementation with Resistance Training (RT) on their antioxidant and ergogenic effects, specifically on their genotoxic protection. Therefore, we evaluated the effect of supplementation of Arg associated with RT 8 weeks about gaining maximum strength and DNA damage in rats. Ten Wistar rats (220-270g, 90 days old) were randomly divided into 3 groups: sedentary (SED, n = 4), Resistance Training (RT, n = 2) and Resistance Training + L-Arginine (RT+Arg, n = 4). Trained rats were submitted to the protocol of RT in a squat apparatus adapted for rats (4 sets of 10-12 reps with 90s interval, 4 times/week, 65-75% of One Maximum Repetition (1MR) for 8 weeks). The supplemented group received Arg (500mg/kg) daily for 8 weeks. After 8 weeks, whole blood was collected from animals to perform the comet assay. It was used CASP software (CASPLabs®) for quantification of DNA damage. To quantify the maximum strength the 1MR test was performed at baseline and after the RT protocol. For comparisons among groups it was performed One-way ANOVA followed by post hoc test of Student-Newman-Keuls. For associations, it was used the Pearson correlation test. We considered the level of significance of 5%.The RT was able to protect DNA damage in rats. However, the association with Arg supplementation was able to promote greater genotoxic protection, compared to sedentary rats. These results indicate remarkable ergogenic action and genotoxic protection of Arg supplementation in association with RT.

Keywords

Arginine; Resistance training; Supplementation; Exercise; DNA damage; Genotoxicity; Rats

Short Communication

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Abbreviations

1MR: One Maximum Repetition; Arg: L-Arginine; cGMP: cyclic Guanosine Monophosphate; DNA: Deoxyribonucleic Acid; GTP: Guanosine Triphosphate; NO: Nitric Oxide; NOS: Nitric Oxide Synthase; OTM: Olive Tail Moment; RONS: Reactive Oxygen and Nitrogen Species; RT: Resistance Training; TM: Tail Moment

Introduction

The supplementation of L-arginine (Arg) has been showed to improve performance in high intensity and short duration exercises, such as weightlifting [1,2], possibly due to its vasodilating action. In this reaction mediated by the conversion of Arg to citrulline, the production of nitric oxide (NO) occurs by the enzymatic catalysis of nitric oxide synthase (NOS). The NO is a Reactive Oxygen and Nitrogen Specie (RONS), found in the form of molecular gas. The responsible enzyme to produce NO is NOS, which may be expressed constitutively in endothelial isoform (eNOS), neuronal (nNOS) and inducible form (iNOS) [3]. The decoupling of NOS constitutive forms lead to the overproduction of two oxidants in endothelium: the superoxide anion-radical ($O_2^{\cdot-}$) and peroxynitrite ($ONOO^-$). These both free radicals are associated with the development of endothelial disfunction [4].

Under normal conditions, endothelium produces NO by Arg in the presence of oxygen catalyzed by eNOS. The NO diffuses into the adjacent smooth muscle, where it is modulated by guanylate cyclase. This enzyme has the function of converting guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP), thereby causing relaxation of vascular wall [5]. In situations where it is established a dysfunction of vascular endothelium, production of NO occurs in the same manner, together with high concentrations of $O_2^{\cdot-}$, which is caused by the decoupling of electrons in the reaction of eNOS. In this case, the $O_2^{\cdot-}$ can be converted into hydrogen peroxide.

The NO may react with $O_2^{\cdot-}$ and form $ONOO^-$, which is a highly cytotoxic compound [6]. Free radicals have high reactivity to adjacent biomolecules. It is well established that reactive species leads to the impairment of important cellular structures such as lipid peroxidation (mainly in plasmatic membranes), damage in

proteins (signaling proteins, enzymes and other proteins) and genomic damage (oxidation of nucleic acids).

The Resistance Training (RT) has been widely utilized by the general population as a very effective method in promoting gains of muscle strength, increase power performance in sports, preventing muscle lesions and increased quality of life [7]. Despite of the scientific literature demonstrate benefits of Arg supplementation in exercised conditions, there are few evidence of associating of supplementation with RT on their ergogenic effects, as well as on genotoxic protection. For that reason, we evaluated the effect of supplementation of Arg associated with RT 8 weeks about gaining maximum strength and DNA damage in rats.

Methodology

Ten Wistar rats (220-270g, 90 days old) were randomly divided into 3 groups: Sedentary (SED, n = 4), Resistance Training (RT, n = 2) and Resistance Training + L-Arginine (RT+Arg, n = 4). The animals were housed under standard conditions (food and water *ad libitum*, temperature between 22 and 24 °C, light - dark cycle of 12 hours). This study was approved by CEUA/UFCSPA, under the protocol number 114/13.

Resistance training protocol

Trained rats were submitted to a RT protocol in a squat apparatus adapted for rats [8,9] (4 sets of 10-12 reps with 90s interval, 4 times/week, 65-75% of One Maximum Repetition (1MR) for 8 weeks). An electrical stimulus (4-5 mA , 0.3 seconds long, with a 3 second interval between each repetition) [10] was applied in the rat's tail using a surface electrode, in order to provoke the extension movement of the lower limbs of the rat.

To quantify the maximum strength, it was performed the 1MR test at baseline and after the RT protocol. To determine the maximum lifted load in one repetition, the One Maximum Repetition (1MR) was utilized. From the obtained value, the load percentage required to perform the training protocol were determined. In response to training, it is expected strength gains along the training, making the realization of retests every two weeks necessary, in order to adjust the training load.

L-Arginine supplementation

It was utilized L-Arginine (presentation form: powder, with purity of 99.9%, Sigma-Aldrich®, Brazil). The supplementation was given by gavage solution, as this resembles human oral consumption and ensures that the desired dosage is achieved. The supplemented group received Arg (500mg/kg) daily for 8 weeks [11]. The animals received the supplement every day before training for the entire period of the protocol (including the days on which they did not train).

Comet assay (alkaline version)

After 8 weeks, whole blood was collected from animals to perform the comet assay. The alkaline Comet Assay in peripheral blood was performed as described in the literature [12,13] with minor changes. It was used 20 µL of whole peripheral blood embedded in 90 µL of 0.75% low-melting point agarose at 37 °C. This mixture was placed into a slide previously coated with 1.5% of normal melting point agarose processed at 60 °C. A cover glass was placed on top and the agar allowed to set at 4 °C and, after gel solidifying, the cover glass were removed. Then, the slides were immersed in iced-cold lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0; containing freshly added 1% (v/v) Triton X-100 and 10% (v/v) dimethylsulfoxide (DMSO) at 4 °C in dark for a minimum of 1 h. Afterwards, to allow DNA unwinding, slides were incubated in a freshly made alkaline electrophoresis buffer (0.3 M NaOH and 1 mM EDTA; pH > 13) at 4 °C for 5 min in a horizontal electrophoresis box. The alkaline electrophoresis was carried out for 15 min at 25 V and 300 mA. Every step was carried in dark. After electrophoresis, slides were washed three times in a neutralization buffer (0.4 M Tris; pH 7.5) for 5 min, rinsed twice in distilled water, and left to dry overnight at room temperature. Then, the slides were fixed for 10 min in trichloroacetic acid 15% w/v, zinc sulfate 5% w/v, glycerol 5% v/v, rinsed three times in distilled water, and dried for 2 h at 37 °C. Finally, the slides were stained with silver nitrate as previously described by Nadin et al. [14].

For DNA damage quantification, it was used CASP software (CASPLabs®) [15]. The software can detect three different types of markers of genotoxic damage, such as percentage of tail DNA, tail moment (represents the distance of DNA migration from the head of the comet) and olive tail moment (represents the product of the tail length and the fraction of DNA in the tail). For image analysis it was scored 100 comets per slide.

Statistical analysis

The results are expressed in mean ± SD. For comparisons among groups it was performed One-way ANOVA followed by post hoc test of Student-Newman-Keuls. For associations, it was used the Pearson correlation test. We considered the level of significance of 5%.

Results

All of the groups showed no differences at the baseline of resistance training protocol ($P>0.05$). After 8 weeks of training, it was observed higher maximum strength in the trained groups, when compared to the sedentary group ($P<0.05$). However, the RT + Arg group showed greater maximum strength than the other groups ($P<0.05$) (Figure 1).

Regarding damage to DNA, % Tail DNA was lower in RT + Arg, compared with RT and SED groups ($P<0.05$) (Figure 2) as well as in other genotoxic markers: Tail Moment (TM) ($P<0.05$) (Figure 3) and Olive Tail Moment (OTM) ($P<0.05$) (Figure 4).

It was observed a strong inversely proportional correlation between different markers of DNA damage with maximum strength. Percentage of Tail DNA with 1MR (r=-0.9719, $P<0.05$), TM with 1MR (r=-0.9046, $P<0.05$) and OTM with 1MR (r=-0.9079, $P<0.05$).

Conclusion

The RT was able to protect DNA damage in rats. However,

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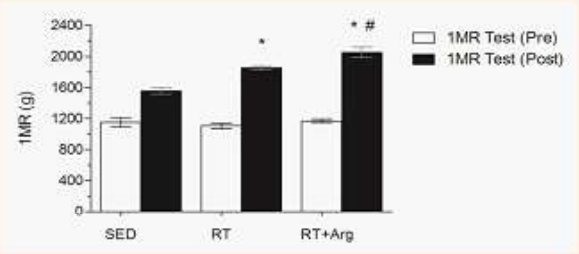

Figure 1: Maximum strength pre and post 8 weeks of study protocol.

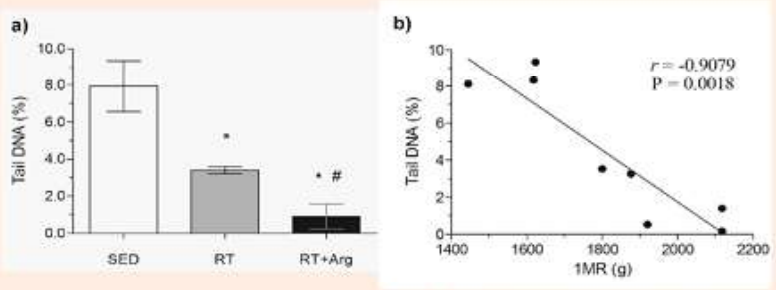

Figure 2: DNA damage in lymphocytes measured by alkaline comet assay. a) % Tail DNA; b) Pearson correlation test between % Tail DNA and the final test of 1MR.

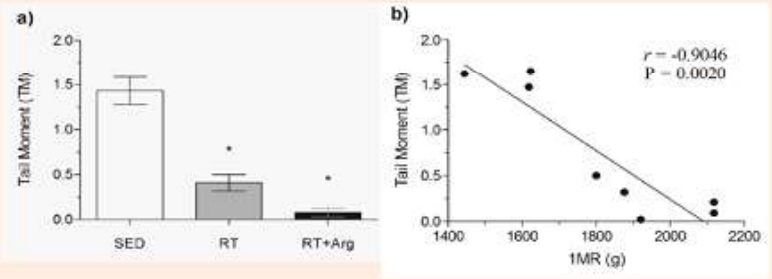

Figure 3: DNA damage in lymphocytes measured by alkaline comet assay. a) Tail Moment (TM); b) Pearson correlation test between Tail Moment (TM) and the final test of 1MR.

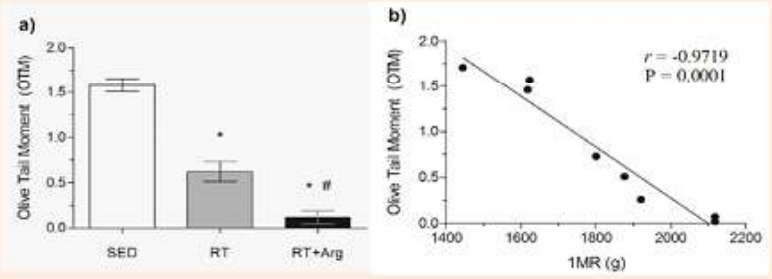

Figure 4: DNA damage in lymphocytes measured by alkaline comet assay. a) Olive Tail Moment (OTM); b) Pearson correlation test between Olive Tail Moment (OTM) and the final test of 1MR.

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the association with Arg supplementation was able to promote greater genotoxic protection, compared to sedentary rats. Also, the association of RT with Arg supplementation increase maximum strength after the training protocol. These results indicate remarkable ergogenic action and genotoxic protection of Arg supplementation in association with RT.

Acknowledgements

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CAPÍTULO II - Artigo 2 em Inglês

L-Arginine supplementation alone influences in genomic stability and cardiac contractility and muscle mass in rats with low L-arginine diet, but is not associated with resistance training adaptations

(Artigo escrito seguindo o formato do *Amino Acids;* Fator de Impacto: 3.653)

L-Arginine supplementation alone influences in genomic stability and cardiac contractility and muscle mass in rats with low L-arginine diet, but is not associated with resistance training adaptations

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Abstract

L-arginine supplementation (Arg) has been related to increase Maximum Strength (MS), improvement of hemodynamic parameters in several diseases. However, there is no clear evidence of its supplementation with Resistance Training (RT) on muscle mass, hemodynamic function, ergogenic effect and genomic stability in leukocytes. The objective was to evaluate the effect of Arg and RT on MS, hemodynamic variables and DNA damage in healthy rats subjected to a low Arg concentration diet. Twenty three Wistar rats (290-320g) were divided into 4 groups: Sedentary (SED,n=6), Sedentary+Arg (SED+Arg,n=6), Resistance Training (RT,n=5), Resistance Training+Arg (RT+Arg,n=6). Trained animals performed RT protocol in a squat apparatus adapted for rats (4 sets of 10-12 repetitions, 90s of interval, 4x/week, 65-75% of One Maximum Repetition (1MR), for 8 weeks). It was used comet assay in blood to measure DNA damage in leukocytes. For comparisons among groups, it was performed two-way ANOVA, post-hoc test of SNK, with a significance level of 5%. The RT induced higher MS in trained groups. The supplementation alone led to increased ratio of gastrocnemius and left ventricle (LV) to body mass, increased LV contractility without changing hemodynamic variables. The SED+Arg group showed higher concentration of extracellular heat shock protein 72 and total testosterone, as well as lower uric acid concentration in blood versus SED group. The administration of isolated Arg and its association with RT promoted less damage in DNA. Arg supplementation with low Arg diet, does not enhance MS gains, however affects increasing muscle mass by increasing testosterone concentration and also demonstrates the positive effect in leukocyte genomic stability in healthy rats.

Key-words: Arginine, Supplementation, DNA Damage, Resistance Training and Rats.

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Introduction

L-arginine is a semi-essential amino acid, a precursor in the synthesis of various bioactive substances, such as nitric oxide (NO), growth hormone and creatine (Boger 2007). The administration of L-arginine blood flow could be enhanced mainly through vasodilatation of the endothelium, and also throughout via angiogenesis stimulation induced by NO (Suzuki 2006). This rationale reinforce the hypothesis that providing increased supply of nutrients to skeletal muscle and higher removal of metabolites, L-arginine might act as a promissory ergogenic supplement in strength exercises (Wax et al. 2012).

Conversely, in diets providing low concentrations of L-arginine, circulating levels of L-arginine decreases in different species, such as rodents, pigs and humans (Castillo et al. 1994; Gross et al. 1991; Wu et al. 2013). Since L-arginine demonstrates notable antioxidant properties, such as reactive oxygen species (ROS) scavenging of superoxide and hydroxil radical (Shan et al. 2013), its deficient intake may be detrimental to the homeostasis of several organs. For instance, in stressful situation, such as sepsis, might affect immune function. The supplementation of L-arginine can be important by increasing the number and mitogen function of T cells (Tong and Barbul 2004). For that reason, it is essential to maintain adequate function of immune system, by increasing antioxidant defenses and/or protecting higher cellular damage.

When the excessive chronic production of ROS and other oxidant agents, and this overcome the cellular antioxidant capacity, cellular lesion to distinct biomolecules may occur, such as lipid peroxidation, protein carbonilation, as well as DNA strand breaks (Ciccia and Elledge 2010; De Bont and van Larebeke 2004). DNA damage plays important role in mutagenesis, carcinogenesis and aging. The accumulation of DNA lesions by ROS has been demonstrated to be a significant risk for the development of cardiovascular diseases and cancer (Azqueta et al. 2014).

Thus, hypothesis had been emerging of L-arginine administration as a nutritional supplement on oxidative parameters in exercise and as a preventive supplement. It has been shown by Huang and colleagues (2008) that L-arginine supplementation improved maximum oxygen consumption, and was able to protect against oxidative damage in skeletal and cardiac muscle, as well as in liver, lungs and kidney by exhaustive exercise in rats.

The low intake of L-arginine has prejudicial effects in animal growth and antioxidant status (Ma et al. 2010; Wu et al. 2010). However, its importance in genomic stability in leukocytes has not been investigated. Also, the association of resistance training protocol with L-arginine supplementation on biochemical parameters and a possible synergic effect of physical exercise has not been investigated yet. Therefore, the objective was to evaluate if L-arginine supplementation and resistance training of eight weeks could enhance maximum strength, cardiac contractility, hemodynamic variables, muscle mass, biochemical parameters in plasma and DNA damage in leukocytes in healthy rats subjected to a low L-arginine diet.

Methods

It was used 23 male Wistar rats from the biothery of the Universidade Federal de Ciências da Saúde de Porto Alegre, with approximately 100 days of age and weighing between 290 and 320 g. The animals were kept in plastic boxes with measures of 220 x 260 x 310 mm, in groups of four animals per cage, in an environment with controlled temperature (21°C) and light-dark cycle of 12 hours. Water and commercial chow (NuviLab CR-1, São Paulo, SP, Brazil) were offered *ad libitum*. The standard chow contained 22% proteins, 61% sugars, 4% fat, 7% fibers, 1% vitamins and 5% minerals. The diet offered low concentrations of L-arginine (0.11%). The development of body weight was monitored weekly.

The handling of animals complied with the provisions of Law No. 11,794 of 08/10/2008, in Law No. 6,899 of 15/07/2009, in Resolution No. 879 of 15/02/2008 (CFMV) and any other rules applicable to the use of animals for teaching and research, especially resolutions of CONCEA. The study was approved by the Ethics Committee and Institution of Animal Use in the process number 114/13.

The animals were randomly assigned to four groups: sedentary group (SED, n = 6), sedentary and supplemented with L-arginine group (SED+Arg, n = 6), resistance training group (RT, n = 5) and resistance training and supplemented with L-arginine group (RT+Arg, n = 6).

Resistance Training Protocol

The animals from trained groups were submitted to a resistance training protocol. It was performed a familiarization period in the adapted squat apparatus of Alves and colleagues (2014) for all animals. The familiarization period consisted in performing 50 to 10 repetitions of 40% to 60% of their body weight three times a week for one week.

The rats were placed in a neoprene vest leaving them in bipedal position in the lower limbs. an electrical stimulus (4-5 mA, 0.3 seconds long, with 3 seconds interval between each repetition) was

applied to the rat's tail through a surface electrode, to cause the extension and flexion movement of the lower limbs of the rat, thus raising the burden in the squat apparatus. This stimulation is of low intensity, not causing any injury to the physical integrity of animals (Tamaki et al. 1992). All training sessions were performed in a dark room.

To determine the maximum load raised in one repetition it was used the One Maximum Repetition test (1MR). From the obtained value, it was determined percentage load for performing the training protocol. In response to training, strength is gained, making it necessary to carry out new test every two weeks to adjust the training load.

The training protocol had total duration of eight weeks, frequency of four times a week, each training session consisted of four sets of 12 repetitions with 65-75% of 1MR load with 90 seconds of rest between each series (Stefani et al. 2014b). The training program followed the recommendations of the American Physiological Society (2006).

L-Arginine Supplementation

The groups were supplemented with a L-arginine solution, which received daily by gavage. The form given the supplement was L-arginine powder with a purity of 99.9% (Sigma-Aldrich, SP, Brazil). It was used 500 mg/kg/day as a supplement dosage, since recent studies showed that this same concentration was able to significantly increase serum levels of L-arginine (Krauss et al. 2012; Shan et al. 2013).

Evaluation of Hemodynamic Function

The records of hemodynamic (blood pressure, heart rate and left ventricular pressure) were performed 24 hours after the end of last training session. The animals were anesthetized with ketamine (80 mg/kg, i.p.) + xylazine (12 mg/kg, i.p.). P50 was used a polyethylene catheter into the left ventricle via cannulation of the right carotid artery. The catheter was inserted into the left ventricle and its position determined by observation of a characteristic of the ventricular pressure waveform.

Record of Blood Pressure, Heart Rate and Left Ventricular Pressure

Before placing the PE50 catheter in the ventricle, the blood pressure was recorded for 5 minutes through the arterial cannula connection to an electromagnetic transducer (P23 Db, Gould-Statham, USA) connected to a signal preamplifier (Hewlett-Packard 8805C, Puerto Rico, USA). Soon after this record, the cannula was positioned in the left ventricle and after 5 minutes of stabilization, the ventricular pressure was recorded for 5 minutes. The analog pressure signals were digitalized (CODAS, 1Kz, DTAQ Instruments, USA) allowing analysis of the pulses of blood pressure moment-to-moment, with a sampling frequency of 1,000 Hz per channel for the study of systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean blood pressure (MBP). The pressure parameters were determined using commercial acquisition software associated with the system. This program allows to detect the maximum and minimum pressure curve beat-to-beat, providing the desirable variables. The MBP was obtained from the integration of pressure values between two consecutive detections of DBP. The heart rate was determined from the systolic interval between two peaks. The left ventricular systolic pressure (LVSP) was determined.

Analysis of Contraction and Relaxation Derivatives of the Left Ventricle

The analysis of the derivatives was based on left ventricular pressure waves recorded in the assessment of hemodynamic function, as performed previously. The analysis was performed using commercial program associated with the acquisition system. This program allows the derivation of the wave of left ventricular pressure and detection of maximal and minimal of these curves beat-to-beat, providing the values of the derivatives of contraction ($+dP/dt_{max}$) and relaxation ($-dP/dt_{max}$) of the left ventricle.

Blood and Tissue Collection

To peform the comet assay and the other biochemical analyzes, it was collected 1.0 ml of whole blood per animal from cannulation through the right carotid artery. After blood collection, the animals were killed by decapitation. At the time of decapitation, the animals were already anesthetized with ketamine (80 mg/kg, i.p.) + xylazine (12 mg/kg, i.p.) for the hemodynamic evaluation. After, it was performed the collection of tissues (heart and gastrocnemius).

Morphological Measures

The morphological measures, namely: the ratio f the mass of the body divided by body mass. This measurement is an indicator of proportionality organs, and the ratio between the left ventricle mass-

to-body mass (LV/BM) is also a reliable marker of left ventricular hypertrophy (Soci et al. 2011). Also, it was performed the ratio of gastrocnemius mass-to-body mass (G/BM).

Biochemical and Molecular Analysis

It was used plasma for determining the concentrations of urea, creatinine, total cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), uric acid and total testosterone. For obtaining the plasma, the samples were centrifuged at 2,500 rpm for 5 minutes. The analysis were performed by spectrophotometry, using commercial kits of Labtest, following the recommendations of the manufacturer, in a semi-automatic apparatus (Metrolab DR 1600®, Wiener). Total testosterone levels were measured by chemiluminescence (Immulite® 1000, Siemens, USA). Total protein was measured by refractometry. Extracellular heat shock protein 72 kDa (eHSP72) levels were assessed in plasma with colorimetric ELISA test (HSP70 high sensitivity ELISA Kit - ADI-EKS-715, Enzo Life Sciences). This test recognizes stress-induced HSP70 (HSP72) with negligible reactivity from other HSP70 family members.

Comet Assay (SCGE) Alkaline Version

The comet assay was performed avoiding direct incidence of light and according to the method of Singh and colleagues (1988). We used 40 µl of whole blood added to 90 µl of low melting point agarose. After lightly mixed, this material was carefully superimposed on a slide previously covered with a thin agarose gel and covered with a coverslip, and kept in a moist chamber at 4˚C for 10 minutes in order to further secure the suspension of blood cells in the gel. Then, the coverslip was carefully removed, and the slide was packaged in a vertical cuvette containing lysis solution for at least 1 hour at 4˚C.

The next step consisted in the unfolding of the cells for 5 min in an alkaline buffer (pH > 10.0). Thereafter, followed by the electrophoresis process, where the lysed cells contained in the agarose gel were subjected to a voltage of 25 mV and 300 mA for 15 minutes in a vat containing alkaline buffer solution (pH > 10.0). Then the slide was neutralized, stained with silver nitrate, rinsed and kept at room temperature to dry for later analysis. The slides of each animal were made in duplicate. The analysis was performed by light microscopy of 20X increase, through quantifying the size of the comet's tail in 100 cells, according to lengths, diameters and dimensions of individual comets. To quantify the damage, it was used as obtained parameters tail moment, Olive tail moment and percentage of tail DNA.

Quantification of DNA Damage

All these parameters were calculated with CASP Labs®, Poland) (Konca et al. 2003). The percentage of DNA in the tail represents the percentage of DNA stands that have migrated from the comet's head. The tail moment is the product of the tail length and of the percentage of DNA in the tail. The OTM is the product of the distance (relative to the *x*-axis) between the center of gravity of the head with the center of the gravity of the tail of the comet and the % of tail DNA. All formulas are available in supplementary data.

Statistical Analysis

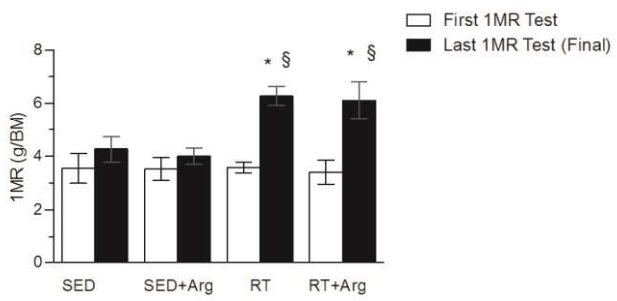
The results are shown as mean ± standard deviation. It was used two-way analysis of variance (ANOVA), followed by the post-hoc of Student-Newman-Keuls, for comparisons among groups. It was considered significant P values < 0.05. Statistical analysis were conducted in SigmaPlot 12.0 software and graphics elaborated in GraphPad Prism 5.0.

Results

Maximum Strength

There was no difference among groups in the first test of 1MR (P>0.05). Regarding the last test of 1MR and its variation between the first and last test, the trained groups showed higher values compared to sedentary groups independent of L-arginine supplementation (P<0.05) (Figure 1a and 1b).

a)



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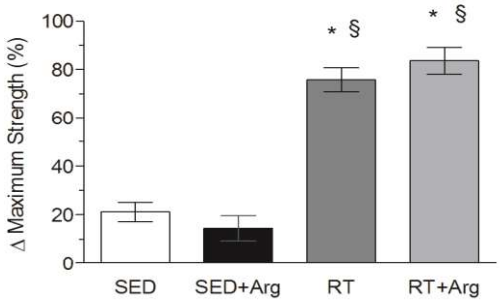

Fig. 1 Maximum strength gain of sedentary animals and of animals submitted to 8 weeks of resistance training supplemented with L-arginine. **a)** Maximum strength by comparing the values of the first and last One Maximum Repetition test (1MR). **b)** Δ Maximum strength (%) of groups. Values in mean ± SD. Two-way ANOVA followed by the post-hoc de Student-Newman-Keuls. n=5-6 animals/group; * P<0,05 vs. SED; § P<0,05 vs. SED+Arg

Gastrocnemius mass and left ventricle mass ratio with final body mass

At the beginning of the study, all groups started equally in body mass (P>0.05). After of eight weeks of experiments, only the group RT remained with the same body mass (P>0.05). All sedentary groups and trained associated with L-arginine supplementation group gained body mass (P<0.05), showing no difference among them(P>0.05). The ratio of mass of the gastrocnemius and body mass was higher in SED+Arg, RT and RT+Arg groups compared to the sedentary group(P<0.05). While the ratio of LV/BM, only the group sedentary and supplemented with L-arginine showed higher values, compared to SED (P<0.05).

Table 1 Morphometric parameters of sedentary, trained and supplemented rats with L-arginine.

	Initial Body Mass (g)	Final Body Mass (g)	Δ Body Mass (%)	LV/BM (mg/g)	G/BM (mg/g)
SED	312.67 ± 18.49	336.60 ± 21.88*	+7.40 ± 5.85*	2.05 ± 0.03	4.60 ± 0.16
SED+Arg	315.20 ± 14.17	343.00 ± 2.35*	+9.01 ± 5.29*	2.34 ± 0.20**	5.13 ± 0.32**
RT	309.40 ± 16.88	312.40 ± 11.41	+1.08 ± 3.34	2.08 ± 0.31	5.07 ± 0.55**
RT+Arg	313.17 ± 15.28	341.33 ± 4.93*	+10.09 ± 5.43*	2.05 ± 0.04	5.32 ± 0.21**

Values in mean ± SD. Two-way ANOVA followed by the *post-hoc* of Student-Newman-Keuls. n=5-6 animals/group. LV/BM, Left Ventricle-to-Body Mass Ratio; G/BM, Gastrocnemius-to-Body Mass Ratio. *, P<0.05 compared to RT group **; P<0.05 compared to SED group

Hemodynamic Variables

The hemodynamic parameters of blood pressure, such as DBP, SBP, MBP, LVSP and HR were not significantly different among groups (P>0.05) (Table 2).

Table 2 Hemodynamic parameters of blood and ventricular pressure of sedentary, trained and supplemented rats with L-arginine

	DBP (mmHg)	SBP (mmHg)	MBP (mmHg)	LVSP (mmHg)	HR (bpm)
SED	79.42 ± 14.72	104.42 ± 18.07	91.68 ± 15.06	113.52 ± 21.52	216.01 ± 23.27
SED+Arg	85.72 ± 14.05	107.71 ± 17.81	97.85 ± 15.72	110.32 ± 15.80	215.16 ± 15.51
RT	75.03 ± 10.77	101.68 ± 17.38	93.25 ± 9.13	100.13 ± 19.19	205.83 ± 19.89
RT+Arg	82.07 ± 17.35	109.69 ± 23.43	90.70 ± 15.63	107.56 ± 21.35	217.18 ± 23.73

Values in mean ± SD. Two-way ANOVA followed by the *post-hoc* of Student-Newman-Keuls. n=5-6 animals/group. DBP, Diastolic Blood Pressure; SBP, Systolic Blood Pressure; MBP, Mean Blood Pressure; LVSP, Left Ventricle Systolic Pressure; HR, Heart Rate

Left Ventricle Derivatives of Contraction and Relaxation ($+dP/dt_{máx}$/ $-dP/dt_{máx}$)

There was an increase in contractility in the groups that received L-arginine compared to sedentary group (P<0.05), with no significant change when compared to the trained group (P>0.05). Regarding the derivative of relaxation, it was observed no difference among groups (P>0.05) (Figure 2).

a) **b)**

Fig. 2 Derivatives of contractility and of relaxation of left ventricle of trained and supplemented rats with L-arginine. **a)** Derivative of contraction ($+dP/dt_{máx}$) in mmHg/s. **b)** Derivative of relaxation ($-dP/dt_{máx}$) in mmHg/s. Values in mean ± SD. Two-way ANOVA followed by the *post-hoc* of Student-Newman-Keuls. n=5-6 animals/group; * P<0,05 vs. SED

Biochemical Parameters

The groups showed no difference regarding the biochemical parameters in plasma (P>0.05). With exception of the sedentary supplemented with L-arginine group, which showed lower levels of uric acid compared to sedentary group (P<0.05).

Table 3 Biochemical parameters of rats submitted to resistance training and rats supplemented with L-arginine

	Urea (mg/dL)	Creatinine (mg/dL)	Total Cholesterol (mg/dL)	Triglycerides (mg/dL)	AST (UI/L)	ALT (UI/L)	Uric Acid (mg/dL)
SED	33.20 ± 4.97	0.59 ± 0.04	46.60 ± 17.81	45.50 ± 20.73	187.33 ± 113.52	66.50 ± 20.33	1.60 ± 0.73
SED+Arg	35.80 ± 4.27	0.54 ± 0.04	49.00 ± 4.95	43.25 ± 17.86	161.00 ± 59.09	67.67 ± 15.98	0.75 ± 0.45*
RT	33.80 ± 3.77	0.56 ± 0.03	41.40 ± 2.70	44.00 ± 4.83	222.20 ± 85.73	65.20 ± 4.27	1.20 ± 0.50
RT+Arg	34.40 ± 4.28	0.56 ± 0.05	50.80 ± 3.11	48.75 ± 5.38	128.67 ± 50.31	57.33 ± 12.79	0.80 ± 0.34

Values in mean ± SD. Two-way ANOVA followed by the *post-hoc* of Student-Newman-Keuls. n=5-6 animals/group. AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase. *, P<0.05 compared to SED group

eHSP72 and Testosterone

The SED+Arg group showed higher concentrations of eHSP72 and total testosterone compared to the sedentary group (P<0.05). All other groups showed no difference among them (P>0.05) (Figure 3).

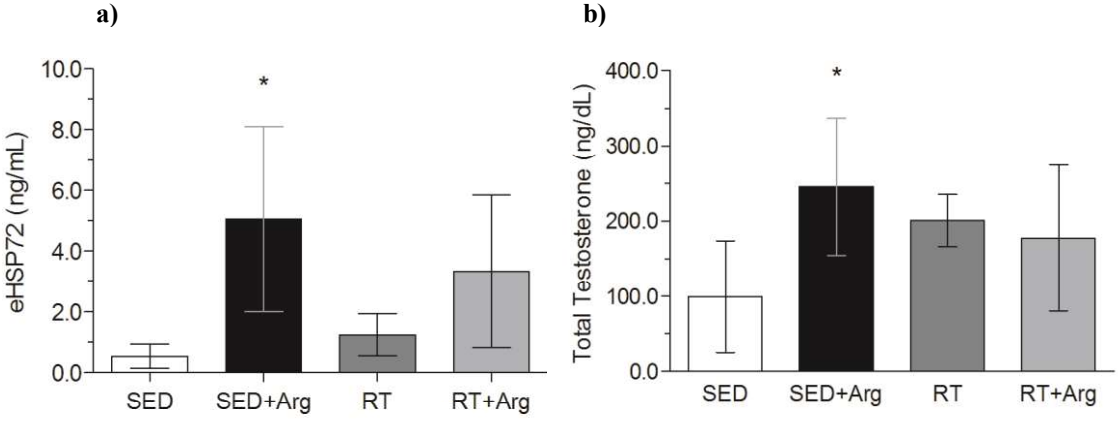

Fig. 3 Concentration of extracellular HSP72 and total testosterone in plasma of sedentary and trained rats supplemented with L-arginine. **a)** eHSP72 concentration in plasma. **b)** Total testosterone concentration in plasma. Values in mean ± SD. Two-way ANOVA followed by the *post-hoc* of Student-Newman-Keuls. n=5-6 animals/group; * P<0,05 vs. SED

DNA Damage

In Figure 4 it is possible to observe the presence and frequency of damaged cells in the experimental groups Trained and supplemented groups showed lower DNA damage compared to the sedentary group in the three variables used to quantify the damage (% tail DNA, tail moment and OTM) (P<0.05), as shown in Figure 5. Nonetheless, when the % tail DNA is evaluated, the supplemented group showed lower damage than the trained and sedentary groups (P<0.05). By analyzing tail moment and OTM, trained and supplemented group showed even lower values, which was significant compared to the trained group (P<0.05).

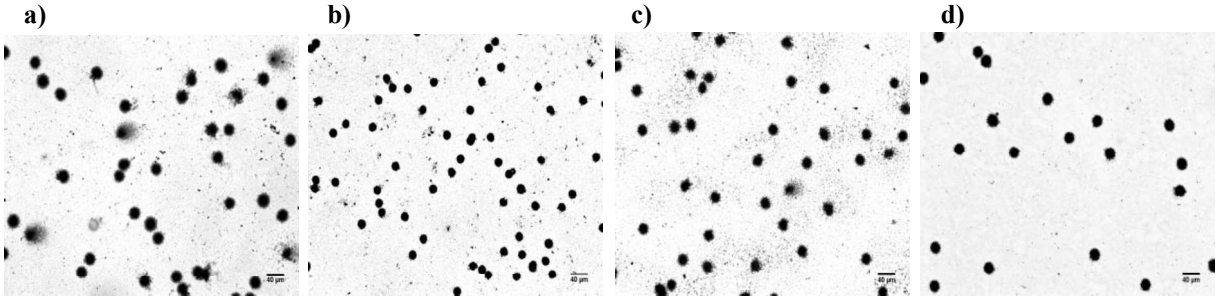

Fig. 4 Images captured by optical microscopy of slides containing isolated leukocytes from whole blood of sedentary and trained rats supplemented with L-arginine. Increase of 10x, image scale of 40µm, slides stained with silver nitrate. n=5-6 animals/group. **a)** Image of cells from SED group. **b)** Image of cells from SED+Arg group. **c)** Image of cells from RT group. **d)** Image of cells from RT+Arg group

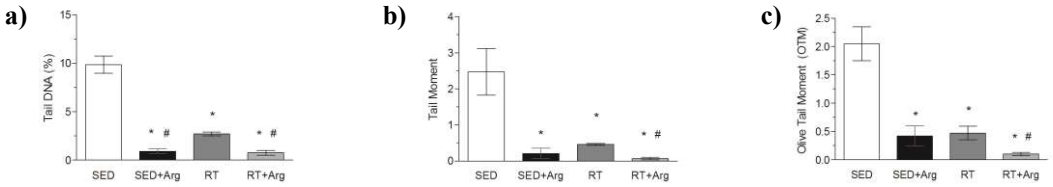

Fig. 5 Quantitative parameters of damage in DNA of sedentary and trained rats supplemented with L-arginine. **a)** DNA percentage present in the comet's tail. **b)** Tail Moment, value of the product of the tail length and the percentage of DNA in the tail. **c)** Olive Tail Moment, value obtained by the difference of center of gravity of the head with the comet's tail. Values in mean ± SD. Two-way ANOVA followed by the *post-hoc* of Student-Newman-Keuls. n=5-6 animals/group; * $P<0,05$ vs. SED; # $P<0,05$ vs. RT

Discussion

Our study shows novel findings regarding the L-arginine supplementation. The supplementation influenced body mass gain, also increased left ventricle and gastrocnemius masses. Our results also exhibit an influence in left ventricle derivatives of systolic pressure and, for the first time, demonstrated to have a relation of HSP72 and testosterone in L-arginine supplemented sedentary animals by increasing its concentrations. In addition, a previous study in our laboratory showed similar findings regarding DNA damage protection by L-arginine supplementation (Stefani et al. 2014a). However, the present study shows a wider scenario of the impact of supplementing L-arginine in biochemical parameters.

Analyzing the results of strength gain, both trained groups showed higher gain compared to sedentary groups. Additionally, the trained with supplementation group did not have any further gain of strength. Therefore, the increase of maximum strength was related to resistance training. A similar study, with healthy animals, shows that L-arginine supplementation did not influence the performance of exercise in rats (Huang et al. 2009). However, an increase in exercise tolerance after L-arginine supplementation is observed only in the presence of pathological conditions accompanied of endothelial dysfunction, such as hypertension and heart failure (Boger 2007; McConell et al. 2006; Schwedhelm et al. 2008). Thus, our results mirrors the condition of healthy animals, and for that reason there was no ergogenic effect of L-arginine in trained groups.

Supplemented groups and trained groups showed higher gastrocnemius mass-to-body mass ratio compared to sedentary group. This higher ratio observed in trained groups can attributed to the hypertrophy generated by the resistance training. Concomitantly, sedentary animals that were supplemented with L-arginine, showed higher G/BM and an increase in the testosterone concentration. This results indicates that the elevated concentration of testosterone may have played a significant role in muscle hypertrophy in the sedentary supplemented group, inducing muscle protein synthesis where it is expressed androgen receptor, such as in skeletal muscle and in cardiac muscle (Marsh et al. 1998). It has been demonstrated that L-arginine can increase mammalian target of rapamycin (mTOR) phosphorilation in skeletal muscle in neonatal piglets (Yao et al. 2008) and increase muscle gain (Tan et al. 2009). Interestingly, it has been already shown that higher L-arginine availability increased growth of neonatal pigs without increasing plasma insulin or growth hormone (Kim and Wu 2004). In our study we used adult rats, this effect may be similar to those observed in neonatal piglets, however, our data suggests that this supplementation affected body composition, not being restricted exclusively to animal growth and development. The trained and supplemented group probably did not showed even higher values of G/BM because of the effects of exercise training. Resistance training increases protein intake requirement (Szedlak and Robins 2012), probably the total intake of L-arginine in chow plus the arginine supplemented could not contemplate total L-arginine requirement for muscle growth.

It is interesting to observe that the sedentary group that received standard chow, which contained low concentrations of L-arginine, showed remarkable differences in left ventricle derivatives of pressure, as well as left ventricle and gastrocnemius mass. Also, a similar investigation of Cremades and colleagues (2004) demonstrated that deficient diets in L-arginine strongly influenced the growth of rats. Deficient diets of only in L-arginine showed to induce remarkable reduction in muscle and cardiac mass development in lower limb, approximately 20% in relation to the group with standard chow. Our results corroborates with this singular investigation. We showed that the L-arginine supplementation in situations without physical training, seemed to exert an important function in animal growth and development, mainly in cardiac and skeletal muscle structures. As for the biochemical parameters, no changes were observed among groups, indicating that low L-arginine diet does not seem to affect negatively markers of liver, kidney damage, as well as alter lipid profile.

We found greater eHSP72 expression only in SED+Arg group compared with the control group. Generally the export of HSP72 denotes a cell signaling and this stress is strongly related to the development and progression of several diseases, including insulin resistance and obesity (Krause et al. 2015). However, in our study we did not observe any changes in biochemical parameters, suggesting that

the increase in the concentration of HSP72 in plasma may be acting as a signaling factor for the increase in the secretion of testosterone. It has been shown that the increase in intracellular expression of HSP70 in enterocytes related to L-arginine supplementation is not always associated with metabolic disorders, but may be acting as intestinal growth signaling factor in early-weaned pigs (Wu et al. 2010).

The higher left ventricle contractility observed in supplemented groups, as demonstrated by the derivative of contractility in left ventricle ($+dP/dt_{max}$), seems to be related to the greater ratio of left ventricular mass-to-body mass. The additional intake of L-arginine seems to have directly influenced the export of HSP72 into the plasma, which was greater in the group that did not performed resistance training. Evidences suggests that cardiomyocyte intracellular HSP70 appears to be associated with the management of myocardial Ca^{2+}. The intracellular HSP70 is capable of modulating by increasing the activity of sarcoplasmatic reticulum Ca^{2+}-ATPase, as well as augmenting the phosphorylation of ryanodine receptor in cardiomyocytes (Lakshmikuttyamma et al. 2006). Therefore, regulating Ca2+ handling factors could increase heart contractility. In addition to this mechanism, the role of HSP72 in the process of cardiac hypertrophy, the same group of sedentary animals showed higher concentration of testosterone which reinforces our findings that possibly exists a relationship between higher export of HSP72 and cardiac hypertrophy. In fact, remains unclear which tissue could be sustaining the export of HSP72 observed in plasma.

The analysis of hemodynamic variable were not statistically different from each other, this finding may be attributed to blood and ventricular pressure levels and heart rate were not changed in the animals. Any alteration of these levels might be observed when there is an endothelial dysfunction, which occurs in several diseases, such as hypertension, heart failure and atherosclerosis (Appleton 2002; Piepoli et al. 2004). As the animals used in our study were healthy, absents of endothelial dysfunction, L-arginine supplementation was not necessary to reverse this situation, since hemodynamic parameters showed no difference among groups.

Our study demonstrates that diets with low concentration of L-arginine could exert major function in genomic stability. It is possible that L-arginine supplementation plays an important role on leukocyte replication. Low L-arginine diets may compromise approximately 90% of sperm density and number, as well as stimulates proliferation in intestinal cells (Tan et al. 2010; Wu et al. 2013). It has been demonstrated protective effects of L-arginine administration by partially protecting liver damage in acute cholestasis (Ozsoy et al. 2011). The results of the analysis of quantification of DNA damage showed higher percentage of DNA damage in untrained group, approximately 10% while the resistance training led values less than 3% of DNA damage. It has been demonstrated that the NO synthesized in blood could act as antioxidant, inhibiting free radical generation from rat peripheral polymorphonuclear leukocytes (Seth et al. 1994). Possibly the lower DNA damage in leukocytes is attributed to lower pro-oxidant generation in the groups that received supplemented L-arginine.

Resistance training has generated protection in DNA damage possibly due to more efficient mechanisms of nuclear DNA repair (Cash et al. 2014), but its association with L-arginine supplementation resulted in an even more powerful protection, since dietary factors appears to be crucial in genomic stability (Brevik et al. 2011). Although of demonstrating for the first time the protective effect of isolated supplementation of L-arginine or associated with resistance training, to our knowledge no study conducted such biochemical approach to assess the damage in DNA. With these results, it is possible to assign a genotoxic protective factor of L-arginine in blood. In addition to enhancing the protection against oxidative stress chronically exercise (training), improving the balance between the formation of reactive oxygen species and antioxidant defenses.

Shan and colleagues (2013) showed that L-arginine levels were reduced when an increase of inducible nitric oxide synthase (iNOS) activity, and also demonstrated that the supplementaton of L-arginine was able to exert antioxidant activity through NO pathway. In the same study it was shown that the supplementation with the same dosage used in our study (500 mg/kg/day) was able to increase the serum bioavailability of L-arginine in sedentary and trained animals. This increase was possibly caused by singnaling higher expression of iNOS, which would justify the protection in sedentary and supplemented groups. It is noteworthy that such work used as endurance training model. This exercise protocol has distincts physiological and biochemical features of the exercise training protocol that we used, however, both works showed antioxidant effect of L-arginine supplementation, even in different biomarkers of oxidative stress.

L-arginine is able to down-regulate the enzymatic activity of xanthine oxidase (XO) in different tissues, such as heart, skeletal muscle, liver and lung, when submitted to exhaustive exercise (Huang et al. 2008) The protective role of NO remains in preventing the formation of superoxide by XO reaction. In XO biochemical reactions uric acid is also formed as by-product, and it is considered a biomarker for chronic heart failure patients monitoring (Braunwald 2008). In our study we observed lower concentration of uric acid in the sedentary supplemented group, compared to sedentary with low L-

arginine diet. This finding reinforces the antioxidant effect observed in the dosage supplemented, assuming it is protecting a diet with low concentration of L-arginine.

This study presents few limitations, for instance, we did not measure the concentration of intracellular HSP72 in heart and in gastrocnemius. These analyzes could justify a possible export of HSP72 for plasma and tissue which could be contributing more significantly to this signaling in muscle hypertrophy.

In summary, our findings shows that L-arginine supplementation did not influenced positively in maximum strength gain. However, showed to increase muscle mass through increasing testosterone concentration. The supplementation was not related to resistance training adaptations in all analyzed parameters, but showed synergistic effect with exercise training in leukocyte genomic stability in healthy rats submitted to low L-arginine diet.

Conflicts of Interest

The authors declare no conflict of interest.

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CAPÍTULO III - Artigo 3 em Inglês

Quantification of DNA damage in different tissues in rats with chronic heart failure

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Quantification of DNA damage in different tissues in chronic heart failure rats

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Abstract

Background Chronic heart failure (CHF) is a complex syndrome which comprises structural and functional alterations in the heart in maintaining the adequate blood demand to all tissues. Few investigations sought to evaluate oxidative DNA damage in CHF. However, it has not been characterized DNA damage in distinct tissues in CHF. Therefore, our objective was to quantify the DNA damage using the comet assay in left ventricle (LV), lungs, diaphragm, gastrocnemius and soleus in rats with CHF.

Methods Twelve male Wistar rats (300 to 330 g) were selected for the study: Sham (n=6) and CHF (n=6). The animals underwent myocardial infarction by the ligation of the left coronary artery. After six weeks, the animals were euthanized. It was performed a cell suspension of the tissues. The comet assay was performed to evaluate single and double strand breaks in DNA.

Results The CHF group showed higher values of left ventricle end-diastolic pressure (LVEDP), pulmonary congestion, cardiac hypertrophy and lower values of maximal positive and negative derivatives of LV pressure, LV systolic pressure ($P<0.05$). CHF group showed higher DNA damage (% tail DNA, tail moment and Olive tail moment) compared to Sham ($P<0.001$). The tissue with the highest damage was the soleus, compared to LV and gastrocnemius in CHF group ($P<0.05$). DNA damage was positively correlated with LVEDP in all tissues ($P<0.05$).

Conclusion Our results indicates that the CHF affects all tissues, both centrally and peripherically, being more affected in skeletal muscle (soleus) and is positively correlated with LV dysfunction.

Keywords: Chronic heart failure, rats, DNA damage, single cell gel electrophoresis, comet assay

Introduction

Heart failure is a complex syndrome which characterizes structural and functional abnormalities in the heart in maintaining the adequate blood demand. The chronic heart failure (CHF) affects approximately 1 to 2% of the population in developed countries and its prevalence increases of at least 10% in older adults [1]. One of the most common causes to heart failure is myocardial infarction (MI), which induces pathologic cardiac remodeling [2].

This syndrome does not affect only the heart, it also broadly affects other organs, such as lungs and skeletal muscle [3]. The hypoperfusion, which is sustained with a ventricular dysfunction in a vicious cycle, induces oxidative stress in the majority of tissues [4]. It has been proposed elsewhere that oxidative stress biomarkers, such as concentration of malondyaldehyde and uric acid, could enlighten the extent of oxidative damage and guide the treatment in patients with CHF [5, 6].

Since the reactive oxygen species (ROS) can damage different biomolecules, such as lipids, proteins and DNA, the damage in the nucleic acids has not been consistently investigated in CHF. One of the biomarkers that has been already target of investigation is the concentration of 8-hydroxy-2'-deoxyguanosine (8-OHdG) [7]. However, its measurement mirrors the oxidative damage in one type of DNA lesion, which does not reflect the total genotoxic damage in the DNA helix [8]. For that, toxicological assays, such as the single cell gel electrophoresis (SCGE), or comet assay, has never been tested in CHF, aiming to assess the global DNA damage in different tissues. This technique is broadly used in environmental toxicology studies and is considered to be consistent, sensitive and highly reproductive in *in vitro* and *in vivo* studies [9-11].

The comet assay directly measures the extent of DNA damage, which is constituted by single strand and double strand breaks of DNA [12]. This method is possible to measure in blood and all tissues of interest, expanding the analysis of local damage and to correlate with physiological and functional parameters [13].

So, the objective of this study was to evaluate the DNA damage in different tissues, such as the left ventricle, lungs, diaphragm and skeletal muscle (gastrocnemius and soleus) in rats with CHF.

Methods

Animals

It was selected 12 male Wistar rats (from 300 g to 330 g) from Animal Breeding Unit of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA, Brazil). The animals were housed in groups of three/animals per cage, which received food and water *ad libitum* in an specific room maintained at 22^{o}C under 12:12 hours light-dark cycle.

The handling of the animals obeyed Law nº 11,794 of 10/08/ 2008, Law nº 6,899 of 07/15/2009, and Resolution nº 879 of 02/15/2008 (CFMV), as well as other provisions applicable to the use of animals for teaching and research, in particular the resolutions of the National Council on Animal Experimentation. This study was approved by CEUA/UFCSPA, under the protocol number 114/13.

Induction of Myocardial Infarction (MI)

The animals were anesthetized with xylazine (12 mg/kg ip) and ketamine (90 mg/kg ip), intubated and artificially ventilated. It was performed the ligation of the left coronary artery. Sham operations were performed according to Pfeffer et al (1979).

After the surgeries, the animals received one injection of cetoprophane (5.4 mg/kg ip) at each 6 hours completing 48 hours and penicillin (70,000 units/ml ip). The surgeries were performed by one surgeon. The mortality rate post-surgery of the animals was of 15%. After the induction of MI, it was designated 6 weeks of recovery, which was necessary to the animals develop chronic heart failure.

Hemodynamic Evaluation

After the sixth week, the animals were anesthetized with xylazine (12 mg/kg i.p.) and ketamine (90 mg/kg i.p.). A polyethylene catheter (PE-50) was placed into the right carotid artery. The arterial pressure was recorded and the catheter was positioned into the left ventricle to perform the ventricular pressure recording. The data was registered by a pressure transducer (strain-gauge, Narco Biosystem Miniature Pulse Transducer RP-155, Houston, Texas, USA), which was coupled to a pressure amplifier (Stoelting, wood dated, Illinois, USA). Pressure analogical signals were digitalised by a data-acquisiton system (CODAS-Data Acquisition System, Akron, Ohio, USA) with a sampling rate of 2,000 Hz. These data were used to determine diastolic blood pressure (DBP), systolic blood pressure (SBP), mean blood pressure (MBP), heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP) and left ventricular maximum positive and negative dP/dt ($+dP/dt_{max}$, $-dP/dt_{max}$), as previously described [15].

Tissue Collection

The animals were euthanized through an intravenous infusion of an overdose of the anesthetic pentobarbital (80 mg/kg i.p.) [16]. After that, the lungs, diaphragm, the right gastrocnemius, the right soleus and the heart were removed. The left ventricle was

separated from the right ventricle for the comet assay. All samples were stored at -80°C for posterior analysis.

Determination of Infarct Size, Cardiac Hypertrophy and Pulmonary and Hepatic Congestion

The hearts were removed and weighted, without blood within the chamber and without atria. The size of the infarct area was determined by planimetry [17]. To evaluate cardiac hypertrophy, the organ mass were expressed as a proportion of body mass (tissue mass/body mass - mg/g) [18]. To determine pulmonary and hepatic congestion, the lungs and liver of each animal were removed, weighted and dehydrated (80°C) for 48 hours, and then weighted again to evaluate the water percentage.

Single Cell Gel Eletrophoresis (SCGE)

The SCGE was performed in alkaline conditions (pH > 13.0), according to Singh and colleagues (1988). All the procedures were performed avoiding any direct incidence of light. For the assay, it was first made a cell suspension of the tissue (left ventricle, lungs, diaphragm, right gastrocnemius and right soleus) in PBS buffer (pH = 7.40) with standard and gentle manual homogenization. For this step it was necessary to observe the density of cells that would be used in each slide. It was used Neubauer's chamber to count, approximately 7.3×10^5 cells/slide.

The suspension of cells (40 µl) was added to agarose of low melting point (90 µl). After gently mixed, this material was carefully superimposed over a slide previously covered with a thin agarose gel layer with a coverslip, and kept in a humid chamber at 4°C for 10 minutes, in order to further secure the suspension of tissue cells

in the gel. Then, the coverslip was carefully removed and the slide, conditioned in a vertical cuvette containing lysis solution for at least 1 hour at 4°C.

The following step consisted in the unfolding of the cells, for 30 minutes in an alkaline buffer (pH > 10.0). Thereafter, was followed by the process of electrophoresis, where the lysed cells contained in the agarose gel were subjected to a voltage of 25 mV and 300 mA for 15 minutes in alkaline buffer solution (pH > 10.0). Then the plate was neutralized, stained with silver nitrate, rinsed and kept at room temperature to dry for later analysis. The slides of each animal were made in duplicate and a positive control of DNA damage with hydrogen peroxide (30 µl/slide). The analysis was conducted under an optical microscope with a 20x increase by quantifying the size of the comet's tail in 50 to 100 cells, according to the lengths, diameters , radii and dimensions of individual comets. To quantify the damage it was used as parameters obtained percentage of tail DNA, tail moment and Olive tail moment.

Quantification of DNA Damage

All the parameters presented in the results session regarding the SCGE were calculated by the software CASP (CASP Labs®, Poland) [20]. The percentage of DNA in the tail, tail moment and Olive tail moment formulas are available for consult in the supplementary data. The tail moment is characterized as the product of tail length and the percentage of DNA in the tail. The Olive tail moment, which is another parameter of representing DNA damage, comprises as the product of the distance (relative to the x-axis) between the center of gravity of the head with the center of gravity of the tail of the comet and the percentage of tail DNA.

Sample Size and Statistical Analysis

For a minimum difference of 23 arbitrary units of tail moment of ± 4 of standard deviation, it was possible to determine a minimum statistical difference in two groups with three animals each [21]. In our investigation, we decided to use six animals in each group. The data are presented in mean ± SD. For comparisons between groups it was performed t test and two-way analysis of variance among different tissues. For associations between hemodynamic variables and pulmonary congestion, it was performed Pearson's correlation test. It was considered significance of 5%. For statistical analysis it was used SigmaPlot version 12.0 for Windows and for creating the graphics it was used GraphPad Prism version 5.0 for Windows.

Results

Morphological Parameters

The animals showed no difference regarding neither initial body mass, nor the final body mass (P >0.05). The animals submitted to MI showed mean of infarction area of 36%. It was observed higher ratio of myocardial mass, right ventricle and left ventricle-to-body mass compared to sham group (P<0.05), indicating cardiac remodeling in both ventricles. Regarding the congestion in lungs and in liver, it was only observed higher congestion in lungs in the CHF group (P<0.05) (Table 1).

Hemodynamic Parameters

When compared to sham group, it was observed lower mean blood pressure in CHF group (P<0.05). Systolic and diastolic blood pressure, as well as heart rate showed no difference between the control group (Table 2). Regarding the ventricular pressure variables, it was observed lower left ventricle systolic pressure (P<0.05) and higher left

ventricle end diastolic pressure in CHF group (P<0.05), when compared to sham group (Table 2).

The maximal positive derivative of ventricular pressure (+dP/dt_{max}) showed alterations in CHF group, presenting lower values (P<0.05), as well as the maximal negative derivative of ventricular pressure (-dP/dt_{max}), which showed lower values, compared to the control group (P<0.05) (Table 2).

DNA Damage Parameters

It was observed higher values of DNA damage in all variables (% tail DNA, tail moment and Olive tail moment) in CHF group, in all analyzed tissues (P<0.05) (Table 3). The DNA damage can be observed in the formation and frequency of comets in the left ventricle, pulmonary, diaphragmatic, gastrocnemius and soleus cells (Figure 1).

Despite the genotoxic damage was remarkably higher in CHF rats in all tissues, when compared to other tissues in the same pathologic condition, it was observed higher damage in soleus compared to gastrocnemius and left ventricle in CHF group (P<0.05). The difference of DNA damage among tissues in sham-operated animals and in CHF animals can be observed in Figure 6. Also, the presence of pulmonary congestion in CHF group showed positive association with DNA damage in lung cells (Figure 3A). It was found significant correlation between DNA damage in the different tissues, with LVEDP (Figures 3B to 3F).

Discussion

To the best of our knowledge, this is the first study to report the total extent of DNA damage in different tissues in a CHF rat model. The major finding of this investigation is the reproductive and applicability of SCGE in experimental model of

MI. We showed that the animals with CHF demonstrated higher extent of DNA damage than the control group in heart, lungs, diaphragm and skeletal muscle. This finding supports the main hypothesis that the CHF affects the stability of DNA not locally, but systemically. We also demonstrated that the DNA damage was positive correlated with pulmonary congestion in lung cells and to the diastolic dysfunction.

Since the CHF is a complex syndrome, it is essential to investigate the extent of damage that the hypoperfusion may promote. We showed an *in vivo* model of CHF that the damage was ranging from two-fold to six-fold higher than in the absence of heart failure. The animals of this study demonstrated traditional alterations observable in the ligation of the left coronary artery model of heart failure in rats [22-24]. It was observed a mean LVEDP above 30 mmHg, which characterizes a ventricular dysfunction [25, 26]. It was also observed traditional hemodynamic alterations in animals with CHF, such as lower LVSP, maximum positive and negative derivatives of ventricular pressure. The morphological parameters also showed noteworthy alterations in left and right ventricle hypertrophy. All these parameters (hemodynamic and morphological) characterize the presence of CHF.

The method of SCGE performed in alkaline conditions allows the evaluation of global genotoxic damage. The damage observed in the comets is formed by single and double strand breaks that are unattached of the cromatin, in DNA fragments [13]. Recently, it has been developed fluorescent probes to detect different types of DNA damage in *in situ* hybridization [27]. However, this method has been used almost entirely in *in vitro* studies.

Recently has been proposed the evaluation of 8-OHdG in patients with CHF. The 8-OHdG is an oxidized purinic base, one of the most frequent oxidative products of DNA [28]. Most of the lesions in DNA may be manifested in single and double strand

breaks, not only in oxidative by-products. Reactive oxygen species may damage DNA and form oxidative bases, such as 8-OHdG, 5-hydroxyuracil, 2-hydroxyadenine and 4,6-diamino-5-formamidopyridine. However, the quantification of 8-OHdG mirrors the oxidative DNA damage, not the global DNA damage [29, 30].

Kono and colleagues (2006) first demonstrated that patients with CHF showed remarkably higher concentration of 8-OHdG in serum. Also, our findings corroborates with the results of higher 8-OHdG concentrations in the left ventricle [31]. Since this initial investigation, other clinical trials aimed to reproduce this finding. Kobayashi and colleagues (2011) sought to evaluate the clinical usefulness of quantification of 8-OHdG as a biomarker in patients with CHF. In this investigation, it was found that the 8-OHdG was higher in CHF patients, when compared to the control group, but also became higher as the New York Heart Association class increased [7]. Also, the authors found negative correlation between 8-OHdG concentration with the left ventricle ejection fraction (r = -0.27). Although in our study we used a different measurement of ventricular dysfunction, LVEDP, we found a significant positive correlation between the total DNA damage in left ventricle and LVEDP (r = 0.85). Our findings supports even more the results of oxidative DNA damage, indicating that other factors could be influencing a wider scenario of DNA damage in CHF.

In a prospective study of Susa and colleagues (2012) with approximately 650 days of follow-up duration, demonstrated that urinary concentration of 8-OHdG patients that had fatal event had higher levels of 8-OHdG than in CHF patients without cardiac events.

In our study, we demonstrated that the left ventricle diastolic dysfunction is positively correlated with the total DNA strand breaks in all tissues (left ventricle, lungs, diaphragm, gastrocenemius and soleus). These findings supports previous results

from Suzuki and colleagues (2011), which firstly demonstrated that the elevation of only one oxidative DNA lesion in serum, 8-OHdG, was an important prognostic information for stratifying risk in patients with heart failure. In addition, it was showed significant changes in left ventricle end-diastolic diameter, creatinine concentration and the cumulative event-free survival in patients with CHF with higher concentrations of 8-OHdG ($\geq$ 40 ng/mL).

In a recent meta-analysis, it was demonstrated that eight studies evaluated the oxidative DNA damage to the specific DNA lesion of 8-OHdG. All of the investigations demonstrated higher concentrations of 8-OHdG in CHF patients [34]. The rationale for higher concentrations of DNA oxidative products indicates that the higher extent of genotoxic damage is highly contributed to the oxidation of mitochondrial DNA [35]. Cardiac myocytes present the highest content of mitochondria, which could indicate higher formation of ROS and contribute significantly to mitochondrial dysfunction. To the best of our knowledge, our study is the first to quantificate the total extent of nuclear DNA damage in CHF.

One interesting finding of this study was the higher damage of DNA in soleus cells, than in left ventricle cells. This results supports the fact that after the MI the ventricle functionally and morphologically adapts and the peripheral muscle suffers histological and biochemical alterations [36-38]. The acute phase of MI is characterized by the necrosis of cardiac myocytes, which expands the area of necrosis of the left ventricle in the following hours, affecting adjacent structures [39]. In this phase of MI, the extent of DNA damage probably is higher than in any other tissue, as can be observed in pro-inflammatory citokines and autophagic mediators [40, 41].

Days after the MI, the left ventricle adapts, showing a different scenario, mainly in developing a scar area in the area affect by the ischemia and thus, modifying its

geometry to a globoid format [42]. In this chronic phase after MI, the left ventricle shows lower apoptotic and necrotic rate, when compared next to the MI [43]. According to the investigation of Yndestad and colleagues (2009), the border of the infarction area of the left ventricle showed higher capacity to repair major oxidative damage, such as formamidopyridine and 5-hydroxycytosine, than the non-infarcted area. Also, in the same study, no difference was observed of the accumulation of 8-OHdG in the border zone of the infarcted area and the non-infarcted area. This finding suggests that the adjacent area that was affect by the myocardial infarction, shows higher adaptability to repair this type of oxidative damage, which contributes to the cardiac remodeling. Our results corroborates with this fact, once the soleus muscle showed higher damage in DNA, compared to the left ventricle.

Since the cardiomyocytes have a renewal rate of approximately of 1% in young people, and about 0.45% in elderly [45], this fact reinforce our findings regarding the difference of DNA damage among the tissues of CHF rats. The left ventricle shows high adaptability to modify its geometry, ability to repair major oxidative products of DNA. It has been demonstrated that the main problem of CHF is not the central alterations in the heart, but it also affects, indirectly, all other organs [46]. The hypoperfusion leads to formation of free radicals of different ways. For instance, several sources of ROS are increased in the failing heart, such as the xantine oxidase pathway, NAD(P)H oxidase, the uncoupling of nitric oxide synthase in cardiomyocyte and the leakage of superoxide of mitochondria [47, 48].

The skeletal muscle in CHF is highly affected by the hypoperfusion which augments the oxidative damage within. Since the oxidative skeletal muscle depends more of the oxidative metabolism, it is expected to observe higher damage in our findings. The animals in our study were sedentary and by that, the antioxidant defense

system in soleus muscle might be constantly decreased by the CHF [49]. Compared to the left ventricle, the soleus muscle does not have the same ability for adaptation, which may explain why the DNA damage was higher.

Limitations

This work shows few limitations, such as the absence of evaluating the DNA damage in other tissues (liver, encephalic structures and other skeletal muscles). Our experimental design to perform the hemodynamic evaluation via carotid artery, does not permit to securely measure DNA damage of encephalic structures, once the procedure showed to induce ischemia in central nervous system. Other limitation that may enrich our findings is the measurement of mutagenesis. Evaluating the mutagenesis of the CHF, along with the SCGE, might lead to a more robust scenario of DNA damage and its lack of repair of DNA lesions. Our design aims to evaluate the longitudinal damage that experimental model of CHF might lead and its differences among tissues, for that, the ability of the DNA to repair its lesions could not be performed.

Conclusion

Our results shows DNA damage using SCGE in CHF experimental model by MI. The left ventricle dysfunction clearly affects the cardiac tissue, lungs, diaphragm, gastrocenemius and soleus and showed to be associated with the extent of DNA damage, being the more affected the soleus muscle than left ventricle and gastrocnemius. The presence of pulmonary congestions showed to be associated with DNA damage in lung cells. We demonstrated that the comet assay can be a reliable tool for quantifying genotoxic damage in different tissues of animals with CHF and that the

soleus muscle is more affected by the heart failure than the left ventricle and gastrocnemius.

Authors Contribution

SGP, NRB, RDD, HVS, DMD, LPD, RCR conception and design of research; SGP, NRB, RDD, HVS, DMD performed experiments; SGP, NRB, RDD, HVS, DMD, LPD, RCR analyzed data; SGP, NRB, RDD, HVS, DMD, LPD, RCR interpreted results of experiments; SGP, NRB, RDD, HVS, DMD, RCR prepared figures; SGP, NRB, RDD, HVS, DMD, LPD, RCR drafted manuscript; SGP, NRB, RDD, HVS, DMD, LPD, RCR edited and revised manuscript; SGP, NRB, RDD, HVS, DMD, LPD, RCR approved final version of manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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Tables

Table 1: Body Mass, morphometric cardiac characteristics, infarct area and pulmonary and hepatic congestion of sham-operated rats and rats with left ventricular dysfunction.

Variables	Sham	CHF
Initial Body Mass (g)	330.25 ± 17.24	328.29 ± 18.12
Final Body Mass (g)	400.50 ± 29.61	356.38 ± 32.23
Infarcted Area (%)	---	36.39 ± 8.11
MM/BM (mg/g)	2.56 ± 0.08	3.29 ± 0.46*
LV/BM (mg/g)	1.88 ± 0.21	2.36 ± 0.47*
RV/BM (mg/g)	0.58 ± 0.12	1.40 ± 1.01*
Pulmonary Congestion (%)	65.67 ± 9.34	87.31 ± 3.36*
Hepatic Congestion (%)	70.46 ± 1.05	71.43 ± 1.07

Values are presented in mean ± SD; n=6 for all groups. Sham, sham-operated rats; CHF, chronic heart failure rats. Myocardial mass-to-body mass ratio (MM/BM), left ventricle mass-to-body mass ratio (LV/BM), right ventricle mass-to-body mass ratio (RV/BM). * $P < 0.05$ compared to Sham group.

Table 2: Mean, diastolic and systolic blood pressure, left ventricle end diastolic pressure, left ventricle systolic pressure and left ventricular maximum/minimum change over time of sham-operated rats and rats with left ventricular dysfunction.

Variables	Sham	CHF
MBP (mmHg)	93.01 ± 14.70	76.78 ± 5.83*
DBP (mmHg)	73.54 ± 16.28	67.54 ± 7.15
SBP (mmHg)	99.75 ± 20.91	85.93 ± 5.51
Heart Rate (bpm)	253.56 ± 70.84	245.19 ± 57.69
LVEDP (mmHg)	5.40 ± 2.26	32.55 ± 5.32*
LVSP (mmHg)	104.24 ± 6.03	89.15 ± 3.15*
+ dP/dt_{max} (mmHg/s)	6264.33 ± 1566.47	4281.63 ± 708.75*
- dP/dt_{max} (mmHg/s)	5209.63 ± 1274.09	2823.80 ± 540.65*

Values are presented in mean ± SD; n=6 for all groups. Sham, sham-operated rats; CHF, chronic heart failure rats. Mean blood pressure (MBP), diastolic blood pressure (DBP), systolic blood pressure (SBP), left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), maximal positive derivative of ventricular pressure ($+dP/dt_{max}$) and maximal negative derivative of ventricular pressure ($-dP/dt_{max}$). * $P < 0.05$ compared to Sham group.

Table 3: DNA quantification in different tissues of sham-operated animals and rats with chronic heart failure.

	Sham			CHF		
	% Tail DNA	Tail Moment	Olive Tail Moment	% Tail DNA	Tail Moment	Olive Tail Moment
Left Ventricle	7.65 ± 3.35	0.77 ± 0.44	1.37 ± 0.59	33.29 ± 7.70*	10.51 ± 3.31*	7.04 ± 1.71*
Lungs	17.86 ± 3.93	6.76 ± 2.59	7.31 ± 2.15	36.20 ± 5.17*	23.30 ± 7.25*	19.10 ± 4.65*
Diaphragm	6.86 ± 2.63	1.40 ± 0.93	1.82 ± 0.79	41.23 ± 13.86*	14.06 ± 6.51*	9.82 ± 3.03*
Gastrocnemius	7.63 ± 4.66	1.04 ± 0.88	1.43 ± 0.70	28.07 ± 15.53*	8.69 ± 5.14*	6.17 ± 3.53*
Soleus	11.54 ± 2.46	1.53 ± 0.96	1.84 ± 0.76	55.79 ± 11.53*	20.90 ± 5.32*	12.83 ± 3.68*

Values are presented in mean ± SD; n=6 for all groups. Sham, sham-operated rats; CHF, chronic heart failure rats.

* = P <0.01 versus Sham in relation to the variable and to its correspondent tissue.

Figure Captions

Figure 1 Image of cells submitted to SCGE (comet assay) of left ventricle, lungs, diaphragm, gastrocnemius and soleus of Sham-operated and rats with CHF.

Panel A: Isolated cells of the respective tissue of Sham-operated rat. Panel B: Isolated cells of the respective tissue of rat with CHF induced by MI. Slides stained with silver nitrate. The image shows no formation of comets in Panel A, but in Panel B the image shows the formation of comets with distinct tails. The length of the tail represents the single strand and double strand breaks of nuclear DNA (magnification of 20x, scale bar of 20 μm).

Figure 2 DNA damage in different tissues, according to % tail DNA, induced by CHF.

Quantification of DNA damage in isolated cells of left ventricle, lungs, diaphragm, gastrocnemius and soleus muscle in Sham-operated rats and rats with CHF. Sham (n=6), CHF (n=6). Quatification of DNA damage performed with CASP® (CASP Labs, Poland) using 50 to 100 cells per slide in duplicate. One-way ANOVA, with post-hoc test of Student-Newman-Keuls. * P <0.05 vs Soleus.

Figure 3 Correlation of DNA damage in different tissues with %H_2O in lungs and with LVEDP in Sham-operated and rats with CHF.

The graphics shows black circles (●) of Sham-operated animals and white circles (○) of animals with CHF. Correlation were performed using Pearson's correlation test. Panel A: The graphic shows positive correlation between lung cells DNA damage (% tail

DNA) with the %H_2O in lungs (r = 0.4815, P<0.05) . Panel B: positive correlation between DNA damage (% tail DNA) with left ventricle end-diastolic pressure (LVEDP) in left ventricle (r = 0.8527, P<0.0001). Panel C: positive correlation between DNA damage (% tail DNA) with LVEDP in lungs (r = 0.6863, P<0.001). Panel D: positive correlation between DNA damage (% tail DNA) with LVEDP in diaphragm (r = 0.7658, P<0.001). Panel E: positive correlation between DNA damage (% tail DNA) with LVEDP in gastrocnemius (r = 0.5586, P<0.01). Panel F: positive correlation between DNA damage (% tail DNA) with LVEDP in soleus (r = 0.9047, P<0.0001).

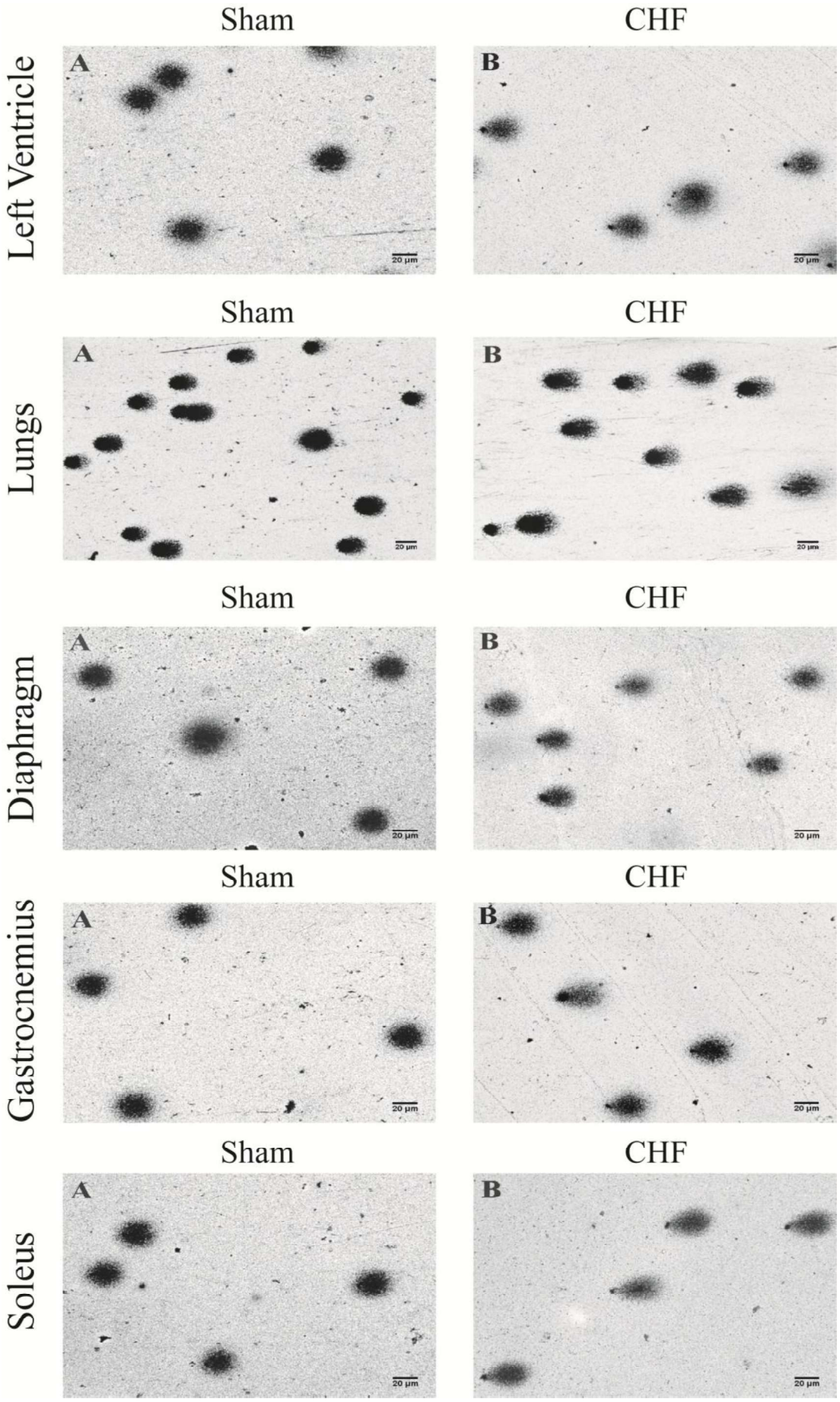

Sham
CHF
Left Ventricle
A
B
Sham
CHF
Lungs
A
B
Sham
CHF
Diaphragm
A
B
Sham
CHF
Gastrocnemius
A
B
Sham
CHF
Soleus
A
B

Fig. 1

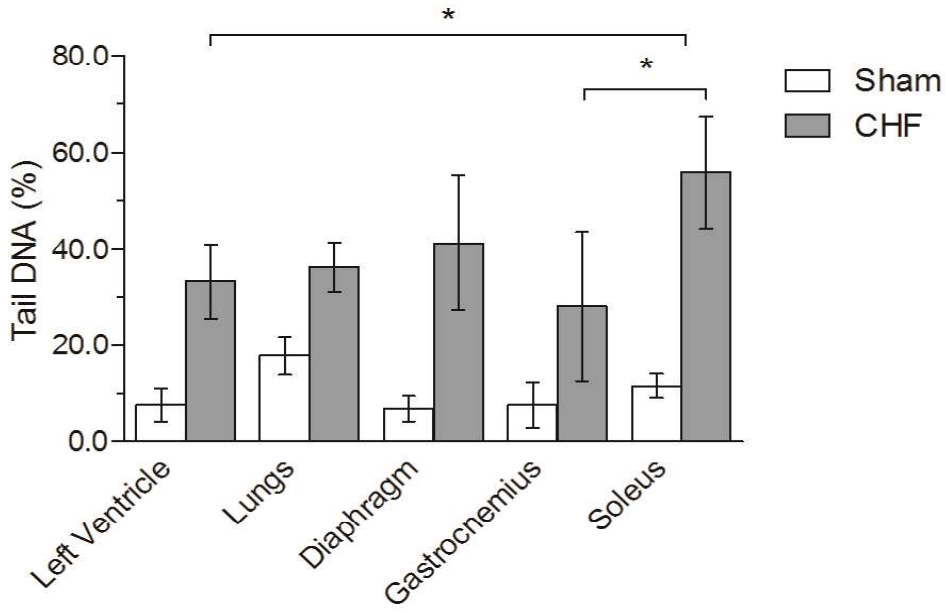

*
*
80.0
60.0
40.0
20.0
0.0
Tail DNA (%)
Sham
CHF
Left Ventricle
Lungs
Diaphragm
Gastrocnemius
Soleus

Fig. 2

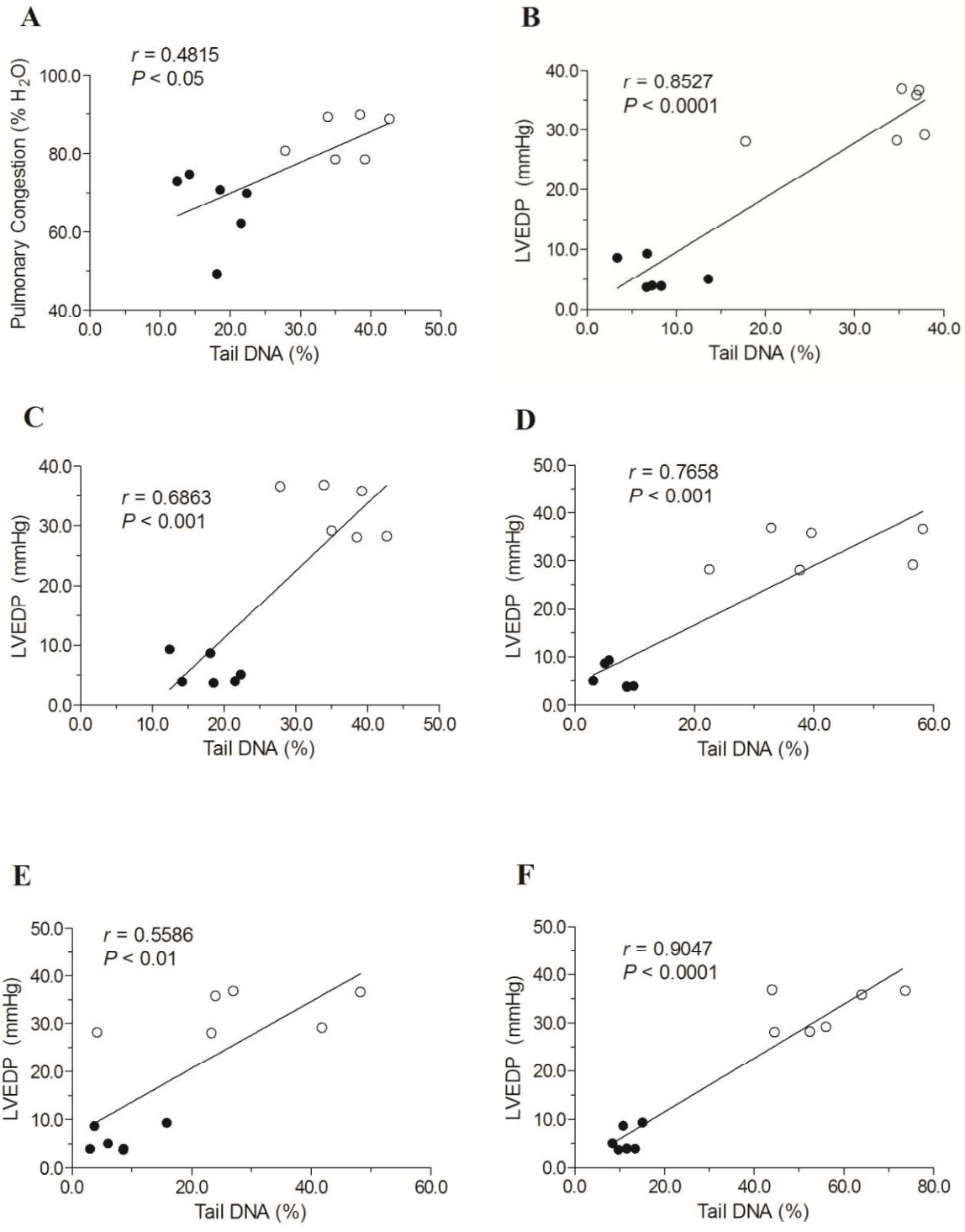
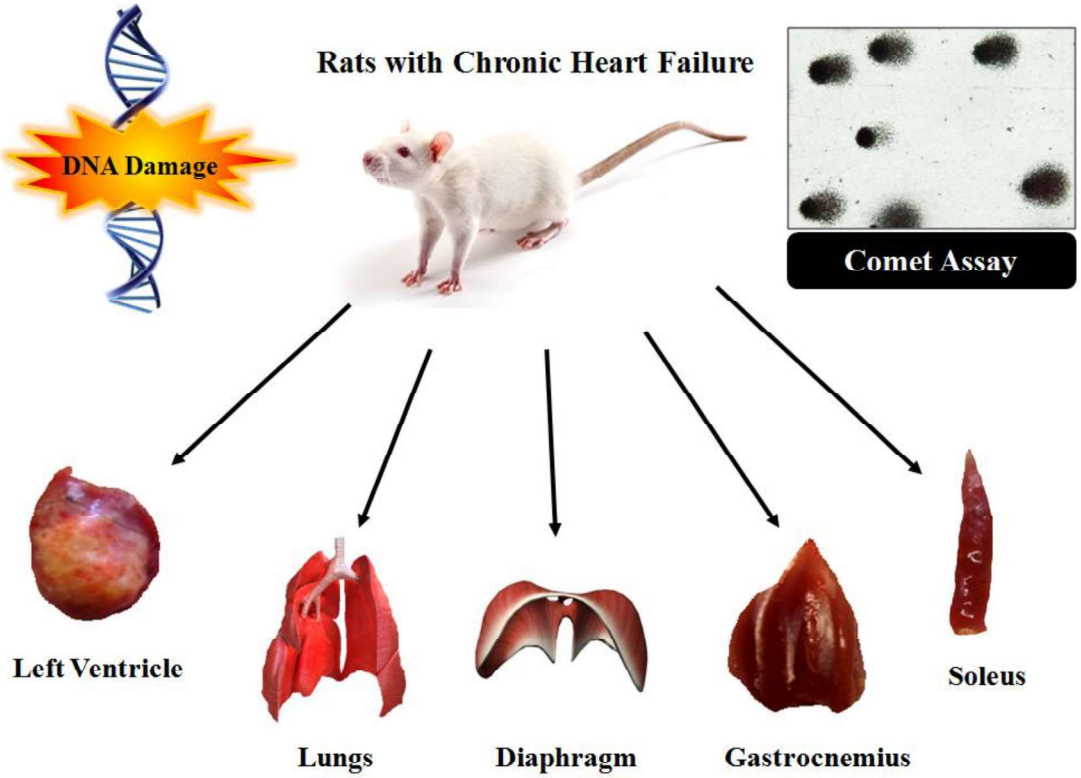

A
r = 0.4815
P < 0.05
Pulmonary Congestion (% H2O)
Tail DNA (%)
B
r = 0.8527
P < 0.0001
LVEDP (mmHg)
Tail DNA (%)
C
r = 0.6863
P < 0.001
LVEDP (mmHg)
Tail DNA (%)
D
r = 0.7658
P < 0.001
LVEDP (mmHg)
Tail DNA (%)
E
r = 0.5586
P < 0.01
LVEDP (mmHg)
Tail DNA (%)
F
r = 0.9047
P < 0.0001
LVEDP (mmHg)
Tail DNA (%)

Fig. 3

Graphical Abstract



CONCLUSÕES

O ensaio cometa tem sido aplicado em diferentes situações, como no monitoramento de dano genotóxico em doenças, por exemplo no câncer, bem como em situações agudas de exercício físico ou até mesmo em situações onde o desfecho principal observado é a proteção de dano.

O treinamento de força isolado e associado à suplementação de um aminoácido semi-essencial (L-arginina) demonstrou efeitos interessantes, ainda não descritos na literatura científica, como a modulação de contratilidade cardíaca e estabilidade genômica. A suplementação de L-arginina demonstrou não atuar sobre marcadores bioquímicos (hepáticos, perfil lipídico, entre outros), tanto quando administrada isoladamente, quanto associada ao treinamento de força.

Em função de ainda ser pouco utilizada em modelos animais, o ensaio cometa demonstrou ser uma ferramenta essencial para responder as hipóteses de efeitos protetores de intervenções crônicas, como o treinamento de força, a suplementação de L-arginina e a associação entre estas intervenções. No futuro, mais estudos devem se apropriar do uso do ensaio cometa em ensaios clínicos em humanos.

Quanto à abordagem transversal fisiopatológica, o ensaio cometa demonstrou ser um parâmetro altamente sensível à alterações observadas em animais com insuficiência cardíaca. Os seus parâmetros (% DNA da cauda, momento da cauda e momento da cauda de Olive) demonstraram índices de correlação positivos com parâmetros de disfunção ventricular esquerda, bem como com o grau de congestão pulmonar.

Portanto, através de desenhos experimentais apropriados é possível utilizar o ensaio cometa como uma ferramenta sensível e enriquecedora da investigação científica a fim de tornar o resultado observado mais robusto.

ANEXO I

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO

UFCSPA
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

COMISSÃO DE ÉTICA NO USO DE ANIMAIS – UFCSPA

A Comissão de Ética no Uso de Animais, analisou o Projeto:

Projeto: 114/13

Pesquisadores:
Cláudia Rhoden
Guiseppe Stefani
Ramiro nunes
Jadson Alves
André Dornelles
Pedro Dal Lago

Título: EFEITOS DA SUPLEMENTAÇÃO DE I-ARGININA ASSOCIADA OU NÃO AO TREINAMENTO DE FORÇA SOBRE O GANHO DE FORÇA MÁXIMA, FUNÇÃO HEMODINÂMICA E ESTRESSE OXIDATIVO EM DIFERENTES TECIDOS EM RATOS COM INSUFICIÊNCIA CARDÍACA.

Este Projeto foi aprovado em seus aspectos éticos e metodológicos. Todo e qualquer alteração do projeto, assim como eventos adversos graves, deverão ser comunicados a esta CEUA.

Porto Alegre, 12 de junho de 2013.

Katya V. Rigatto
Coordenadora do CEUA
UFCSPA

Rua Sarmento Leite, 245 • Porto Alegre, RS • CEP 90050-170 • Fone: 0 xx 51 3303 8000 • Fax: 0 xx 51 3303.8810 • www.ufcspa.edu.br

ANEXO II

Author Guidelines Journal of Nutritional Health and Food Engineering

(ISSN 2373-4310)

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Words: Up to 300
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Abstract should include a brief content of the article.

2. Keywords

Words: Up to 10
Font: Cambria
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Introduction should provide background, comprehensive insight on the purpose of the study and its significance.

5. Conclusion

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Conclusion should elucidate how the results communicate to the theory presented as the basis of the study and provide a concise explanation of the allegation of the findings.

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Provide list of individuals who contributed in the work and grant details.

7. Conflict of Interest

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Note* If there are any sub headings in the body text, sub-categorize them accordingly under the heading in which they fall.

For example: 1. Heading

1.1. Sub-heading

1.1.1. Sub-sub-heading

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Note* Provide the link for the listed references

General style of reference

1. **Journal References**

Author name/s (Year) Title of article. Journal short name Volume(Issue): Full inclusive page numbers.

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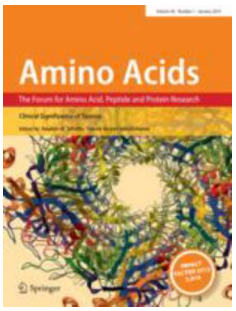

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- Use tab stops or other commands for indents, not the space bar.
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- LaTeX macro package (zip, 182 kB)

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Acknowledgments of people, grants, funds, etc. should be placed in a separate section before the reference list. The names of funding organizations should be written in full.

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- Generic names of drugs and pesticides are preferred; if trade names are used, the generic name should be given at first mention.

References

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995; Kelso and Smith 1998; Medvec et al. 1999).

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The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.
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- Article by DOI

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- Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

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- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

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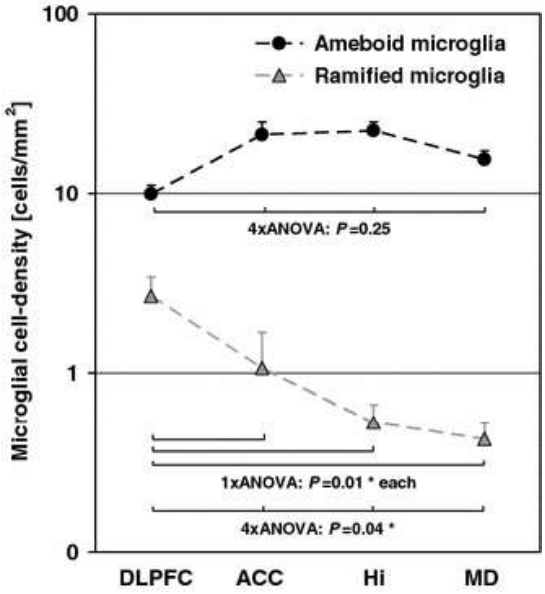

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- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
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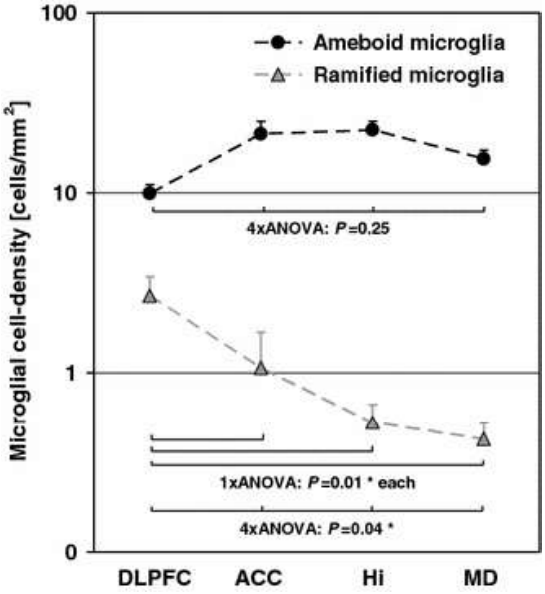

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- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
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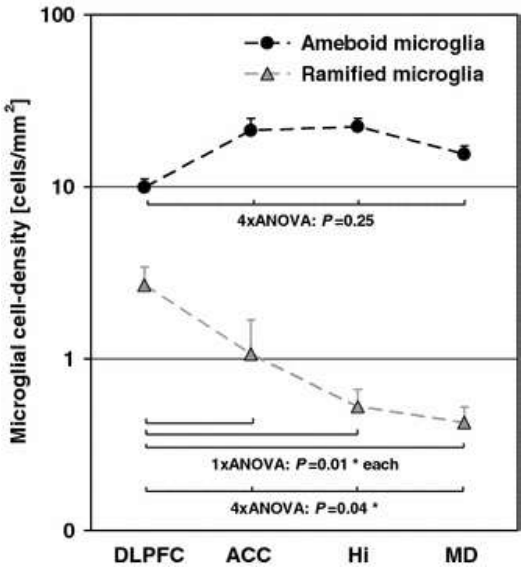
Combination Art



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(ISSN 0167-5273)

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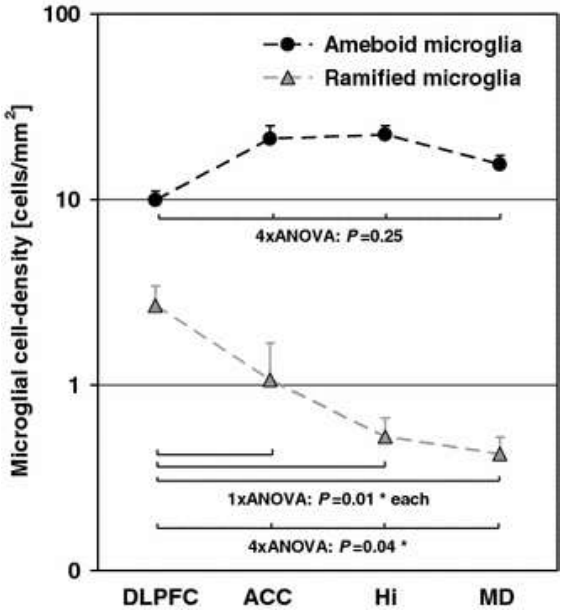

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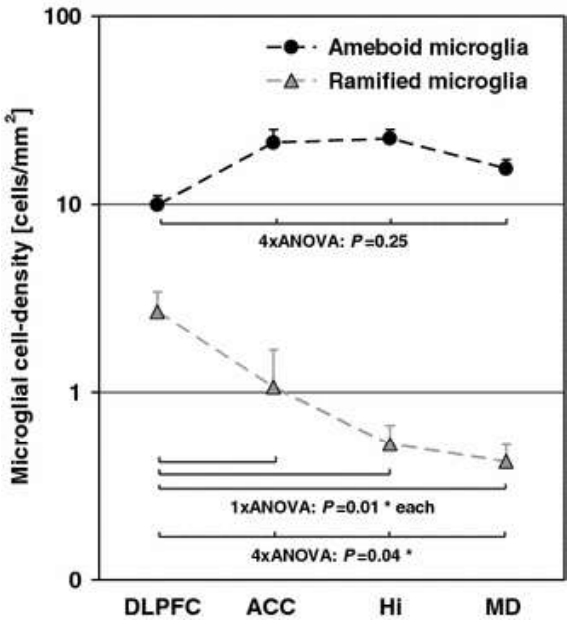

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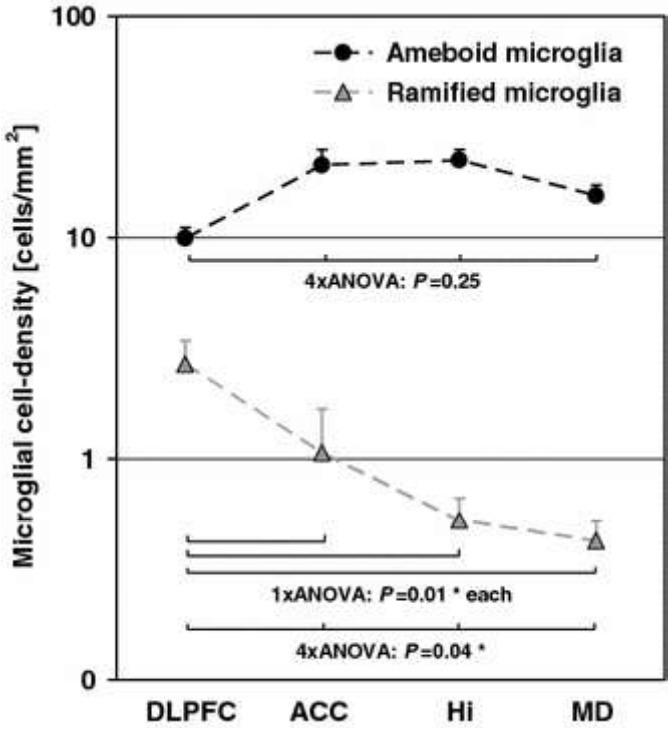

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