**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL**
**INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE**
**PROGRAMA DE PÓS-GRADUAÇÃO DE CIÊNCIAS BIOLÓGICAS: NEUROCIÊNCIAS**

# O PAPEL DA RESTRIÇÃO CALÓRICA NA NEUROPROTEÇÃO

TESE DE DOUTORADO

**Letícia Carina Ribeiro**

Porto Alegre
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**Letícia Carina Ribeiro**

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Neurociências, do Instituto de Ciências Básicas da Saúde, da Universidade Federal do Rio Grande do Sul – UFRGS, como requisito parcial para obtenção do título de Doutor.

Porto Alegre
2009

***“Posso todas as coisas naquele que me fortalece”.***

***(Filipenses 4:13)***

***Dedico este trabalho à minha princesinha, Melissa (que Deus me enviou para alegrar e iluminar a minha vida); ao meu marido, Cleber; e aos meus pais, Sílvio e Maria. Obrigada por tudo!!!***

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À minha família: aos meus pais, Sílvio e Maria; aos meus sogros, João Celi e Vilma; pelo amor, pela preocupação, pelo apoio e por compartilharem comigo mais essa vitória!

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**SUMÁRIO**

**ABREVIATURAS**

| | |
|---|---|
| AGES | Produtos finais avançados de glicação |
| AMPA | Ácido $\alpha$-amino-3-hidróxi-5-metil-isoxazolenopropionato |
| CAT | Catalase |
| DCF | Diclorodiidrofluoresceína |
| EAAT | Transportadores de aminoácidos excitatórios |
| ERO | Espécies reativas de oxigênio |
| GFAP | Proteína ácida fibrilar glial |
| GLAST | Transportador de glutamato/aspartato |
| GLT | Transportador de glutamato |
| GluRs | Receptores glutamatérgicos |
| GPx | Glutationa peroxidase |
| GS | Glutamina sintetase |
| GSH | Glutationa reduzida |
| GSSG | Glutationa oxidada |
| IGF-1 | Fator de crescimento semelhante à insulina |
| MDA | Malondialdeído |
| mGluRs | Receptores metabotrópicos |
| NMDA | N-metil-D-aspartato |
| NO | Óxido nítrico |
| RC | Restrição calórica |
| SGZ | Zona subgranular |
| SNC | Sistema nervoso central |
| SVZ | Zona subventricular |
| TBA | Ácido tiobarbitúrico |

**RESUMO**

A dieta de restrição calórica (RC) tem apresentado efeitos que diminuem a progressão, ou previnem completamente, uma variedade de doenças associadas com o envelhecimento, incluindo doenças cardiovasculares, diabetes e doenças neurodegenerativas. O prejuízo da função celular astroglial, incluindo captação de glutamato, atividade da glutamina sintetase (GS) e secreção de S100B, além de parâmetros de estresse oxidativo alterados podem contribuir para a progressão de doenças neurológicas. Este estudo objetivou avaliar alterações astrocíticas hipocampais em resposta à RC, medindo parâmetros astrogliais, tais como: captação de glutamato, atividade da GS e imunoconteúdo de GFAP e S100B. Nós investigamos também, os efeitos da RC nos parâmetros hipocampais (Hc) e corticais cerebrais (Cx) de estresse oxidativo e de comportamento em ratos Wistar machos. Parâmetros bioquímicos sanguíneos também foram avaliados. Os ratos (60 dias de idade) foram alimentados com dieta *ad libitum* ou com RC por 12 semanas. Os animais do grupo RC apresentaram um ganho de peso menor, de aproximadamente 16% em relação ganho de peso do grupo controle. A RC induziu um aumento significativo (23%) na captação de glutamato e na atividade da GS (26%). Não houve diferença significativa no imunoconteúdo de S100B ou GFAP. Os parâmetros bioquímicos avaliados não diferiram entre os grupos, indicando bom estado de saúde. Além disso, a RC não alterou parâmetros de ansiedade, mas aumentou significativamente a atividade locomotora, elevou os níveis de GSH e diminuiu a atividade de GPx, diminuiu a produção de ERO. Além disso, não alterou a peroxidação lipídica, conteúdo de óxido nítrico e a atividade da catalase. A RC também diminuiu o nível basal de dano oxidativo ao DNA, medido pelo ensaio cometa. Em resumo, este estudo indica que a RC modula as funções astrocíticas, através do aumento da captação de glutamato e da atividade da GS, sugerindo que a dieta pode exercer seus efeitos neuroprotetores contra patologias do SNC através da modulação destas funções. A RC também pode induzir a modulação hipocampal e cortical cerebral, resultando em alterações metabólicas, que por sua vez, melhoram as condições basais de importantes parâmetros de defesa celular, como por exemplo, o aumento dos níveis de GSH e diminuição do dano oxidativo ao DNA.

## ABSTRACT

The dietary caloric restriction (CR) has been shown to slow the progression of, or even prevent entirely, a range of age-dependent pathologies, including cardiovascular disease, diabetes and neurodegenerative diseases. The impairment of astroglial cell function, including glutamate uptake, glutamine synthetase (GS) activity and S100B secretion, besides oxidative stress parameters altered, may contribute to the progression of neurological disorders. The present study aimed to evaluate hippocampal astrocytic changes in response to CR diet, measuring astroglial parameters, such as glutamate uptake, GS activity and the immunocontent of GFAP and S100B. We investigated also the effects of CR on hippocampal (Hc) and cerebral cortical (Cx) oxidative stress and behavioral parameters in male Wistar rats. Blood biochemical parameters were also analyzed. Rats (60-day old) were fed ad libitum or on CR diets for 12 weeks. CR-fed rats showed approximately 16% less body weight gain than control rats. The CR diet was able to induce a significant increase in glutamate uptake (23%) and in GS activity (26%). There were no statistically significant differences in the immunocontent of either GFAP or S100B. No differences were observed in the biochemistry parameters evaluated, indicating normal healthy. CR did not alter anxiety parameters but increased locomotion performance; increased glutathione content; decreased glutathione peroxidase activity; decreased reactive oxygen species production and did not altered lipid peroxidation, nitric oxide content and catalase activity. Also, CR diet decreased basal DNA damage index, measured by comet assay. In summary, the present study indicates that CR modulates astrocyte functions by increasing glutamate uptake and GS activity, suggesting that CR might exert its neuroprotective effects against brain illness by modulation of astrocytic functions. The CR diet also induced hippocampal and cerebral cortical modulation, resulting in metabolic changes which in turn improve the basal status of important parameters of cellular defenses, such as the increased glutathione content and decreased DNA damage.

## INTRODUÇÃO

### Restrição Calórica

As manipulações dietéticas são as formas mais antigas e comuns de tratamento, e têm sido prescritas através da história para as mais diversas patologias dentro da medicina. Historicamente, numerosas sociedades reconheceram os efeitos benéficos, para a saúde e o bem estar geral, da ingestão limitada de alimentos por certos períodos de tempo. Estas restrições alimentares ocorriam geralmente em função de motivos religiosos ou em épocas de escassez de comida, como em guerras ou alterações climáticas extremas.

O primeiro estudo científico utilizando uma dieta de restrição calórica (RC) foi realizado em 1935 (McCay, Crowell, & Maynard, 1935), demonstrando que a RC, quando implementada após a puberdade, aumenta a expectativa média e máxima de vida, além de prevenir ou atenuar a severidade de doenças crônicas em ratos.

Desde então, os possíveis benefícios provenientes da dieta de restrição calórica tem sido tema de grande atenção. A grande maioria dos estudos preconiza uma restrição de 20 a 40% da densidade energética consumida *ad libitum* em 24 horas, com a garantia da ingestão adequada de todos os nutrientes (lipídios, proteínas e carboidratos). A introdução da dieta de restrição calórica passa por uma fase inicial de adaptação, na qual ocorrem ajustes metabólicos em resposta à diminuição energética, sendo seguida por uma fase estável que apresenta alteração do estado fisiológico, incluindo características como diminuição da temperatura corporal, glicemia, insulinemia, gordura corporal e de

peso, que será responsável pelo aumento do tempo de vida (Koubova & Guarente, 2003).

Alguns mecanismos de ação da restrição calórica sobre o aumento da expectativa de vida têm sido estudados e três deles se destacam: redução da sinalização do fator “Insulin-like Growth Factor-1” (IGF-1), diminuição do estresse oxidativo e ativação das sirtuinas 1 e 2 (Guarente, 2006; Horska et al., 1999; Merry, 2005; Sanz et al., 2005; Young & Kirkland, 2007) .

Estudos demonstram que a restrição calórica, quando realizada de maneira consistente e em longo prazo, retarda o processo de envelhecimento e expande a expectativa máxima de vida em diferentes espécies. Estes dados sugerem que a RC previne ou retarda a ocorrência de doenças crônicas como diabetes, cardiomiopatias, doenças autoimunes, respiratórias e renais, além de câncer. Além disso, a RC diminui a neurodegeneração, previne a perda neuronal, bem como uma variedade de doenças neurodegenerativas que estão relacionadas com a idade (Bishop & Guarente, 2007; Levenson & Rich, 2007).

No que diz respeito ao seu papel e sua atuação no sistema nervoso central (SNC), estudos revelam que os efeitos antienvelhecimento estão relacionados com a estimulação da neurogênese em adultos e diminuição da expressão de genes relacionados com a inflamação e com a resposta ao estresse, protegendo o SNC de patologias crônicas e agudas. Estudos epidemiológicos em humanos sugerem que a RC reduz o risco de neuropatologias crônicas como Alzheimer e Parkinson, bem como insultos agudos como acidente vascular cerebral (Contestabile & Ciani, 2004).

Apesar dos grandes avanços já obtidos nas pesquisas direcionadas para o esclarecimento dos mecanismos de ação que proporcionam o aumento da expectativa máxima de vida com a administração da restrição calórica, muito ainda precisa ser estudado, principalmente no que tange à ação da restrição calórica no SNC.

**O Envelhecimento**

A compreensão dos mecanismos envolvidos no processo de envelhecimento também contribui para a elucidação sobre como a RC está envolvida no aumento da expectativa de vida.

O envelhecimento é um processo fisiológico e natural que ocorre no organismo com o avanço da idade. Ele pode ser dividido basicamente em duas etapas: primária e secundária. Cabe salientar que esta classificação não é universalmente aceita em função da impossibilidade de separar completamente cada fator. A fase primária é caracterizada pela deterioração progressiva da estrutura física e da função biológica que ocorre com o avanço da idade por si só, independentemente de outros fatores. Por exemplo, mudanças na composição corporal (diminuição da densidade mineral óssea, da massa muscular, e acúmulo de gordura abdominal), declínio das funções cardíaca, pulmonar, renal e imune. A fase secundária consiste numa deterioração acelerada na função e estrutura dos órgãos, que é mediada por patologias, como diabetes e hipertensão, ou por

fatores ambientais ou estilos de vida prejudiciais tais como exposição excessiva ao sol ou tabagismo (Fontana & Klein, 2007).

Os mecanismos biológicos e celulares precisamente responsáveis pelo surgimento das etapas do envelhecimento não são conhecidos, mas envolvem uma constelação de fatores complexos e inter-relacionados que incluem: estresse oxidativo (indução de danos em proteínas, lipídeos e DNA – com reparo inadequado ao dano em DNA, instabilidade genética mitocondrial e de genomas nucleares) (Beckman & Ames, 1998; Gilchrest & Bohr, 1997; Lombard et al., 2005; Sohal, Mockett, & Orr, 2002; Sohal & Weindruch, 1996); inflamação crônica (causada pelo aumento da produção de adipocinas e citocinas) (Krabbe, Pedersen, & Bruunsgaard, 2004); alterações no metabolismo de ácidos graxos, incluindo o aumento de ácidos graxos livres circulantes e ocasionando resistência à insulina (Basu et al., 2003); prejuízo da função celular (acúmulo anormal de proteínas, produtos finais de glicação avançada – AGES) (Cefalu et al., 1995; Frye, Degenhardt, Thorpe, & Baynes, 1998; Gafni, 1997; Verzijl et al., 2000); ativação do sistema nervoso simpático e do sistema angiotensina além de alterações do sistema neuroendócrino (Basso et al., 2005; Seals & Esler, 2000; Smith, Betancourt, & Sun, 2005); perda neuronal; deterioração na função e estrutura de células em todos tecidos e órgãos (Campisi, 2005).

**Sinalização neuroglial**

As células gliais estão envolvidas em diversas funções para manter a integridade do sistema nervoso central, incluindo a captação de glutamato, síntese

de glutamina e defesa antioxidante (Chen & Swanson, 2003; Magistretti, 2006; McKenna, 2007; Raps, Lai, Hertz, & Cooper, 1989). Os astrócitos constituem aproximadamente 50% do número total de células do SNC. Eles são divididos em dois tipos: os protoplasmáticos, na substância cinzenta (freqüentemente ramificados e com largas expansões) e os fibrosos, na substância branca (com menos ramificações, cilíndricas e longas) (Wang & Bordey, 2008). Sabe-se que os astrócitos e neurônios apresentam um sofisticado sistema de comunicação recíproca que pode regular a liberação de neurotransmissores, a excitabilidade neuronal e a transmissão sináptica (Araque, Carmignoto, & Haydon, 2001; Araque, Parpura, Sanzgiri, & Haydon, 1999) no conjunto da sinapse de três elementos (Figura 1).

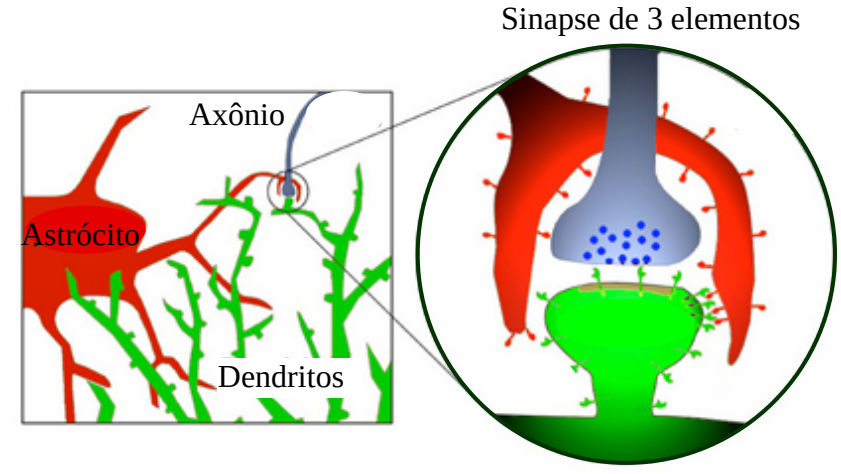


**Figura 1. Imagem ilustrativa de uma sinapse de três elementos, contendo o neurônio pré-sináptico, o neurônio pós-sináptico e o astrócito**. Adaptado de (Bourgin et al., 2007).

As funções desempenhadas pelos astrócitos no SNC são diversas, tais como (1) manutenção da homeostase de íons extracelulares, especialmente o potássio;

(2) metabolismo de neurotransmissores, particularmente o glutamato; (3) atuam como guias na migração de neurônios nos estágios iniciais do desenvolvimento; (4) síntese e liberação de fatores tróficos e substâncias neuroativas para os neurônios e para os outros astrócitos; (5) participação na resposta imune no cérebro; (6) participação na resposta a danos ao SNC, tornando-se reativos (gliose reativa[1]); (7) regulação do espaço extracelular através da variação do volume astrocítico (Araque, 2008; Chen & Swanson, 2003; Wang & Bordey, 2008).

Alguns marcadores periféricos têm sido propostos para a avaliação do comprometimento neuronal e glial, particularmente a proteína glial S100B (Van Eldik & Wainwright, 2003).

**S100B**

A S100B é um membro da família de proteínas S100, encontrada principalmente na forma homodimérica (com cerca de 21 kDa). É uma proteína ligante de cálcio do tipo EF-hand (hélice-loop-hélice) com dois sítios de ligação ao cálcio por monômero (Van Eldik & Wainwright, 2003). Foi isolada há mais de 40 anos (Moore, 1965) a partir de um extrato de cérebro bovino. Posteriormente, verificou-se que este extrato continha duas proteínas muito similares, a S100A1 e a S100B, e a mesma foi identificada também em tecidos extracerebrais e hoje são conhecidos 21 membros da família S100, presentes nos mais diversos tipos celulares de vertebrados (Donato, 2003) os quais possuem aproximadamente 50%

[1] Gliose reativa se refere à hiperplasia e/ou hipertrofia das células gliais após injúrias ao SNC formando uma "cicatriz" no local da lesão (Dahl & Bignami, 1974; Shao & McCarthy, 1994).

de homologia na sua seqüência de aminoácidos (Van Eldik & Wainwright, 2003). As proteínas da família S100 receberam esta denominação por serem solúveis mesmo em sulfato de amônio 100%.

A S100B é produzida e secretada principalmente por astrócitos e exerce efeitos autócrinos e parácrinos sobre outras células gliais e neurônios apresentando funções intra e extracelulares como fosforilação de GFAP e manutenção da integridade do citoesqueleto (Donato, 2003; Frizzo et al., 2004).

A proteína S100B tem um efeito duplo dependente da concentração, ou seja, em concentrações na ordem nM exerce efeito neurotrófico, promovendo crescimento de neuritos, aumentando a sobrevivência de neurônios durante o desenvolvimento e em situações de injúria ao SNC (Gottfried et al., 2002; Leite et al., 2004; A. C. Tramontina et al., 2008; F. Tramontina et al., 2006) e protegendo neurônios, contra a excitotoxicidade do glutamato (Ahlemeyer, Beier, Semkova, Schaper, & Krieglstein, 2000; F. Tramontina et al., 2006). O efeito trófico também é exercido em astrócitos. Já em concentrações na ordem µM exerce efeito neurotóxico induzindo apoptose (Van Eldik & Wainwright, 2003).

**Glutamato, Glutamina Sintetase e Glutationa**

O glutamato é o principal neurotransmissor excitatório do SNC de mamíferos e exerce um importante papel na plasticidade neural e neurotoxicidade (Hertz, 2006; McKenna, 2007; Pellerin & Magistretti, 1994). O glutamato medeia vários processos vitais, tais como: desenvolvimento das células nervosas, incluindo proliferação e migração (Hertz, 2006), modulação de mecanismos de aprendizado

e memória (Izquierdo & Medina, 1997) e envelhecimento (Segovia, Porras, Del Arco, & Mora, 2001).

O glutamato, após ser sintetizado, é estocado pelo sistema de transporte presente nas vesículas que se encontram no terminal pré-sináptico. Quando ocorre a despolarização dos terminais sinápticos glutamatérgicos, o glutamato que se encontra nas vesículas é liberado para o meio extracelular (fenda sináptica) para interagir com seus receptores ionotrópicos e/ou metabotrópicos que estão localizados nas membranas pré e pós-sinápticas e também nas membranas gliais (Gallo & Ghiani, 2000; Scannevin & Huganir, 2000).

Os receptores glutamatérgicos (GluRs) têm papel fundamental na plasticidade e no desenvolvimento neural, bem como nos processos de neurodegeneração e transmissão sináptica. A ativação excessiva dos GluRs durante episódios de estresse cerebral, tais como: isquemia, traumatismo craniano, surtos epilépticos e doenças neurodegenerativas levam à morte de neurônios (neurotoxicidade). Os GluRs estão envolvidos intimamente na fisiopatologia das funções cerebrais (Anderson & Swanson, 2000; Loureiro et al., 2005; Nakanishi et al., 1998; Ozawa, Kamiya, & Tsuzuki, 1998).

Os GluRs são divididos em duas classes distintas: receptores ionotrópicos e metabotrópicos (Stanimirovic, Ball, Small, & Muruganandam, 1999). Os receptores ionotrópicos são assim denominados, pois são canais iônicos permeáveis a cátions e foram subdivididos em N-metil-D-aspartato (NMDA) e não NMDA, que compreende os receptores ácido $\alpha$-amino-3-hidróxi-5-metil-isoxazolenopropionato

(AMPA) e cainato. Os receptores metabotrópicos (mGluRs) pertencem a uma família de receptores que estão acoplados às proteínas G. A concentração de glutamato no espaço extracelular determina o grau de ativação de seus receptores, por isso, é essencial manter os níveis extracelulares desse neurotransmissor normais.

Após, o glutamato é removido da fenda sináptica principalmente por sistemas de transporte (EAAT), que são dependentes de sódio, localizados nos neurônios (EAAT3/EAAC1; EAAT4-5) e principalmente nas células gliais (EAAT1/GLAST e EAAT2/GLT1) (Amara & Fontana, 2002; Anderson & Swanson, 2000; Danbolt, 2001; Gottfried et al., 2002; Robinson & Dowd, 1997). A captação astrocítica é a mais eficiente para manter normal a concentração extracelular de glutamato. Os astrócitos captam glutamato e intracelularmente ele é convertido em glutamina pela enzima glutamina sintetase (GS) (EC 6.3.1.2) (Figura 2). A glutamina é liberada pelos astrócitos e captada por neurônios para ser novamente convertida em glutamato, ciclo este conhecido como glutamina-glutamato (Matthews et al., 2005).

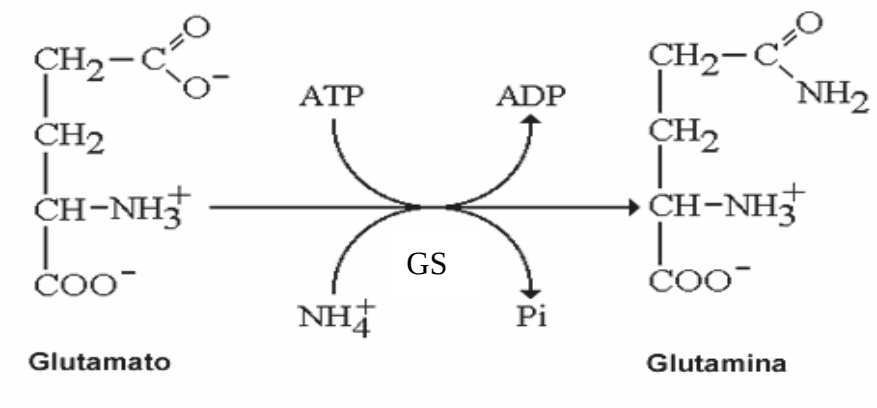


**Figura 2. Reação catalisada pela enzima Glutamina Sintetase**

A GS é uma enzima chave em duas importantes vias bioquímicas: no fígado a GS catalisa a detoxificação da amônia e no cérebro, além deste papel, garante a reciclagem do neurotransmissor glutamato, estando localizada nos astrócitos (Matthews, Gould, & Vardimon, 2005).

O antioxidante glutationa (GSH) é um tripeptídeo (Gli-Cis-Glu) essencial para a detoxificação celular de espécies reativas de oxigênio nas células gliais, sendo encontrado na forma reduzida (GSH) (Figura 3) ou oxidada (GSSG) (Figura 4). Na forma oxidada, ocorre a formação de uma ligação dissulfeto entre os grupos tiol das cisteínas. A importância deste par é tal que a razão GSH/GSSG é normalmente utilizada para estimar o estado redox dos sistemas biológicos. Em situações normais a GSSG representa apenas uma pequena fração da glutationa total (Dringen, 2000).

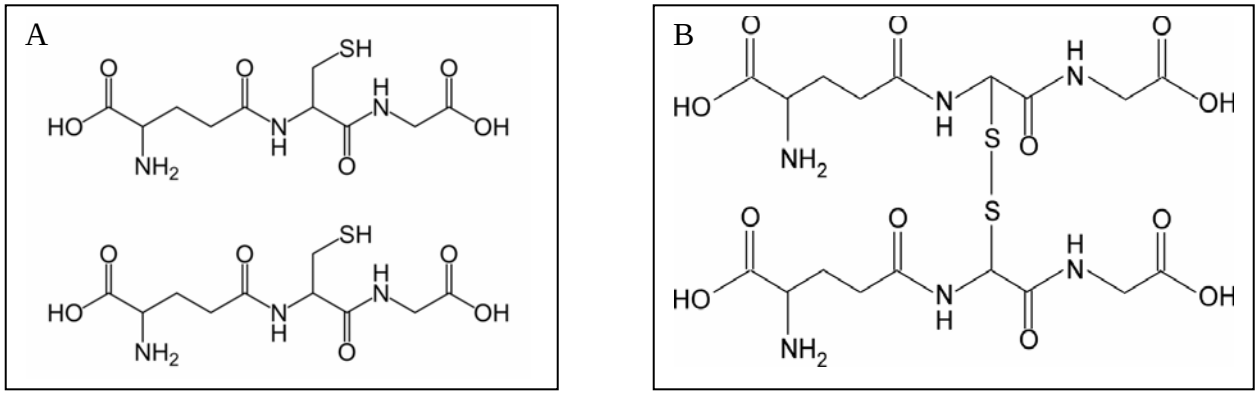


**Figura 3. Estrutura de duas moléculas de glutationa (A) na forma reduzida (GSH) e (B) na forma oxidada (GSSG).**

O grupo tiol da GSH contribui para manutenção do meio intracelular reduzido. A reciclagem do GSH depende do ciclo das pentoses e, portanto, do fornecimento de glicose. No SNC, os astrócitos secretam GSH, contribuindo para diminuir a produção de radicais livres (ex. auto-oxidação da glicose que resulta em formação de superóxido). Além disso, a GSH extracelular serve como precursor e indutor para síntese de GSH neuronal, cuja redução tem sido associada à morte celular em muitas doenças neurodegenerativas. Um sistema GSH comprometido, no cérebro, tem sido relacionado com o estresse oxidativo que ocorre em doenças neurobiológicas (Dringen, 2000).

**Óxido nítrico**

O óxido nítrico (NO) é um radical livre que medeia respostas fisiológicas, funcionando como segundo mensageiro e dessa forma, atua em mecanismos de sinalização celular. A biossíntese de NO ocorre a partir do aminoácido arginina, sendo catalisada pela enzima óxido nítrico sintase. Existem três isoformas da óxido nítrico sintase: a neuronal, a endotelial e a induzível. A forma induzível está presente nos astrócitos (Shibata & Kobayashi, 2008).

**Estresse Oxidativo**

O radical livre é uma estrutura química que possui um elétron desemparelhado, ou seja, ocupando um orbital atômico ou molecular sozinho. Isso o torna instável, reativo e com enorme capacidade para combinar-se inespecificamente com as

diversas moléculas integrantes da estrutura celular e derivados de cada uma delas, levando a oxidação de proteínas, lipídios e DNA (Gutteridge & Halliwell, 2000).

Os radicais livres formam-se em condições fisiológicas em proporções controladas pelos mecanismos de defesa celular. Entretanto, em situações patológicas, essa produção pode aumentar substancialmente. O estresse oxidativo pode resultar de uma situação em que há uma diminuição nos níveis das enzimas antioxidantes, uma elevada velocidade de produção de espécies reativas de oxigênio ou uma combinação de ambas as condições (Halliwell, 1998).

A peroxidação lipídica é um processo complexo e ocorre em múltiplos estágios. Por isso, muitas técnicas são utilizadas para medir a peroxidação de lipídios de membrana, lipoproteínas ou ácidos graxos. Cada técnica mede um produto resultante diferente, não sendo correto afirmar que uma só técnica mede a peroxidação lipídica total. O teste do ácido tiobarbitúrico (TBA) é o método mais antigo e mais utilizado para medir peroxidação de ácidos graxos. Nesse método, o malondialdeído (MDA) formado durante a peroxidação lipídica, reage com o TBA para gerar um composto colorido que é detectado espectrofotometricamente ou fluorimetricamente (Halliwell, 2006).

**Espécies Reativas de Oxigênio (ERO)**

As ERO e outros radicais livres podem ser produzidos por fontes endógenas e exógenas. Entre as principais fontes endógenas de radicais livres estão a cadeia de transporte de elétrons mitocondrial, a degradação de ácidos graxos nos peroxissomos, os mecanismos de detoxificação mediados pelo complexo

enzimático citocromo P-450, o processo de fagocitose; entre as exógenas destacam-se as radiações, o cigarro e solventes orgânicos (Gutteridge & Halliwell, 2000).

O ensaio com diclorodiidrofluoresceína (DCF) é o mais frequentemente usado para a determinação da formação de ERO. Consiste em uma técnica fluorimétrica altamente sensível, que pode ser usada em células vivas. As células são pré-tratadas com um composto não fluorescente, o qual se transforma em um fluoróforo, após a reação com o radical. A diclorodiidrofluoresceína (DCF-DA) é administrada como um éster diacetado (DA), que é rapidamente captado pelas células sofrendo desesterificação. O DCFH resultante sofre oxidação, predominantemente com espécies altamente oxidantes como radicais hidroxila e peroxinitrito (Shibata & Kobayashi, 2008).

Embora o $H_2O_2$ não seja estritamente um radical livre por definição, ele é uma ERO importante por sua capacidade de gerar radical hidroxila ($OH^{\bullet}$) em presença de metais como ferro. Ele é formado principalmente na matriz mitocondrial, durante o processo de redução do oxigênio, ou pela dismutação do radical superóxido pela enzima superóxido dismutase (Fridovich, 1998).

**Dano ao DNA e Ensaio Cometa**

O dano ao DNA produzido por oxidação é considerado o mais significante dano oriundo do metabolismo celular. Estima-se que aproximadamente $2x10^4$ lesões oxidativas ao DNA ocorram no genoma humano por dia (Ames & Shigenaga, 1992). Acredita-se que, desta maneira, o reparo destas lesões possua um papel

central na prevenção do aumento de mutações nos organismos vivos (Maluf, 2004).

O excesso de ERO (por ex. $H_2O_2$) pode levar a oxidação de lipídios, proteínas e DNA, causando dano as membranas celular e nuclear. O dano irreparável ao DNA está envolvido na carcinogênese, envelhecimento e outras doenças degenerativas (Cozzi et al, 1997). Porém, devemos levar em consideração que o dano ao DNA pode ser induzido por hábitos alimentares e estilo de vida (McCord & Edeas, 2005).

O Ensaio Cometa é uma técnica simples e rápida para verificarmos de modo quantitativo, quebras simples e duplas ao DNA (Faust et al., 2004; Maluf & Erdtmann, 2000). Um estudo prévio (Ostling & Johanson, 1984) detectou o dano ao DNA induzido por radiação ionizante após eletroforese, sob condições de pH neutro, verificando apenas quebras duplas ao DNA. Em outro estudo (Singh, McCoy, Tice, & Schneider, 1988) propuseram realizar a técnica sob condições alcalinas e assim, detectaram quebras simples e duplas ao DNA. Atualmente, esta técnica sofreu pequenas modificações (Tice et al., 2000), e vem sendo amplamente utilizada para detecção de dano ao DNA.

## OBJETIVOS

### Objetivos gerais

Este trabalho tem como objetivo geral avaliar parâmetros que contribuam para a elucidação do efeito neuroprotetor da restrição calórica em modelo experimental em ratos Wistar.

### Objetivos específicos

**a)** Estabelecer um modelo animal de restrição calórica em ratos Wistar.

**b)** Avaliar o estado nutricional: dosagem em soro de proteínas totais, albumina, triglicerídios, colesterol total e HDL, glicose, creatinina e uréia.

**c)** Avaliar parâmetros gliais: captação de glutamato, glutamina sintetase (GS), secreção de S100B, imunoconteúdo de GFAP em hipocampo.

**d)** Avaliar parâmetros comportamentais: ansiedade, atividade locomotora e níveis de corticosterona em soro.

**e)** Avaliar parâmetros de estresse oxidativo: enzimas antioxidantes (catalase e glutationa peroxidase), produção de radicais livres, lipoperoxidação, além de óxido nítrico (NO) e glutationa (GSH) em hipocampo e córtex cerebral; dano oxidativo ao DNA em sangue total e hipocampo.

## RESULTADOS

### Capítulo I

Os resultados obtidos neste capítulo estão demonstrados no artigo “Caloric restriction increases hippocampal glutamate uptake and glutamine synthetase activity in Wistar rats”. Ribeiro LC, Quincozes-Santos A, Leite MC, Abib RT, Kleinkauf-Rocha J, Biasibetti R, Rotta LN, Wofchuk ST, Perry ML, Goncalves CA, Gottfried C. (2009) Neurosci Res 64:330-334.

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# Caloric restriction increases hippocampal glutamate uptake and glutamine synthetase activity in Wistar rats

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ABSTRACT

Recent studies indicate that caloric restriction (CR) protects the central nervous system from several pathological conditions. The impairment of astroglial cell function, including glutamate uptake, glutamine synthetase (GS) activity and S100B secretion, may contribute to the progression of neurological disorders. The present study aimed to evaluate hippocampal astrocytic changes in response to CR diet, measuring astroglial parameters, such as glutamate uptake, GS activity and the immunocontent of GFAP and S100B. Blood biochemical parameters were also analyzed. Rats (60-day old) were fed *ad libitum* or on CR diets for 12 weeks. CR-fed rats showed approximately 16% less body weight gain than control rats. The CR diet was able to induce a significant increase in glutamate uptake (23%) and in GS activity (26%). There were no statistically significant differences in the immunocontent of either GFAP or S100B. In summary, the present study indicates that CR also modulates astrocyte functions by increasing glutamate uptake and GS activity, suggesting that CR might exert its neuroprotective effects against brain illness by modulation of astrocytic functions.



## 1. Introduction

Dietary calorie restriction (CR) has been applied for more than 70 years and has wide-ranging health benefits, including extended longevity (Hulbert et al., 2007; Roth et al., 2007). Some recent studies indicate that CR protects the central nervous system (CNS) from age-related diseases, i.e. preventing the symptoms associated with Alzheimer's, Huntington's and Parkinson's diseases (Mattson, 2005). While the neurobiological mechanisms responsible for the role of caloric restriction in protection of the CNS are not fully understood, recent studies suggest that CR regulates adult neuronal stem cells, increase adult neurogenesis in young adults rats, and reduce the age-related decline in neurogenesis in older animals (Levenson and Rich, 2007).

Astrocytes play an active role in brain function by affecting the activity of neurons (Fields and Stevens-Graham, 2002), especially in neuronal development, activity, plasticity, differentiation and maturation (Stevens, 2008; Volterra and Meldolesi, 2005). They may also promote neurogenesis, showing that they are widely involved in neuroprotection (Markiewicz and Lukomska, 2006). Other biochemical parameters have been used to characterize astroglial involvement in neural plasticity and injury, including glutamate uptake, glutamine synthetase (GS) and S100B protein.

Glutamate is the major excitatory neurotransmitter in the central nervous system and its accumulation is implicated in neurodegenerative disorders. Astroglial cells are responsible for major glutamate transport and regulate extracellular levels of glutamate (Hertz, 2006; Magistretti, 2006). The impairment of glutamate transporters causes excitotoxicity and leads to increased ROS production and consequent cell damage (Had-Aissouni et al., 2002). The glutamate homeostasis in the brain is maintained by its well-balanced release, uptake and metabolism. Moreover, astrocytes have a specific enzyme glutamine synthetase (GS) (EC 6.3.1.2) that catalyses the amidation reaction of glutamate to form glutamine. Astroglial glutamate uptake and GS are, respectively, responsible for the removal of glutamate from the synaptic cleft and synthesis/replacement of glutamine to neurons (Bak et al., 2006). Thus, malfunction of astrocytic glutamate transporters will lead to an excessively high extracellular glutamate concentration which may result in neurodegeneration

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caused by the excitotoxic action of glutamate (Schousboe and Waagepetersen, 2005).

In addition to GS, two other proteins are used to characterize astrocytes: glial fibrillary acidic protein (GFAP) and S100B. GFAP is a specific marker of mature astrocytes; CNS injuries are commonly accompanied by astrogliosis, characterized by an increase in GFAP (O'Callaghan et al., 1991). S100B is a $Ca^{2+}$-binding protein expressed and secreted by astrocytes, having a trophic activity on neuron and glial cells (Tramontina et al., 2006; Van Eldik and Wainwright, 2003). In the developing brain, and following acute glial activation, a glial-derived protein, S100B, acts as a neurotrophic factor and neuronal survival protein. In contrast, overproduction of S100B by activated glia can lead to exacerbation of neuroinflammation and neuronal dysfunction. This duality supports the potential of S100B as a biomarker for brain damage, implicates glial activation as a possible treatment target in acute and chronic CNS disorders, and highlights the dual role of glia in the reparative and pathologic responses to neurologic injury (Van Eldik and Wainwright, 2003).

In this study, we investigated possible specific astrocyte alterations in the hippocampi of Wistar rats in response to CR diet, investigating glutamate uptake, glutamine synthetase activity, GFAP and S100B immunocontent. Blood biochemical parameters were also evaluated.

## 2. Materials and methods

### 2.1. Materials

N-methyl-D-glucamine, HEPES and all the other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). L-[$^3$H]-glutamate (specific activity 30 Ci/mmol) was purchased from Amersham International, UK.

### 2.2. Animal research and diets

Thirty male 60-day-old Wistar rats came from the local breeding colony (ICBS–UFRGS). Animals were maintained in a ventilated room at 21 °C, with free access to water on a 12-h light/dark cycle. The experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local authorities. Animals were weight matched and divided into two groups: control (*ad libitum*) and calorie restricted rats (CR) (Table 1) that received regular laboratory chow (Nuvilab-CR1, from Nuvital, Brazil) for 12 weeks. The caloric restriction was progressive, initiated at 10% restriction in the first week, changed to 20% at second week, and to 30% at third week until the end of treatment. The food intake was monitored daily, and the animals were weighed weekly (Chang et al., 2007; Horska et al., 1999).

### 2.3. Blood sampling and analysis

After treatment, animals were overnight-starved (6th hour) and anesthetized with an intramuscular injection of ketamine and xylazine (75 and 10 mg/kg, respectively). The blood samples were obtained with intracardiac punction, and the animals were killed by decapitation. The blood samples were incubated at room temperature (25 °C) for 5 min and centrifugated at 3200 rpm for 5 min. Serum was stored at −70 °C until the day of the analysis. Biochemical analyses were carried out in a multi-test analyzer (Mega; Merck, Darmstadt, Germany), using specific kits supplied by Merck as follows: total protein (protein-SMT, 1.19703.0001, biuret method); albumin (albumin-SMT, 1.19722.0001, bromocresol method); glucose (GLUC-DH, 1.07116.0001); urea (urea-SMT, 1.19702.0001, UV test); creatinine (creatinine-SMT, 1.19726.0001, UV test); triglycerides (SMT-triglyceride, 1.19706.0001, GPO-PAP method); cholesterol (cholesterol-SMT, 1.19738.0001, CHOD-PAP method). HDL cholesterol was determined using a kit (HDL cholesterol direct FS) from DiaSys (Diagnostic Systems International, Holzheim, Germany).

**Table 1**
Composition of the laboratory chow.

| Composition | (g/kg) |
|---|---|
| Total fat | 110 |
| Sunflower oil | 5 |
| Proteins | 220 |
| Fibers | 30 |
| Ash | 60 |
| Vitamins | 20 |
| Carbohydrates | 520 |

Commercial non-purified diet, Nuvilab-CR1 (Curitiba, PR, Brazil).

### 2.4. Hippocampal dissection

The brains were removed and placed in cold saline medium with the following composition (in mM): 120 NaCl; 2 KCl; 1 $CaCl_2$; 1 $MgSO_4$; 25 HEPES; 1 $KH_2PO_4$; 10 glucose, adjusted to pH 7.4 and previously aerated with $O_2$. The hippocampi were dissected and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper for subsequent analysis.

### 2.5. Gutamate uptake assay

Hippocampal slices were then transferred immediately to 24-well culture plates, each well containing 0.3 mL of physiological medium and only one slice. Glutamate uptake was performed, as previously described (Thomazi et al., 2004). Medium were replaced by Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl; 0.63 $Na_2HPO_4$; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 $KH_2PO_4$; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$; 5.55 glucose, in pH 7.4. The assay was started by the addition of 0.1 mM L-glutamate and 0.66 μCi/mL L-[2,3-$^3$H]-glutamate. Incubation was stopped after 5 min by removal of the medium and rinsing the slices twice with ice-cold HBSS. Slices were then lysed in a solution containing 0.5 M NaOH. Sodium-independent uptake was determined using N-methyl-D-glucamine instead of sodium chloride. Sodium-dependent glutamate uptake was obtained by subtracting the non-specific uptake from the specific uptake. Radioactivity was measured with a scintillation counter.

### 2.6. Glutamine synthetase activity

The enzymatic assay was performed, as previously described (dos Santos et al., 2006). Briefly, homogenized tissue samples (0.1 mL) were added to 0.1 mL of reaction mixture containing (in mM): 10 $MgCl_2$; 50 L-glutamate; 100 imidazole–HCl buffer (pH 7.4); 10 2-mercaptoethanol; 50 hydroxilamine–HCl; 10 ATP and incubated for 15 min at 37 °C. The reaction was stopped by the addition of 0.4 mL of a solution containing (in mM): 370 ferric chloride; 670 HCl; 200 trichloroacetic acid. After centrifugation, the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate treated with ferric chloride reagent.

### 2.7. ELISA for S100B

ELISA for S100B was carried out as described previously (Leite et al., 2008). Briefly, 50 μL of sample (containing between 5 and 10 ng/μL of total protein) plus 50 μL of Tris buffer were incubated for 2 h on a microtiter plate previously coated with monoclonal anti-S100B. Polyclonal anti-S100B was incubated for 30 min and

then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min (serum samples) or both antibodies were incubated together for 1 h (tissue samples). The color reaction with o-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.02 to 10 ng/mL.

### 2.8. ELISA for GFAP

ELISA for GFAP was carried out, as described previously (Tramontina et al., 2007). Briefly, the microtiter plate was coated with 100 μL samples containing 500 ng of protein for 24 h at 4 °C. Incubation with a polyclonal anti-GFAP from rabbit for 1 h was followed by incubation with a secondary antibody conjugated with peroxidase for 1 h, at room temperature. A colorimetric reaction with o-phenylenediamine was measured at 492 nm. The standard human GFAP (from Calbiochem) curve ranged from 0.1 to 5 ng/mL.

### 2.9. Statistical analysis

Data are reported as mean ± standard error mean (S.E.M.) and were analyzed by Student's *t*-test. Values of $P < 0.05$ were considered significant. All analyses were performed using the SPSS program, Version 12.0 (SPSS, Chicago, IL).

### 2.10. Protein content

The total protein content was determined by the modified method of Lowry (Peterson, 1977), using BSA as standard.

## 3. Results

### 3.1. Effect of CR diet on body weight and serum biochemistry

Sixty-day-old rats were fed *ad libitum* or on CR diets for 12 weeks. As illustrated in Fig. 1, CR rats showed an approximately 10% (208 ± 5 g, $P < 0.05$) reduction in body weight gain within 2 weeks, compared to control rats (229 ± 8 g). This difference was achieved after 4 weeks (by 16%, $P < 0.001$) and remained relatively stable until the end of treatment. Interestingly, the biochemistry analysis of serum (Table 2) demonstrated that urea content was lower (35 ± 4.34, $P < 0.05$) in CR-rats than in controls (48 ± 2.51). Other variables, including glucose, lipemia, creatinin and protein indicated normal health.

### 3.2. Effect of CR diet on glutamate uptake and glutamine synthetase in hippocampal slices

As shown in Fig. 2, basal glutamate uptake was 0.77 ± 0.014 nmol/(mg protein/min) and CR diet was able to induce a significant increase (23%) in hippocampal glutamate uptake (0.95 ± 0.015 nmol/(mg protein/min), $P < 0.001$). The basal GS activity (0.46 ± 0.016 μmol/(mg protein/h)) was also increased (26%) under CR diet (0.58 ± 0.023 μmol/(mg protein/h), $P = 0.025$), as shown in Fig. 3.

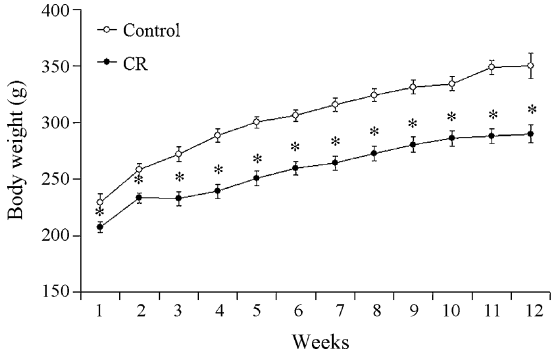


**Fig. 1.** Evolution of body weight in rats fed *ad libitum* (control) and caloric restriction (CR) diets. Figure shows the evolution of body weight of control rats (open symbols) and CR diet-fed rats (closed symbols) over 12 weeks. Values are mean ± S.E.M. ($n = 15$). *Significantly different from controls (Student's *t*-test, $P < 0.05$).

**Table 2**
Serum biochemistry of rats fed *ad libitum* (control) and caloric restriction (CR) diets for 12 weeks.

| | Control | CR |
|---|---|---|
| | (mg/dL) | |
| Glucose | 233 ± 16.44 | 196 ± 31.08 |
| Cholesterol | 65 ± 2.10 | 60 ± 2.24 |
| Triacylglicerol | 100 ± 11.35 | 83 ± 14.38 |
| HDL | 42 ± 2.47 | 36 ± 4.12 |
| Creatinin | 0.54 ± 0.02 | 0.50 ± 0.05 |
| Urea | 48 ± 2.51 | 35* ± 4.34 |
| Albumin | 2.30 ± 0.05 | 2.25 ± 0.31 |
| Protein | 6.28 ± 0.26 | 5.47 ± 0.69 |

Values are means ± S.E.M., $n = 8$.
* Statistically significant from control by Student's unpaired *t*-test ($P < 0.05$).

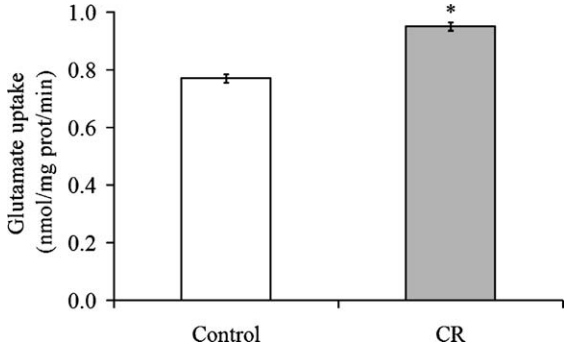


**Fig. 2.** Glutamate uptake in the hippocampus of rats submitted to caloric restriction (CR) for 12 weeks. Hippocampal slices of 0.3 mm were incubated with [$^3$H]-glutamate for 5 min. Values are mean ± S.E.M. ($n = 8$). *Significantly different from controls (Student's *t*-test, $P < 0.001$).

### 3.3. Effect of CR diet on the astrocytic protein markers, S100B and GFAP

Next, we examined the effect of CR diet on S100B (from serum and hippocampus) and GFAP (from hippocampus). As shown in Table 3, there were no statistical differences in the immunocontent of either S100B or GFAP, as compared to control groups.

## 4. Discussion

Many experiments have established that CR markedly decreases body weight gain and fat mass in mammals (Wanagat et al., 1999). As illustrated in Fig. 1, CR rats showed approximately 16% lower body weight gain than controls. Moreover, these animals are not undernourished, as observed by serum parameters in Table 2, indicating normal healthy status. Interestingly, the biochemistry analysis of serum showed decreased urea content in CR-fed rats. This result deserves further investigation regarding renal glutaminase activity to better explain this effect.

We demonstrated herein, that CR was able to modulate important astroglial functions in Wistar rats. It is now well established, in every species tested to date (yeast, roundworm, rodents and monkeys), that dietary caloric restriction confers beneficial health effects, such as the slowing down of many age-dependent processes and extending lifespan (Gillette-Guyonnet and Vellas, 2008).

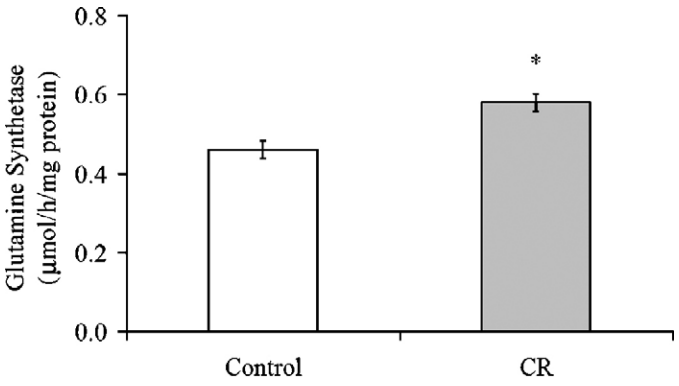


**Fig. 3.** Glutamine synthetase (GS) in the hippocampus of rats submitted to caloric restriction (CR) for 12 weeks. Homogenized tissue samples were incubated as described in Section 2. Values are mean ± S.E.M. ($n$ = 8). *Significantly different from controls (Student's $t$-test, $P < 0.05$).

**Table 3**
S100B and GFAP immunocontent.

| | S100B | | GFAP |
|---|---|---|---|
| | Serum | Hippocampus | Hippocampus |
| Control | 0.22 ± 0.09 | 1.09 ± 0.07 | 12.4 ± 2.1 |
| CR | 0.20 ± 0.10 | 0.93 ± 0.11 | 12.9 ± 3.8 |

Rats were fed with *ad libitum* (control) and caloric restriction (CR) diets for 12 weeks. S100B and GFAP immunocontent were measured by ELISA. Values are means ± S.E.M., $n$ = 3–4.

Metabolic and cellular signalling pathways potentially involved in caloric restriction-mediated changes for brain health have been integrated, increasing neurotrophic factors (Maswood et al., 2004), protein chaperones and antiapoptotic proteins in many different regions of the brain (Mattson, 2008; Mattson et al., 2003). While accumulating experimental evidence suggests that CR may offer protection against age-related neuronal loss and neurodegenerative disorders (Gillette-Guyonnet and Vellas, 2008), little is known about the influence of astroglial cells on CR effects, especially with regard to glutamate metabolism. Astrocytes are intimately associated with glutamatergic transmission and, thus, with synaptic plasticity and neuroprotection (Chen and Swanson, 2003; Danbolt, 2001).

We have described, in this study, a significant increase in hippocampal glutamate uptake from extracellular media, indicating modulation in the activity and/or number of the transporters. The concentration of glutamate rises dramatically during neurotransmission (to ~100 μM to 1 mM), and failure of astrocytes to remove this excess glutamate leads to excitotoxic damage and ultimately neuronal death (Danbolt, 2001). Disposal of glutamate from the synaptic cleft in order to maintain the low resting concentration (~1–10 μM) required for neurotransmission is one of the most important functions of astrocytes in brain, since they have a very powerful quantity load of glutamate transporters (McKenna, 2003). In addition, *in vitro* studies on cultured astrocytes and *in vivo* studies on rodents have provided evidence that glutamate and $Na^+$ uptake in astrocytes is a key triggering signal, regulating glucose use in the brain (Escartin et al., 2006).

In order to investigate another important function of astrocytes related to glutamate metabolism, we measured hippocampal GS activity. In agreement with the glutamate uptake increase, we also demonstrated a 26% increase in GS activity under the CR diet. Astrocytes are the only cells in the brain that have the important ability to convert glutamate into glutamine via GS. Glutamine, in turn, is taken up by neurons and used for the synthesis of glutamate (and then GABA, in GABAergic neurons). These findings provide new insights into how the brain functions under the CR diet as well as new targets for clinical interventions in brain pathologies.

The astrocyte-derived neurotrophic factor, S100B, has been measured by several immunoassays in biological fluids and tissue (Leite et al., 2008; Michetti and Gazzolo, 2002). In the current study, we have demonstrated that hippocampal levels of S100B and GFAP were not influenced by CR diet, indicating as expected, absence of astrogliosis. In addition, serum S100B immunocontent was also similar between diets.

CR has been shown to extend life span and ameliorate aging-related functional impairments in a variety of body systems by imposing a homeostatic state. Here, we demonstrate that CR diet can modulate important neural functions in brain homeostasis. However, there are important limitations of the study design to be pointed out: first, this study was performed in adult animals; secondly, the animals used in the study were healthy and thirdly, the diet was administered for 12 weeks to investigate acute effects. Further investigations will be necessary to demonstrate the effects of chronic administration of CR diet on aged animals and also to investigate astrocytes from different brain areas, especially under pathological conditions such as dementia.

In summary, the present study indicates that CR also modulates astrocyte functions by increasing glutamate uptake and GS activity, suggesting that CR might exert its neuroprotective effects against brain illness, by a mechanism that involves modulation of astrocytic functions.

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**Capítulo II**

Os resultados obtidos neste capítulo estão demonstrados conforme manuscrito a seguir:

CALORIC RESTRICTION IMPROVES BASAL REDOX PARAMETERS ON HIPPOCAMPAL AND CORTEX FROM WISTAR RATS

Leticia C. Ribeiro[1], Letícia Rodrigues[1], André Quincozes-Santos[2], Ana C. Tramontina[2], Victorio Bambini[2], Caroline Zanotto[1], Luisa A. Diehl[1], Regina Biasibetti[2], Juliana Kleinkauf-Rocha[2], Gabriela S. Baum[2], Wagner Nunes[2], Carla Dalmaz[1, 2], Carlos-Alberto Gonçalves[1, 2], Carmem Gottfried[1, 2]

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# CALORIC RESTRICTION IMPROVES BASAL REDOX PARAMETERS ON HIPPOCAMPAL AND CORTEX FROM WISTAR RATS

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**ABSTRACT**

The dietary caloric restriction (CR) has been shown to slow the progression of, or even prevent entirely, a range of age-dependent pathologies, including cardiovascular disease, diabetes and neurodegenerative diseases. In addition, we recently demonstrated that CR modulates astrocyte functions by increasing glutamate uptake and GS activity, suggesting that CR might exert its neuroprotective effects against brain illness, by a mechanism that involves modulation of astrocytic functions. In the present study, we investigated the effects of CR on hippocampal (Hc) and cortical (Cx) oxidative stress parameters in male Wistar rats. Behavioral and blood biochemical parameters were also evaluated. CR fed rats had 17% less body weight gain after 12 weeks of treatment. No differences were observed in the biochemistry parameters evaluated, indicating normal healthy. CR did not alter anxiety parameters but increased locomotion performance; increased glutathione content; decreased glutathione peroxidase activity; decreased reactive oxygen species production and did not altered lipid peroxidation, nitric oxide content and catalase activity. Also, CR diet decreased basal DNA damage index, measured by comet assay. In summary, CR diet induced hippocampal and cortical modulation, resulting in metabolic changes which in turn improve the basal status of important parameters of cellular defenses, such as the increased glutathione content and decreased DNA damage.

## INTRODUCTION

The dietary caloric restriction (CR), a limitation of food intake below the *ad libitum* level without malnutrition, can extend the mean and maximum lifespan in a wide range of organism in which it has been tested. CR has also been shown in animal models to slow the progression of, or even prevent entirely, a range of age-dependent pathologies, including cardiovascular disease (1), multiple types of cancer (2) and diabetes (3). CR may also reduce neuronal damage and consequently offer protection against neurodegenerative diseases (4, 5). Some recent studies demonstrate that the CR induces neurogenesis (6) and enhances the synaptic plasticity (7). Moreover, we have been recently demonstrated that CR also modulates astrocytic functions by increasing glutamate uptake and GS activity, suggesting that CR might exert its neuroprotective effects against brain illness, by a mechanism that involves modulation of astrocytic functions (8). These findings suggest that the brain under CR can be more able to resist aging process and restore function following injury.

With aging, the brain undergoes neuronal loss in many areas. Besides, there are cognitive declines with age, as well as decreases in brain structure size and white matter integrity (9). There are evidences that one brain structure, the hippocampus, seems to be particularly sensitive to aging and, at least in part, responsible for the age-related cognitive decline that occurs during normal aging (10). In despite of this, many age-related changes within hippocampus have been documented, for example, altered mitochondrial function, oxidative stress, and alterations in glutamate transmission and synaptic plasticity (7). Some studies

recognized that the cerebral cortex, in particular the frontal cortex, also have an important loss of cells with aging, and the influence of synaptic losses has a larger impact on cognitive decline (11).

Also, age has a powerful effect on enhanced susceptibility to neurodegenerative diseases. Problems occur when the production of Reactive Oxigen Species (ROS) exceeds the ability of cells to defend themselves against these substances. The oxidative stress is referred to as the imbalance between cellular production of ROS and the ability of cells to defend themselves against them. Oxidative stress can cause cellular damage and ROS oxidize cellular components such as membrane lipids, proteins, and DNA (12). There is substantial evidence that the brain, which consumes large amounts of oxygen, have abundant lipid content, and relative paucity of antioxidant enzymes compared with other organs, is particularly vulnerable to oxidative damage.

The oxidative damage is strongly implicated in the pathogenesis of neurodegenerative diseases including Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease and stroke (brain ischemia/reperfusion injury) (13). Cells normally have a number of mechanisms to resist against free radicals-induced cell damage. The major antioxidant defenses consist of antioxidant scavengers such as glutathione (GSH), Vitamin C (ascorbic acid), Vitamin E ($\alpha$-tocopherol), carotenoids, flavonoids, polyphenols, and antioxidant enzymes (Superoxide dismutase, Catalase and Glutathione peroxidase). These antioxidant defense mechanisms can be upregulated in

response to increased ROS or peroxide production. Although it may confer protection against ROS, they are not completely effective in preventing oxidative damage in aging (12, 13). Some recent studies demonstrate that age-related increase in oxidative brain damage is best exemplified by products of lipid peroxidation, protein oxidation and oxidative modifications in nuclear and mitochondrial DNA, beyond a decrease in brain and plasma antioxidants (GSH and antioxidants enzymes activity) (14) .

In the present study, we investigated the effects of caloric restriction on oxidative stress parameters, including basal antioxidant enzymes; lipid peroxidation and DNA damage in hippocampus and cerebral cortex of Wistar rats. Behavioral and blood biochemical parameters were also evaluated.

## MATERIAL AND METHODS

### Animal research and diets

Thirty male 60-day-old Wistar rats came from the local breeding colony (ICBS-UFRGS). Animals were maintained in a ventilated room at 21$^{o}$C, with free access to water on a 12 h light/dark cycle. The experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local authorities. Animals were weight matched and divided into two groups: Control (*ad libitum*) and calorie restricted rats (CR). The CR group received the same regular laboratory chow (Nuvilab-CR1, from Nuvital, Brazil) diet except for a lower caloric intake. The caloric restriction was progressive, initiated at 10% restriction in the first week, changed to 20% at second week, and to 30% at third

week until the end of treatment (12 weeks). The food intake was monitorated daily, and the animals were weighed weekly (8).

**Behavioral tests**

***Elevated plus-maze test.*** The elevated plus-maze task consisted of placing the animal at the center of a maze with two closed arms and two open ones (44.5 cm × 11.5 cm for each arm). During a 2-min period, the number of entries into the closed arms, and the time spent in the open arms were registered (15).

***Exposure to the open field.*** The open-field task was studied using a 50 cm high, 60 cm × 40 cm plywood box with a frontal glass wall and a linoleum floor divided into 12 equal rectangles. Animals were left there for 2 min both in the training and the test session, and the number of rearings and crossings between sectors were registered (15).

**Blood sampling and analysis**

After treatment, animals were overnight-starved ($6^{th}$ hour) and anesthetized with an intramuscular injection of ketamine and xylazine (75 and 10 mg/kg, respectively). The blood samples were obtained with intracardiac punction, and the animals were killed by decapitation. The blood samples were incubated at room temperature (25°C) for 5 min and centrifuged at 3200 rpm for 5 min. Serum was stored at -70°C until the day of the analysis. Biochemical analyses were carried out in a Multi-test Analyzer (Mega; Merck, Darmstadt, Germany), using specific kits supplied by Merck as follows: total protein (protein-SMT, 1.19703.0001, biuret method); albumin (albumin-SMT, 1.19722.0001, bromocresol method); glucose

(GLUC-DH 1.07116.0001); cholesterol (cholesterol-SMT, 1.19738.0001, CHOD-PAP method); triglycerides (SMT-triglyceride, 1.19706.0001, GPO-PAP method). For corticosterone determination, plasma was extracted with ethyl acetate, and the extract evaporated and dissolved for the hormone evaluation with an ELISA kit (Cayman Chemical Co., Ann Arbor, MI, USA). The sensitivity of the assay is 24 pg/mL and the intra assay coefficient of variation was 15%.

**Brain tissue dissection**

The brains were removed and placed in cold saline medium with the following composition (in mM): 120 NaCl; 2 KCl; 1 $CaCl_2$; 1 $MgSO_4$; 25 HEPES; 1 $KH_2PO_4$ and 10 glucose, adjusted to pH 7.4 and previously aerated with $O_2$. The hippocampi (Hc) and cerebral cortices (Cx) were dissected and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper for subsequent analysis.

**Glutathione (GSH) assay**

The GSH content was determined as described before (16). Briefly, hippocampal and cortical slices were homogenized in sodium phosphate buffer (0.1 M, pH 8.0) containing 5 mM EDTA and protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with *o*-phthaldialdehyde (1 mg/mL of methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard glutathione solutions (0–500 µM). The GSH concentrations are expressed as nmol/mg protein.

**Glutathione peroxidase (GPx) activity**

GPx activity was measured by the method of (17) using *tert*-butyl-hidroperoxide as substrate. GPx activity was determined by monitoring NADPH (0.1 mM) disappearance at 340 nm in a medium containing 2 mM GSH, 0.15 U/ml glutathione reductase, 0.4 mM azide and 0.5 mM *tert*-butyl-hidroperoxide. One GPx unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as U/mg protein.

**Catalase (CAT) activity**

CAT activity was assayed by the method of (18) by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM $H_2O_2$, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 50 μg protein. One unit (U) of the enzyme is defined as 1 μmol of $H_2O_2$ consumed per minute and the specific activity is reported as U/mg protein.

**Evaluation of intracellular reactive oxygen species (ROS) production**

Intracellular ROS production was detected using the nonfluorescent cell permeating compound, 2'–7'-dichlorofluorescein diacetate (DCF-DA). Samples homogenized in sodium phosphate buffer, pH 7.4 with 140 mM KCL were treated with DCF-DA (10 μM) for 30 min at 37°C. The fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm, as described previously (19), with modifications. Values are obtained as unit of fluorescence/mg protein and are expressed as percentage of control.

**Thiobarbituric acid-reactive substances (TBARS) measurement**

Lipid peroxidation can be evaluated by the thiobarbituric acid reactive substance assay. This method evaluates the lipid peroxidation assayed for malondialdehyde, the last product of lipid breakdown caused by oxidative stress. The assay was performed, as previously described (20). Briefly, 100 µL of homogenate was added to 200 µL of cold 10% trichloroacetic acid and 300 µL of 0.67% TBA in 7.1% sodium sulfate in a boiling water bath for 15 min. The mixture was placed in cold water for 1 min. Afterwards 400 µL of butyl alcohol were added and samples were centrifuged at 5000 x g for 5 min. Pink-stained TBARS was determined in resulting supernatants in a spectrophotometric microtiter plate reader at 532 nm. Data were expressed as nmol TBARS/mg protein.

**Nitric oxide (NO) production**

NO metabolites, $NO_3$ (nitrate) and $NO_2$ (nitrite), were determined according to (21). Briefly, homogenates from hippocampal slices were mixed with 25% trichloroacetic and centrifuged at 1800 g for 10 min. The supernatant was immediately neutralized with 2 M potassium bicarbonate. NO-3 was reduced NO-2 by nitrate reductase. The total NO-2 in the incubation was measured by a colorimetric assay at 540 nm, based on the Griess reaction. A standard curve was performed using sodium nitrate (0–80 µM). Results were expressed as µmolar of nitrite/mg protein.

**Single cell gel electrophoresis — comet assay**

A standard protocol for comet assay preparation and analysis was adopted (22). The slides were prepared by mixing 5 µL whole blood, or homogenate hippocampus (cold PBS), with 90 µL low melting point agarose (0.75%). The

mixture (cells/agarose) was added to a fully frosted microscope slide coated with a layer of 500 µL of normal melting agarose (1%). After solidification, the coverslip was gently removed and the slides were placed in a lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0–10.5, with freshly added 1% Triton X-100 and 10% DMSO) for 1 day. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 10 min. The DNA was electrophoresed during 20 min at 25 V (0.90 V/cm) and 300 mA, after electrophoresed the slides was neutralized with Tris buffer (0.4 M; pH 7.5). Finally, the DNA was stained with ethidium bromide. After electrophoresis, neutralized and stained nuclei (from random 100-cells fields) were blindly analyzed by fluorescence microscopy (200x). Cells were scored from 0 (undamaged) to 4 (maximally damaged), according to the tail intensity (size and shape), resulting in a single DNA damage score for each cell, and, consequently, for each group. Therefore, a group damage index could range from 0 (all cells no tail, 100 cells x 0) to 400 (all cells with maximally long tails, 100 cells x 4) (23). The index of DNA damage was calculated by multiplying the number of cells by it's respectively index score and than summed up.

**Protein content**

The total protein content was determined by the modified method of Lowry (24), using BSA as standard.

**Statistical analysis**

Data are reported as mean ± standard error mean (S.E.M.) and were analyzed by Student's t-test. Values of $P< 0.05$ were considered significant. All analyses were performed using the SPSS program, Version 12.0 (SPSS, Chicago, IL).

## RESULTS

### Body weight

Sixty-day old rats fed laboratory chow *ad libitum* (control) or on CR for 12 weeks and were weighed weekly. Both groups gained weight during this period. However, CR fed rats exhibited a significantly lower body weight gain than controls. As illustrated by Figure 1, CR fed rats had 12% (215±5 g, $P<0.05$) less body weight gain after 1 week of treatment, compared to the control rats (245±8 g). The difference after 12 weeks was 17%, ($P<0.001$).

### Serum content of glucose, cholesterol and triacylglycerol

No differences were observed in the serum content of glucose, cholesterol, triacylglycerol, corticosterone and protein between groups (Table 2).

### Behavior parameters

In order to investigate behavioral parameters (anxiety and locomotion) in control and CR fed rats, it was performed elevated plus maze and open field tasks after 12 weeks. As showed in Figure 2A, both diets did not alter anxiety parameters. Based on the Kolmogorov–Smirnov goodness-of-fit test, these data were expressed as mean and standard deviation. Number of crossings and rearings was not different between control and CR groups (Student's *t*-test). However, CR-fed

rats increased locomotion performance (Figure 2B). Time spent in the open or closed arms was significantly increased (*$P < 0.05$) in CR groups, compared to the control rats (Student's *t*-test).

**Oxidative stress parameters in hippocampus and cerebral cortex: GSH, GPx, CAT, ROS, TBARS and NO.**

Glutathione content was higher in CR than in control groups (Figure 3), increasing 26% in Hc (from 19.3±0.85 to 24.4±1.25 nmol/mg protein) and 29% in Cx (from 19.3±0.85 to 24.4±1.25 nmol/mg protein) ($P<0.05$). As shown in Figure 4, basal GPx was 15.33±0.79 and 17.10±0.32 U/mg protein in Hc and Cx, respectively. CR diet was able to significantly decrease (about 18%) GPx activity in both cerebral structures ($P<0.005$). The CAT activity was not different between groups as showed in Figure 5.

CR diet-fed rats significantly decreased 26% and 14% ROS production in Hc and Cx, respectively, as compared to the control groups (Figure 6, $P<0.05$) and did not produce any statistically significant differences in TBARS levels (Table 3) and NO production (Table 4).

**DNA damage in blood and hippocampal cells**

The index of DNA damage was not different in blood cells between groups (Figure 7A). However, in hippocampal cells, there was a decrease in basal DNA damage index, from 12±2.2 to 8±1.4 ($P<0.01$) as shown in Figure 7B.

## DISCUSSION

This study was undertaken because the effects of dietary CR (without malnutrition) in brain are poorly understood.

In agreement with our previous data (8), we observed that rats fed with CR diet gained weight but at less rapid rate than control rats. In fact, a decreased body weight gain was observed in CR group compared to control group from the first week and more than 17% reduction was observed after 12 weeks of treatment. We observed a normal proteinemia, which does not support the possibility of inadequate protein intake to explain less weight gain. Also, CR diet did not induced anxiety in the rats, demonstrated by the corticosterone levels and behaviour in the plus maze tasks, which did not differ between groups. Interestingly, CR-fed rats significantly increased general activity levels and exploration habits in the open field tasks, indicating more locomotor activity than the controls.

The current study shows that hippocampal and cortical GSH content was significantly higher in CR than in control groups. These data provide some pieces of evidence for delineating the mechanisms of the protective action of CR by increasing an important non-enzymatic antioxidant for CNS.

The basal values of CAT activity, TBARS levels and NO production were not different between groups. However, CR diet-fed rats induced significantly decrease in ROS production, 26% in Hc and 14% in Cx, as compared to the control groups. High levels of ROS can induce lipid, protein and DNA damage in cells . Hydrogen peroxide, although it is not a free radical, can generate the hydroxyl and other reactive radicals, extending oxidative damage (25). In this context, a decrease in basal ROS production may be an important strategy to the

maintenance of a healthy brain. Glutathione peroxidase is a selenoenzyme family capable of eliminating peroxides by reducing them to $H_2O$ or alcohols, with GSH as reducing substrate (26). CR diet was also able to significantly decrease (about 18%) GPx activity in both Hc and Cx structures.

The present data contributes to the understanding of the CR modulation of neural cells as the followed hypothesis (Figure 8). Dietary calorie restriction results in less metabolic and mitochondrial activity (27) with a subsequent decrease in mitochondrial ROS production as we have demonstrated. A decreased ROS production induced by CR could negatively modulate GPx activity, justifying an increase in GSH levels.

Also, we have demonstrated that CR group had 30% less hippocampal DNA damage than control group. This data is in agreement with recent works showing that CR has been able to reverse the age-related alterations in DNA damage/repair and mutations (28). Protection of DNA is believed to be a key element in preventing cancer as well as in delaying or preventing the aged phenotype. When DNA damage persists in the genome, through replicative processes and/or through transcription-associated mutagenesis, this damage becomes permanent, in the form of mutations and/or chromosomal breakage and instability (28). Studies by Richardson's laboratory suggest that CR is an "intervention" that alters the activation of specific "stress response genes", key enzymes in DNA repair pathways, which then results in upregulation of "DNA repair" capacity (28, 29). Thus, CR diet could be enhancing DNA repair followed by

a decrease in DNA damage, consequently reducing mutation frequency, which would result in maintenance of genomic stability.

In summary, by examining calorie restriction's effects we were able to identify hippocampal and cortical modulation, resulting in metabolic changes in turn improve the basal status of important parameters of cellular defenses, such as an increase in GSH and a decrease in DNA damage. The maintenance of metabolic and physiological stability during aging is a prime determinant of longevity and brain function.

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## TABLES

### Table 1. Composition of the laboratory chow

| Composition | (g/Kg) |
|---|---|
| Total fat | 110 |
| Sunflower oil | 5 |
| Proteins | 220 |
| Fibers | 30 |
| Ash | 60 |
| Vitamins | 20 |
| Carbohydrates | 520 |

Commercial nonpurified diet, Nuvilab-CR1 (Curitiba, PR - Brazil)

**Table 2. Serum biochemistry of rats fed *ad libitum* (control) and caloric restriction (CR) diets for 12 weeks.**

| (mg/dL) | Control | CR |
|---|---|---|
| | (mg/dL) | |
| **Glucose** | 220 ± 13.24 | 201 ± 29.51 |
| **Cholesterol** | 61 ± 3.10 | 60 ± 3.06 |
| **Triacylglicerol** | 100 ± 15.23 | 95 ± 16.45 |
| **Corticosterone** | 36.50 ± 5.20 | 30.40 ± 4.80 |
| **Albumine** | 2.41 ± 0.41 | 2.32 ± 0.58 |
| **Protein** | 5.81 ± 0.33 | 5.53 ± 0.73 |

Values are mean ± S.E.M. (*n*=8).

**Table 3. Lipid peroxidation**

| | Control | CR |
|---|---|---|
| | (nmol/mg protein) | |
| **Hc** | 36.8 ± 6.0 | 35.9 ± 4.9 |
| **Cx** | 26.9 ± 1.8 | 26.7 ± 1.9 |

Rats were fed with control or CR diet for 12 weeks. Lipid peroxidation was evaluated by the thiobarbituric acid reactive substance assay (TBARS), assayed for malondialdehyde. Homogenized tissue samples were incubated as described in the material and Methods section. Values are mean ± S.E.M. (*n*=6). Hc, hippocampus; Cx, cortex.

**Table 4. Nitric oxide (NO) production**

| | Control | CR |
|---|---|---|
| | (µmolar/mg protein) | |
| **Hc** | 16.6 ± 1.7 | 16.4 ± 1.4 |
| **Cx** | 15.6 ± 1.3 | 14.4 ± 0.9 |

Rats were fed with control or CR diet for 12 weeks. Homogenized tissue samples were incubated as described in the material and Methods section. Values are mean ± S.E.M. (*n*=6). Hc, hippocampus; Cx, cortex.

## LEGENDS OF FIGURES

**Figure 1. Evolution of body weight.** Male Wistar rats were fed with laboratory show *ad libitum* (control) or with caloric restriction (CR). Figure shows the evolution of body weight of control (open symbols) and CR diet-fed rats (closed symbols) throughout 12 weeks. Values are means ± S.E.M. ($n$=15). *Significantly different from controls (Student's $t$-test, $P < 0.05$).

**Figure 2. Behavior in open-field and elevated plus-maze.** Rats were fed with control or CR diet for 12 weeks. (A) Open-field task. (B) Elevated plus-maze. Data expressed as mean ± S.E. $n$=15, *$P < 0.05$.

**Figure 3. Glutathione (GSH) content in the hippocampus and cerebral cortex.** Rats were fed with control or CR diet for 12 weeks. Homogenized tissue samples were incubated as described in the Material and Methods section. Values are mean ± S.E.M. ($n$=6). *Significantly different from controls (Student's $t$-test, $P < 0.05$).

**Figure 4. Glutathione peroxidase (GPx) activity in the hippocampus and cerebral cortex.** Rats were fed with control or CR diet for 12 weeks. Homogenized tissue samples were incubated as described in the Material and Methods section. Values are mean ± S.E.M. ($n$=6).

**Figure 5. Catalase (CAT) activity in the hippocampus and cerebral cortex.** Rats were fed with control or CR diet for 12 weeks. Homogenized tissue samples were incubated as described in the Material and Methods section. Values are mean ± S.E.M. ($n$=6).

**Figure 6. Reactive oxygen species (ROS) production in the hippocampus and cerebral cortex.** Rats were fed with control or CR diet for 12 weeks. Homogenized tissue samples were incubated as described in the Material and Methods section. Values are mean ± S.E.M. (*n*=6). *Significantly different from controls (Student's *t*-test, $P < 0.05$).

**Figure 7. Comet assay in the blood and hippocampal cells**. Rats were fed with control or CR diet for 12 weeks. Samples were prepared as described in the Material and Methods section. Values are mean ± S.E.M. (*n*=6).

**Figure 8. Effect of *ad libitum* and calorie restriction diets on ROS generation via respiratory chain.** (A) Under *ad libitum* conditions, glucose is metabolized (1) and during respiration, electrons move down the electron-transport chain. It is thought that hyper-polarization of the mitochondrial membrane can lead to the stalling of electrons in the electron-transport chain (2) and the generation of ROS (3). (B) A physiological hypothesis that might occur during calorie restriction (CR) is initiated by lower calorie intake. This will decrease glucose oxidation (1), decrease the activity of electron-transport chain with (2) decrease generation of ROS (3). A decreased ROS production could negatively modulate GPx activity (4), with increased GSH levels (5).

**Figure 1**

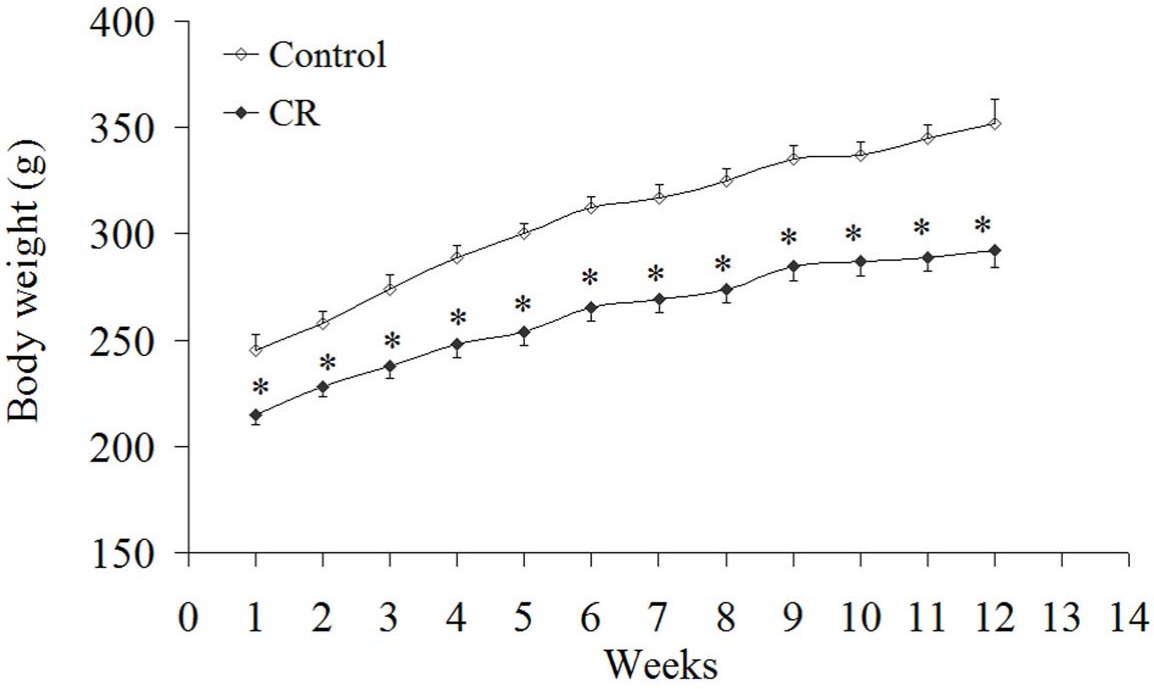

Control
CR
Body weight (g)
400
350
300
250
200
150
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Weeks

**Figure 2**

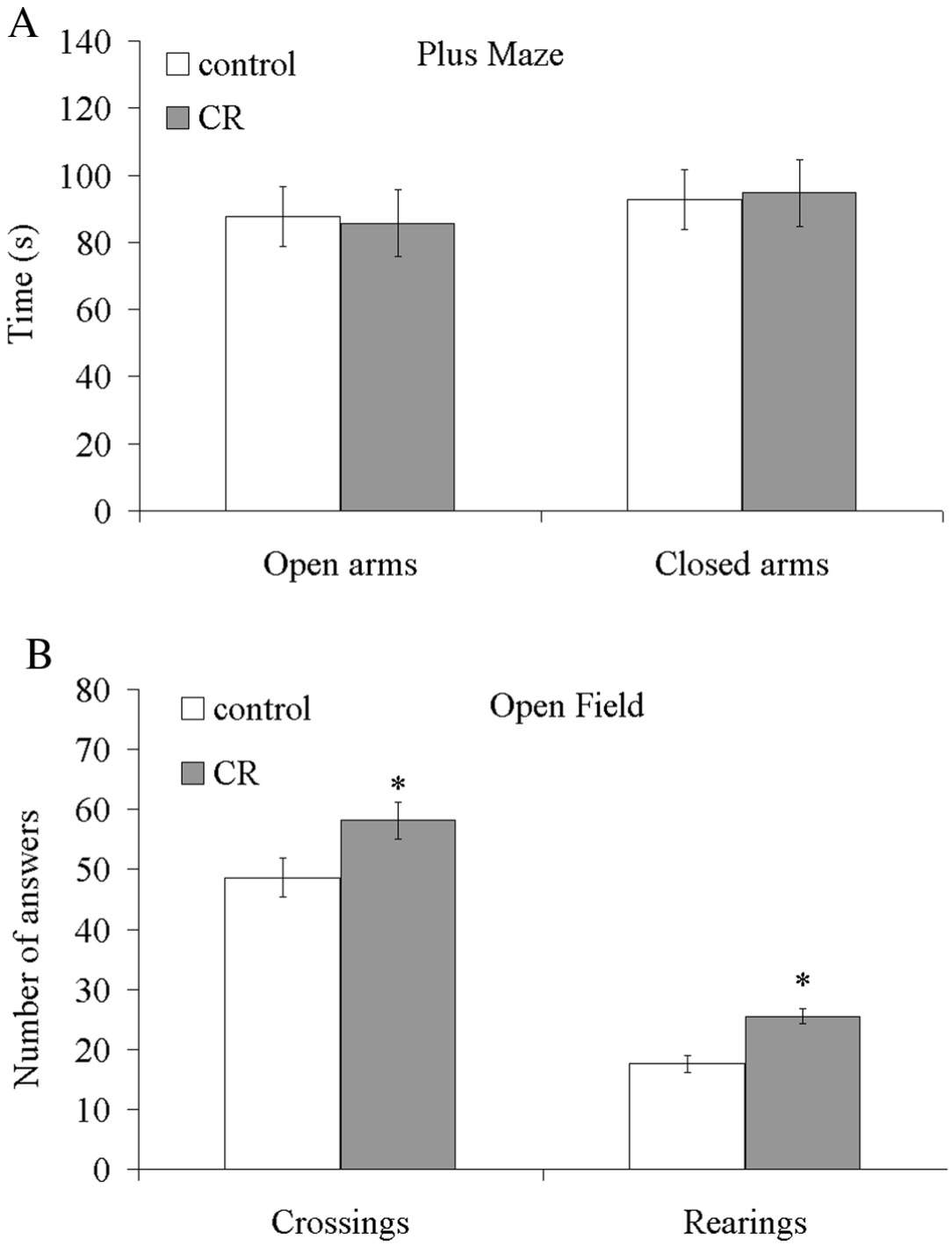

A
Plus Maze
control
CR
Time (s)
140
120
100
80
60
40
20
0
Open arms
Closed arms
B
Open Field
control
CR
Number of answers
80
70
60
50
40
30
20
10
0
*
*
Crossings
Rearings

**Figure 3**

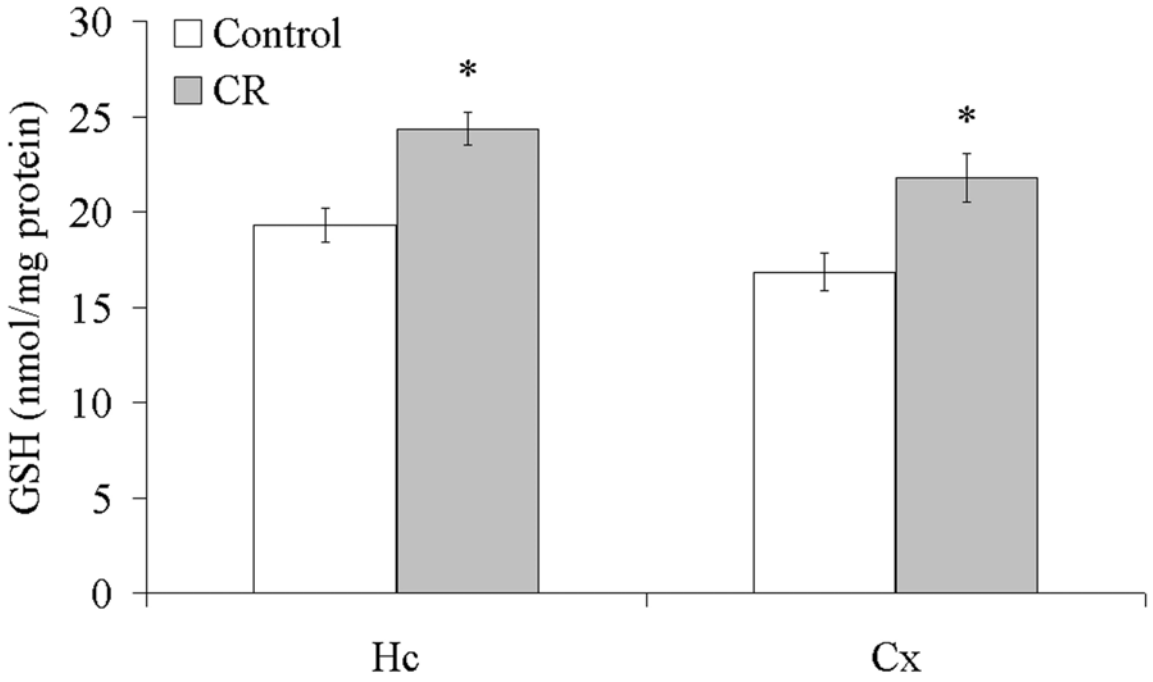


**Figure 4**

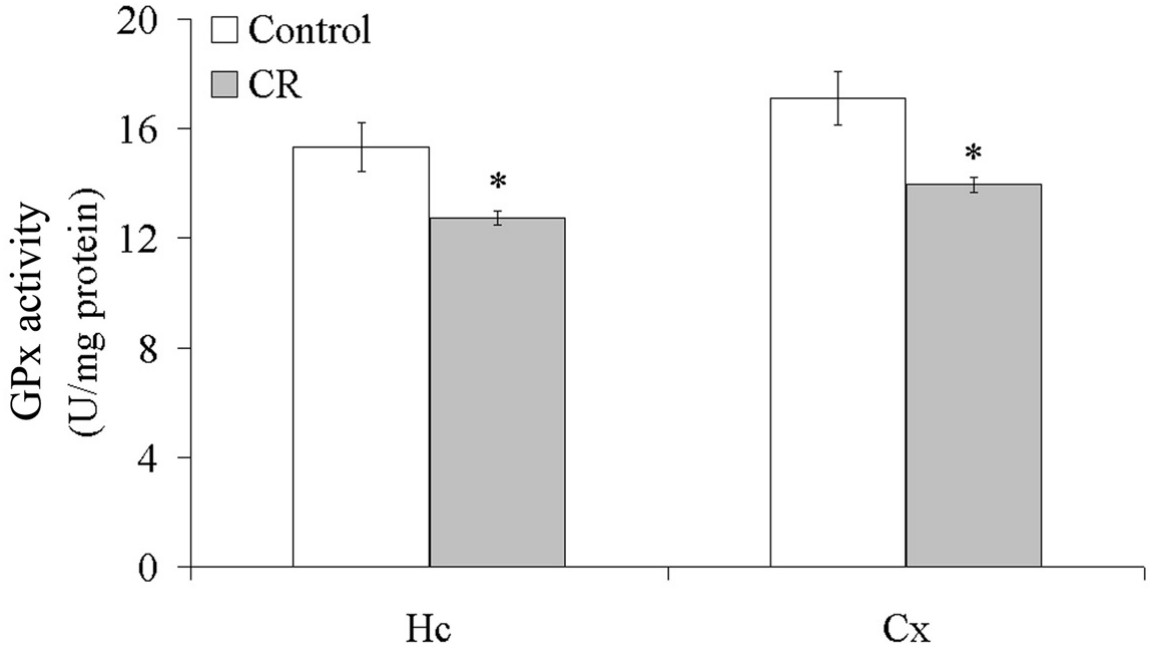


**Figure 5**

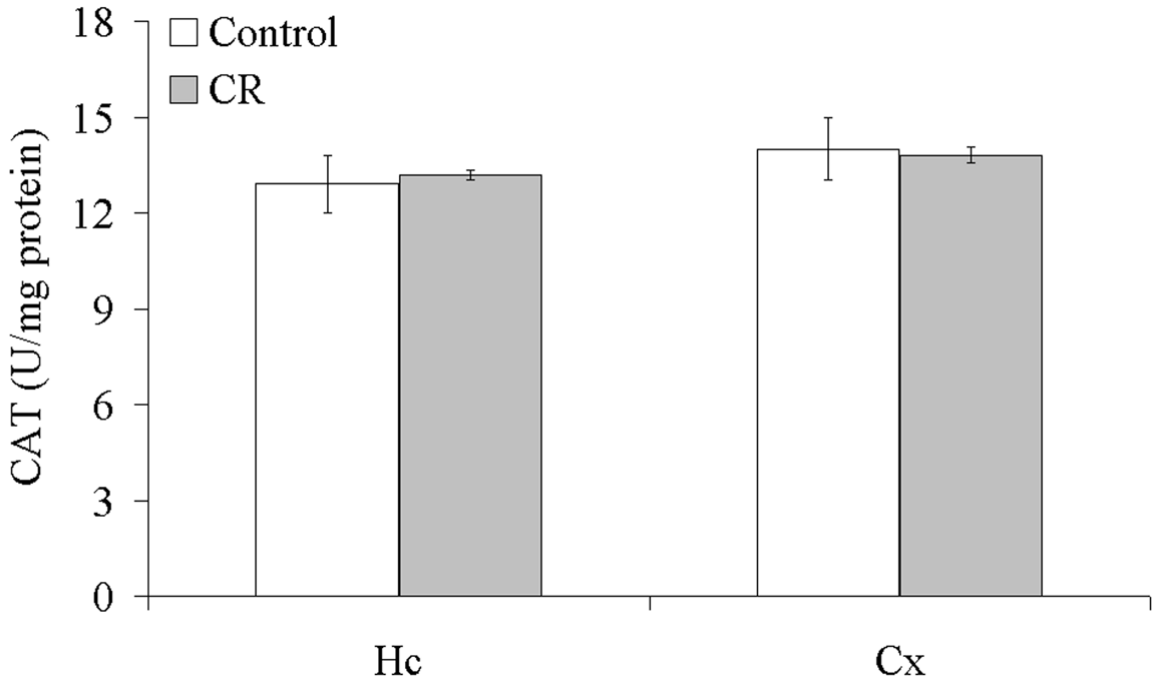


**Figure 6**

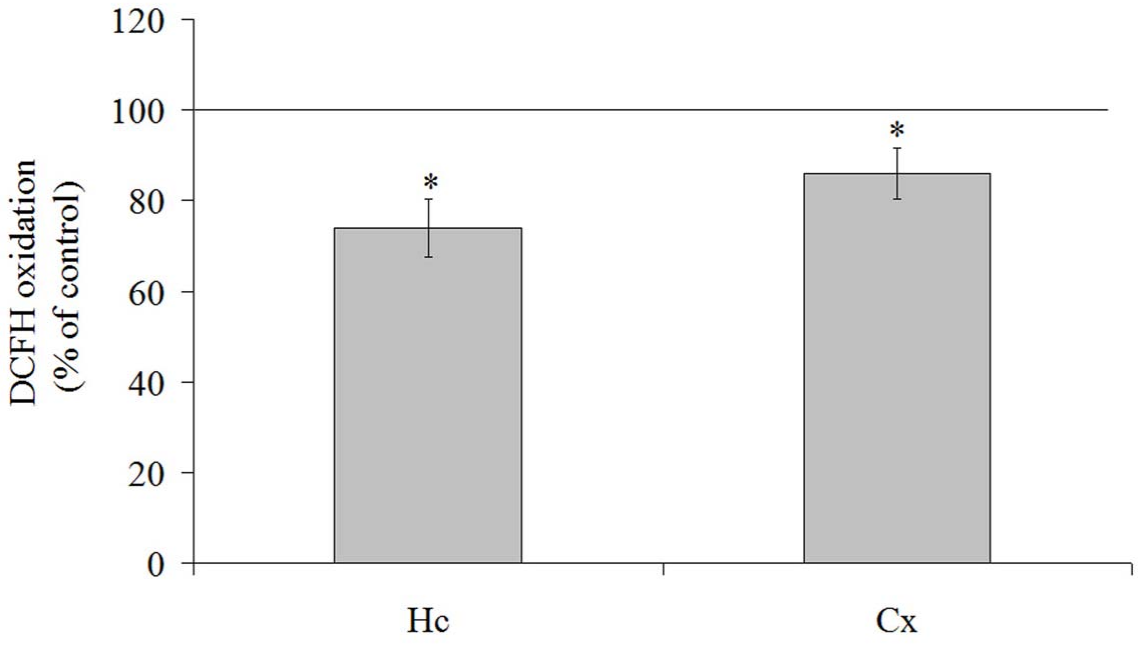


**Figure 7**

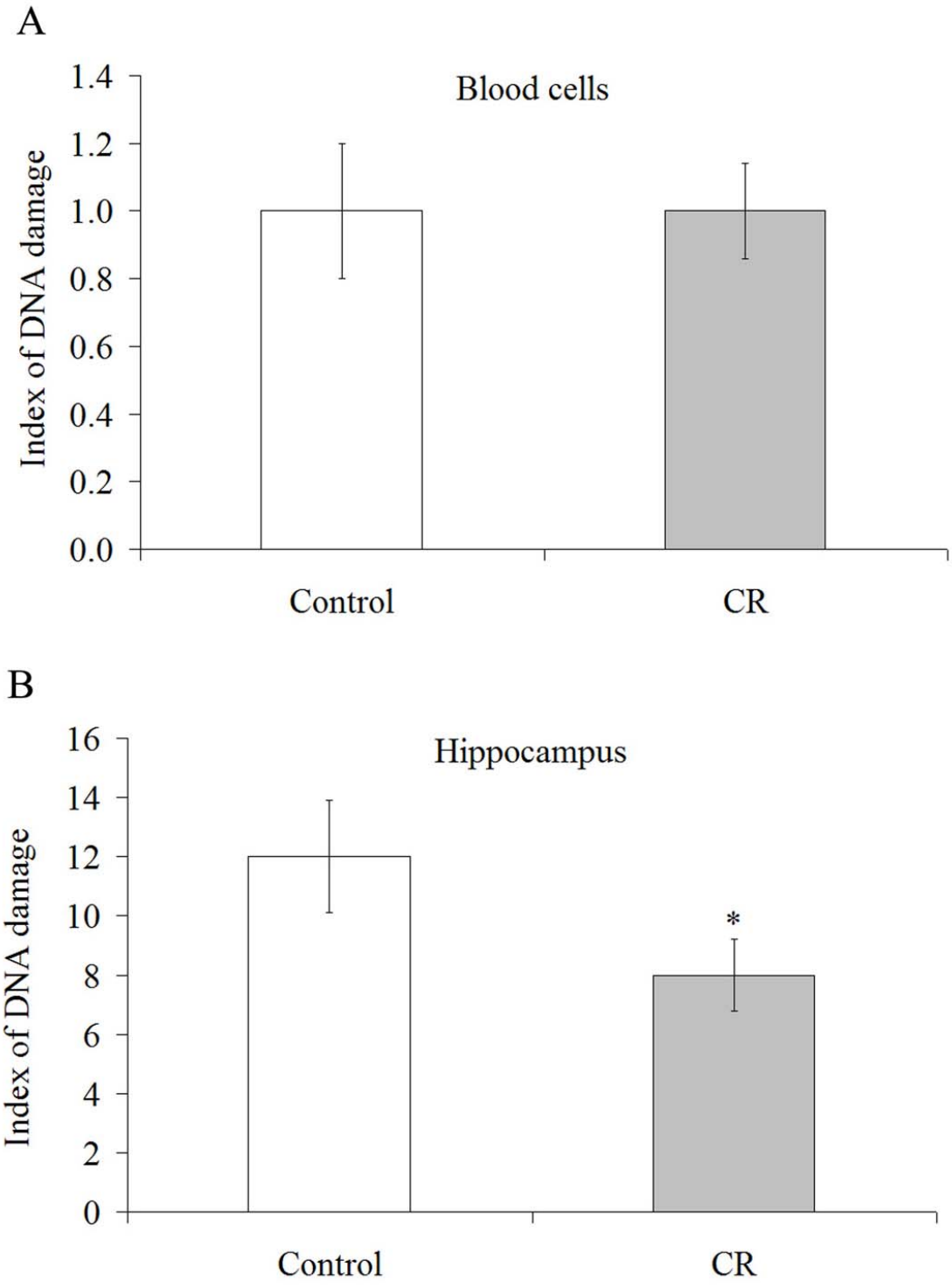
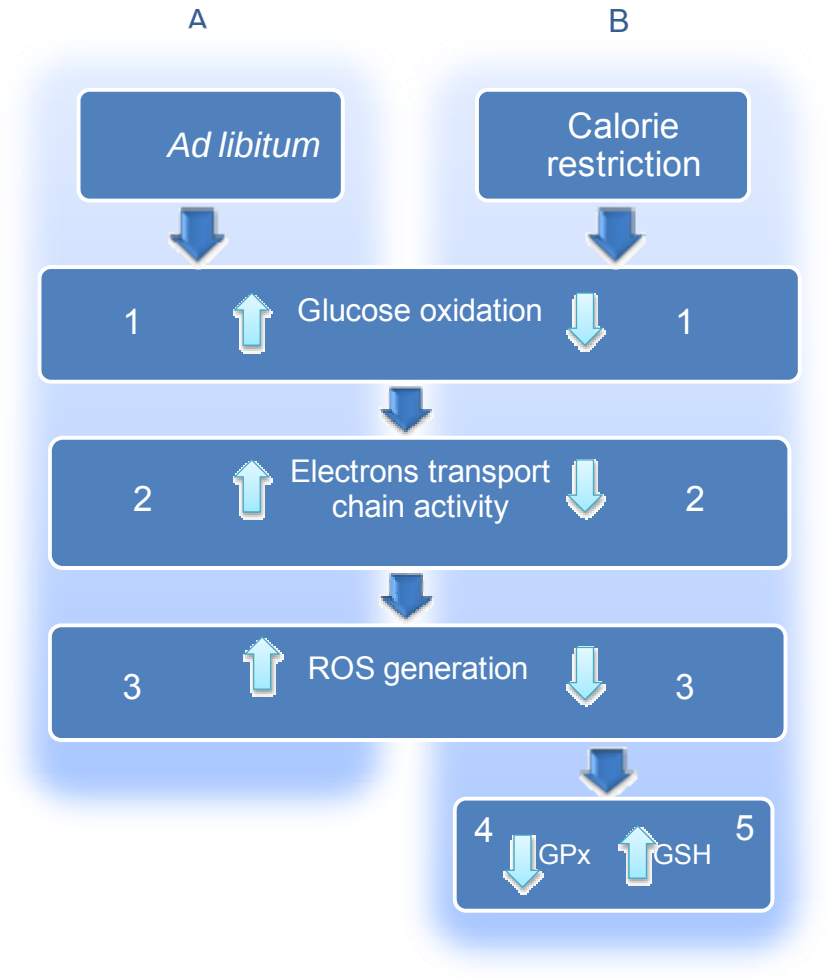

A
Blood cells
Index of DNA damage
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
CR
B
Hippocampus
Index of DNA damage
16
14
12
10
8
6
4
2
0
*
Control
CR

**Figure 8**


A
B
Ad libitum
Calorie restriction
1
Glucose oxidation
1
2
Electrons transport chain activity
2
3
ROS generation
3
4
GPx
GSH
5

## DISCUSSÃO E CONCLUSÕES

Os efeitos benéficos da RC estão bem estabelecidos na literatura, entretanto os mecanismos de ação pelos quais a dieta proporciona o aumento da expectativa de vida, ainda não estão bem elucidados. Este trabalho contribui para a elucidação de alguns desses efeitos, especialmente no SNC.

Os resultados obtidos com a avaliação nutricional dos animais demonstraram que o modelo animal de restrição calórica alcançou o objetivo estabelecido, ou seja, limitou a ingestão de calorias, mas não causou desnutrição. Isso fica bem evidente na análise bioquímica sanguínea desses animais, onde os parâmetros avaliados, principalmente proteínas totais, não diferiram do grupo controle, indicando, sobretudo um bom estado de saúde desses animais. Além disso, os dados da evolução ponderal estão de acordo com a literatura, indicando uma evolução semelhante à do grupo controle, porém com um ganho de peso menor (16 a 17%), o que se explica pela limitada ingestão de calorias. Ainda no que tange às análises bioquímicas, os dados de corticosterona mostraram que os animais submetidos à RC não apresentam elevação deste glicocorticóide quando comparados aos controles, o que se encontraria se estes animais estivessem sido submetido a algum tipo de estresse. Com isso podemos sugerir que a RC por si só, apesar de apresentar um período de respostas metabólicas adaptativas, não se apresentou como agente estressor.

Já está bem descrito que a RC confere efeitos benéficos integrados para a saúde do SNC, como aumento de fatores neurotróficos (Maswood et al., 2004), chaperonas e proteínas anti-apoptóticas em diferentes regiões do cérebro

(Mattson, 2003, 2008). Muitas evidências sugerem que a RC pode conferir proteção contra perda neuronal relacionada com a idade e também contra doenças neurodegenerativas (Gillette-Guyonnet & Vellas, 2008). Entretanto, pouco se sabe sobre a influência da RC nas células astrogliais, especialmente no que diz respeito ao metabolismo do glutamato. Os astrócitos estão intimamente ligados com a transmissão glutamatérgica e, dessa forma, com a plasticidade sináptica e a neuroproteção (Chen & Swanson, 2003; Danbolt, 2001). Este trabalho demonstrou que a RC é capaz de modular importantes funções gliais de ratos tratados com RC.

O ensaio de captação de glutamato demonstrou um aumento de 20% (estatisticamente significativo pelo Teste *t* de Student), na captação de glutamato pelas células neurais hipocampais, provenientes dos animais que foram submetidos à RC. Este dado sugere uma modulação na atividade e/ou no número de transportadores de glutamato. A concentração de glutamato aumenta dramaticamente durante a transmissão (de ~100 μM para 1 mM), e qualquer falha dos astrócitos na remoção desse excesso ocasiona dano excitotóxico e consequentemente morte neuronal (Danbolt, 2001). A remoção eficiente do glutamato secretado na fenda sináptica aos níveis de repouso após a neurotransmissão é uma das mais importantes funções do astrócito no SNC (McKenna, 2007). Portanto, este resultado indica um efeito benéfico da dieta para este parâmetro, sugerindo que estes animais possam estar mais preparados para se proteger da excitotoxicidade do glutamato, aumentando a recaptação glutamatérgica glial.

Os astrócitos são os únicos tipos celulares no SNC que possuem a importante habilidade de converter glutamato em glutamina através da enzima GS. A glutamina, por sua vez, é captada pelos neurônios e utilizada para síntese de glutamato, estabelecendo desta forma um ciclo (Bak, Schousboe, & Waagepetersen, 2006; McKenna, 2007). Em conformidade com o aumento da captação de glutamato nos animais submetidos à RC, este estudo apresentou também um aumento (26%) na atividade da GS, indicando que a dieta beneficia a eficiência do ciclo glutamato-glutamina.

A proteína S100B é secretada pelos astrócitos e também pelo tecido adiposo. Ela é secretada pelos astrócitos em resposta a injúrias. A secreção em baixas concentrações pode exercer efeito neurotrófico, promovendo crescimento de neuritos, aumentando a sobrevivência de neurônios durante o desenvolvimento e em situações de injúria ao SNC e protegendo neurônios contra a excitotoxicidade do glutamato (F. Tramontina et al., 2006). O contrário ocorre quando a secreção acontece em elevadas concentrações, ou seja, ela se torna neurotóxica (Van Eldik & Wainwright, 2003). A proteína ácida fibrilar glial (GFAP) é uma proteína marcadora de astrócitos. Os dados desse trabalho, como se esperava, não apresentaram diferenças no imunoconteúdo de S100B e de GFAP entre os grupos, não alterando esses parâmetros gliais.

As tarefas comportamentais realizadas neste estudo mostraram que os animais alimentados com a RC apresentaram um aumento significativo nos níveis gerais de atividade locomotora e hábitos de exploração na tarefa do campo aberto, provavelmente relacionado com a busca pelo alimento. Já na tarefa que avalia ansiedade, o labirinto em cruz elevado, não houve diferença

entre os grupos, nos permitindo inferir que estes animais não estão ansiosos em função da RC.

A glutationa exerce um papel fundamental na manutenção do balanço fisiológico entre fatores pró-oxidantes e antioxidantes, que são essenciais para a vida e também para a morte de uma célula (Bilska, Kryczyk, & Wlodek, 2007). A GSH é encontrada no organismo em diversas formas redox, dentre elas a sua forma reduzida. No SNC, é caracterizada como a principal defesa antioxidante do astrócito (Droge, 2002). Deficiências de GSH ou da relação GSH/GSSG indicam uma suscetibilidade ao estresse oxidativo, e os danos resultantes estão associados a patologias como câncer e doenças de Parkinson e de Alzheimer. Além disso, alterações nos níveis de GSH afetam o sistema imune, e apresenta papel importante no processo de envelhecimento. Portanto, níveis baixos de GSH, diminuem a capacidade antioxidante celular e a resistência ao estresse oxidativo, e níveis elevados aumentam a capacidade antioxidante celular bem como a resistência ao estresse oxidativo. Este estudo mostrou que os conteúdos de GSH do córtex cerebral e do hipocampo foram significativamente maiores nos ratos alimentados com RC. Estes resultados indicam evidências de um mecanismo protetor da RC em função de um aumento de um fator antioxidante não-enzimático para o SNC.

Os valores basais obtidos nos ensaios da atividade da CAT, produção de NO, e dos níveis de lipoperoxidação, não demonstraram diferenças significativas entre os grupos. Entretanto, os animais submetidos à RC apresentaram diminuição significativa na produção de radicais livres, de 26% em hipocampo e 14% em córtex cerebral, quando comparados ao grupo

controle. Altos níveis de ERO podem induzir danos celulares em proteínas lipídeos e DNA. O hidrogênio peróxido, embora não seja um radical livre, pode gerar o radical hidroxil, e outros radicais, ampliando o dano oxidativo (Halliwell, 2006). Neste contexto, uma diminuição na produção basal de ERO pode ser uma estratégia importante para a manutenção da saúde do SNC. Com a limitação da ingestão calórica, ocorre também uma redução da atividade metabólica e mitocondrial (Heydari, Unnikrishnan, Lucente, & Richardson, 2007) isso sustenta a diminuição da produção de radicais livres encontrada. Com menos radicais livres, há também uma diminuição dos insultos oxidativos, com isso se explica a normalidade nos níveis de CAT, NO e TBARS.

A GPx é uma enzima antioxidante capaz de eliminar o radical peróxido, reduzindo-o a $H_2O$ ou álcool, através da redução da GSH como substrato (Dringen, 2000). A RC foi capaz de reduzir significativamente (cerca de 18%) a atividade da GPx tanto em hipocampo quanto em córtex cerebral. Essa modulação negativa pode ser explicada pela redução da produção de ERO induzida pela RC, e justifica também o aumento dos níveis de GSH.

Nossos resultados mostram ainda que o grupo submetido à RC apresentou 30% de redução do dano oxidativo ao DNA no hipocampo, quando comparado aos controles. Estes dados estão de acordo com trabalhos publicados recentemente, que mostram que a RC é capaz de reverter alterações como dano e mutações ao DNA, relacionadas com a idade (Heydari et al., 2007). Acredita-se que a proteção ao DNA seja um elemento chave na prevenção de diversas patologias, dentre elas o câncer e as doenças neurodegenerativas,

retardando ou prevenindo desta maneira o desenvolvimento do fenótipo do envelhecimento.

## CONCLUSÕES FINAIS

Dito isto, este trabalho indica que a RC modula parâmetros das células gliais (astrócitos), aumentando a captação de glutamato e a atividade da GS, sugerindo que a RC pode exercer efeitos neuroprotetores contra patologias neurais, através de mecanismos que envolvam modulação de funções astrocíticas. Além disso, a RC é capaz de modular estruturas como córtex cerebral e hipocampo, resultando em alterações metabólicas capazes de melhorar parâmetros basais de importantes defesas celulares como aumento de GSH, diminuição da produção de ERO e do dano oxidativo ao DNA. Através destes resultados podemos inferir que a RC permite a manutenção da estabilidade metabólica e fisiológica durante o processo de envelhecimento, sendo assim um fator determinante para a longevidade e funcionalidade do SNC.

## PERSPECTIVAS

Entretanto, mais estudos são necessários para o esclarecimento dos efeitos de longevidade obtidos com a administração da RC, avaliando possíveis efeitos neuroprotetores resultantes da administração desta dieta em animais expostos a modelo de estresse crônico, analisando parâmetros comportamentais e neurogênese. Especificamente:

- Avaliar neurogênese na zona subgranular (SGZ) do giro denteado do hipocampo e na zona subventricular (SVZ).
- Avaliar parâmetros comportamentais relacionados com aprendizado e memória (labirinto aquático de Morris), ansiedade (labirinto em cruz elevado), atividade locomotora (habituação em campo aberto) e depressão (teste do nado forçado).

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