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A RESTRIÇÃO CALÓRICA INTRAUTERINA ALTERA O STATUS ANTIOXIDANTE EM ENCÉFALO DE RATOS

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Trabalho de conclusão de curso de graduação apresentado como requisito parcial para obtenção de grau de bacharel em Nutrição, à Universidade Federal do Rio Grande do Sul, Faculdade de Medicina, Curso de Graduação em Nutrição

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A comissão Examinadora, abaixo assinada, aprova o Trabalho de Conclusão de Curso "**A RESTRIÇÃO CALÓRICA INTRAUTERINA ALTERA O STATUS ANTIOXIDANTE EM ENCÉFALO DE RATOS**", elaborado por Vinícius Stone Silva, como requisito parcial para obtenção do grau de Bacharel em Nutrição.

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“Si os dan papel pautado, escribid por el otro lado.” – Juan Ramón Jiménez

RESUMO

A restrição calórica é considerada um fator de intervenção ideal na promoção da saúde. Diversos autores demonstraram sua capacidade de aumentar a expectativa de vida e proteger diversos organismos de uma série de doenças, inclusive as que estão relacionadas com o aumento do estresse oxidativo. Intervenções no período gestacional induzem uma reprogramação metabólica nos descendentes, podendo alterar o risco de desenvolver doenças crônicas na vida adulta. Nosso objetivo nesse trabalho foi avaliar os efeitos da restrição calórica materna de 40% durante o período gestacional sobre a produção de espécies reativas, modulação das defesas antioxidantes enzimáticas e não enzimáticas e parâmetros de dano oxidativo no cerebelo e córtex cerebral total de ratas mães e seus filhotes. Uma diminuição na concentração de espécies reativas no cerebelo de mães e filhotes foi encontrada, bem como, uma diminuição nos níveis de superóxido mitocondrial no cerebelo dos filhotes. Por outro lado, o córtex cerebral dos filhotes apresentou uma redução nos níveis de superóxido mitocondrial e um aumento na concentração de óxido nítrico. Foi encontrada uma diminuição da capacidade antioxidante enzimática e não enzimática dos filhotes, onde a superóxido-dismutase apresentou atividade aumentada; enquanto a catalase, a glutationa-peroxidase e a glutarredoxina foram negativamente afetadas. As ratas prenhes também apresentaram diminuição da capacidade antioxidante enzimática, entretanto, de maneira mais sutil. As defesas antioxidantes não enzimáticas não sofreram alterações no córtex das mães, enquanto que no cerebelo, demonstraram um padrão adaptativo. Apesar disso, parâmetros de dano oxidativo não sofreram alterações. Nossos resultados são um ponto de partida para esclarecer o impacto da má nutrição na vida intrauterina sobre parâmetros de estresse oxidativo. Acreditamos que a restrição calórica durante a gestação aumenta a vulnerabilidade de ratas prenhes e seus filhotes a futuros danos oxidativos. Entretanto, mais estudos são necessários para explicar por quais mecanismos a restrição calórica afeta as defesas antioxidantes.

ABSTRACT

Caloric restriction has been considered the cornerstone of health, considering its capacity of increasing life span and protecting distinct organisms against a series of diseases, among which, those related to oxidative stress. Interferences in the maternal environment are known to reprogram the offspring metabolism response, impacting in the risk of chronic diseases development in adulthood. Our aim was to assess the effect of 40% caloric restriction on reactive species levels, enzymatic and non-enzymatic antioxidant defenses, besides the oxidative damage parameters in the cerebellum and the total cerebral cortex of pregnant rats and their offspring. Both dams and pups showed an intense oxidative modulation caused by caloric restriction in the cerebellum and cerebral cortex. Dichlorofluorescein oxidation was reduced in the cerebellum of calorie restricted dams and their offspring, while the cerebral cortex was not affected. Decreased mitochondrial superoxide levels were found in the cerebellum and cerebral cortex of pups, while nitric oxide was increased in cortex. Considering that reactive oxygen species were probably altered in brain of diet restricted rats, we measured the activities of the most important antioxidant enzymes responsible by its elimination. In a comprehensive way, superoxide dismutase activity was increased in the cerebellum of dams and in both structures of pups, while it was decreased on dams' cerebral cortex. Both brain structures were strongly affected concerning to catalase, glutathione peroxidase, and glutaredoxin activities, which were decreased in pups and their mothers. Furthermore, non-enzymatic defenses were significantly decreased in pups, despite the fact that dams showed an adaptive pattern in the cerebellum and no alteration in the cerebral cortex. Even though the results suggest increased oxidative status, lipids and proteins were not oxidatively affected. Our data clarify, at least in oxidative aspects, the effects of poor nutrition on brain metabolism in critical periods of life: fetal development and pregnancy. In view of our results, we believe that caloric restriction during pregnancy increases the susceptibility of dams and their offspring to a future oxidative aggression.

LISTA DE FIGURAS:

LISTA DE ABREVIATURAS

CAT - Catalase

ERN – Espécie reativa de nitrogênio

ERO – Espécie reativa de oxigênio

GPx – Glutationa-peroxidase

Grx – Glutarredoxina

GSH – Glutationa reduzida

GSSG – Glutationa oxidada

NO – óxido nítrico

NOS – óxido-nítrico-sintase

Nrf2 – Fator de transcrição nuclear eritroide 2 p45-relacionado

PGC1-α – Coativador-1 α do receptor ativador da proliferação de peroxissomos

SIRT1 – Sirtuína 1

SNC – Sistema Nervoso Central

SOD – Superóxido-dismutase

Sumário

1. REVISÃO DA LITERATURA

1.1. Introdução

A exposição à fome na vida intrauterina seguida de plenitude na vida pós-natal gera uma adaptação metabólica inapropriada aos indivíduos, com reflexos na saúde na vida adulta [1]. Segundo estudos realizados após a invasão alemã na Holanda em 1944, grávidas expostas à desnutrição, com uma dieta de 400 a 800 calorias diárias deram a luz a indivíduos que tiveram uma dieta com aporte calórico pleno após a invasão, e esses indivíduos apresentaram maior prevalência de obesidade e doenças cardiovasculares na vida adulta [2]. Em indivíduos que tiveram a mesma privação calórica na vida intrauterina, porém, não tiveram plenitude após o nascimento, como ocorreu no cerco a Leningrado em 1941, as mesmas complicações não foram observadas [3].

Diversos aspectos nutricionais estão relacionados à prevenção ou desencadeamento de doenças que contém em sua fisiopatogenia o estresse oxidativo [4]. O interesse em encontrar estratégias para diminuir o estresse oxidativo e melhorar o estado redox se dá pelo fato de que com a indução do estresse oxidativo, o indivíduo está sujeito a uma série de complicações relacionadas à oxidação de lipídeos de membranas, proteínas e DNA [5]. O interesse particular pelo sistema nervoso central (SNC), nesse estudo, se dá pelo fato de que esse órgão apresenta um grande consumo de oxigênio, que está relacionado à formação de espécies reativas e o alto conteúdo lipídico, o qual é um alvo das mesmas, bem como uma quantidade relativamente baixa de enzimas antioxidantes, tornando-o um tecido vulnerável ao estresse oxidativo [6, 7].

1.2. Estresse oxidativo

1.2.1 Definições e conceitos básicos

Além da clássica definição de estresse oxidativo cunhada por Sies e Cadenas [8], definido como um desequilíbrio entre a produção de espécies reativas e as defesas antioxidantes [6, 7], uma revisão de literatura trouxe uma nova definição de estresse oxidativo, defendendo a hipótese de que o aumento do mesmo é definido pelo desequilíbrio na relação glutationa reduzida/glutationa oxidada (GSH/GSSG) [9].

Um equilíbrio entre a produção de espécies reativas e as defesas antioxidantes é essencial para a manutenção da homeostase [10]. O papel dos antioxidantes não é eliminar por completo as espécies reativas, mas mantê-las em uma concentração na qual possam exercer suas funções fisiológicas sem promover dano tecidual excessivo [7]. O excesso de espécies reativas está associado à oxidação e consequente disfunção proteica, peroxidação lipídica e dano oxidativo ao DNA [7]. O resultado do aumento de espécies reativas pode levar ao dano celular extenso e irreversível; ou à adaptação celular, quando os níveis de espécies reativas forem moderados, induzindo ativação de vias de sinalização que controlam a expressão de enzimas antioxidantes e de reparo ao DNA [7, 11-13].

1.2.2 Espécies reativas e defesas antioxidantes

O termo espécie reativa engloba diversas moléculas reativas, radicais livres ou não, provenientes de oxigênio, nitrogênio, cloro e bromo [7]. Neste trabalho,

o foco serão as espécies reativas de oxigênio (ERO) e nitrogênio (ERN), cujo metabolismo pode ser observado na figura 1.

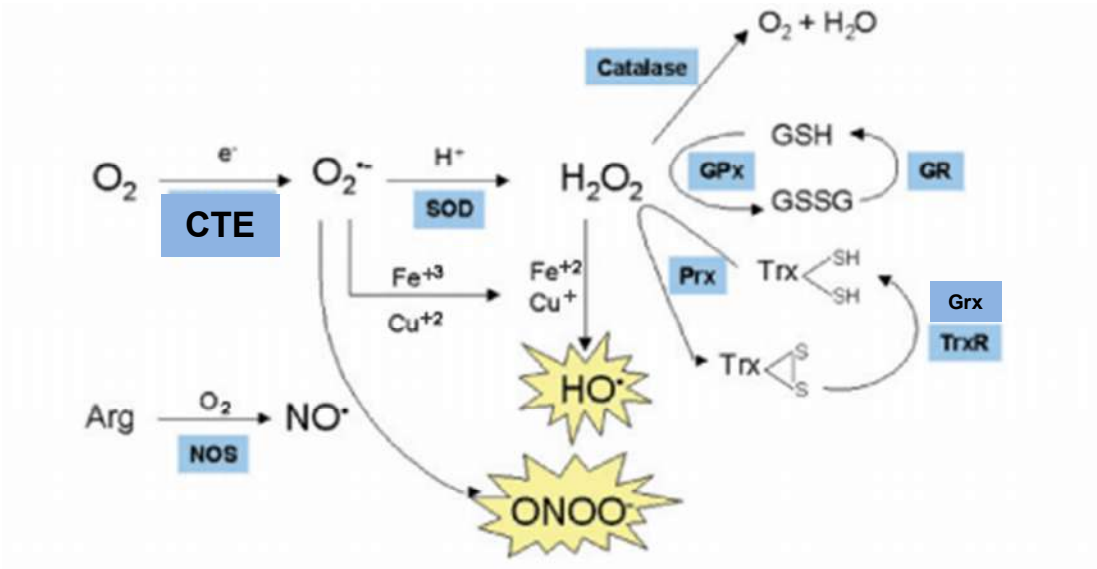

Figura 1. Esquema das reações e respectivas enzimas envolvidas na produção de espécies reativas de oxigênio e nitrogênio. NOS: óxido-nítrico-sintase; SOD: superóxido-dismutase; GPx: glutationa-peroxidase; Prx: peroxirredoxinas; GR: glutationa-redutase; Grx: glutarredoxina; Trx: tiorredoxina; GSH: glutationa reduzida; GSSG: glutationa oxidada. Adaptado de Lívea Fujita Barbosa*, Marisa H.G. de Medeiros e Ohara Augusto, *Quim. Nova,* Vol. 29, No. 6, 1352-1360, 2006.

Dentre as ERN, o óxido nítrico (NO) é a mais relevante, exercendo uma ampla gama de papeis no metabolismo fisiológico e patológico, tais como de vasodilatador, neurotransmissor e modulador inflamatório [14, 15]. O NO é sintetizado pela óxido-nítrico-sintase (NOS), em uma reação que converte

arginina e oxigênio em citrulina e NO. Essa enzima possui diferentes isoformas, neuronal, induzível e endotelial, com regulações e localização tecidual diferenciadas [16]. A característica química desse gás permite que ele atravesse facilmente a membrana celular e possa atuar via ativação da enzima guanilato-ciclase em células adjacentes à produzida, bem como reagir com outras espécies reativas, tais como superóxido, gerando o radical peroxinitrito [7, 15].

Embora o oxigênio seja essencial para a vida, ele também é um gás potencialmente tóxico, já que seu metabolismo envolve a produção de diversas espécies reativas, que podem alterar a estrutura química de biomoléculas [7]. A formação de superóxido se dá pela redução monoeletrônica da molécula de oxigênio, o que pode ocorrer na cadeia respiratória mitocondrial, em reações catalisadas por xantina-oxidase, NADPH-oxidase e NOS desacoplada, bem como pela auto-oxidação de algumas moléculas de baixo peso molecular, tais como dopamina e homocisteína. O radical superóxido pode reagir como outras espécies reativas, tais como o NO, produzindo peroxinitrito, ou ser detoxificado pela enzima superóxido-dismutase (SOD), presente na matriz mitocondrial na isoforma MnSOD, e no espaço intermembranas e citosol como CuZnSOD [17], produzindo peróxido de hidrogênio, uma ERO não radicalar. A eliminação do peróxido de hidrogênio se dá pelas enzimas catalase (CAT), encontrada em altas concentrações no peroxissomo, glutationa-peroxidase (GPx), a qual apresenta quatro isoformas diferentemente localizadas em mamíferos, todas dependentes de selênio como cofator [18]. Ainda agem sobre o peróxido de hidrogênio e peróxidos orgânicos, as peroxirredoxinas, sendo seis isoformas distribuídas na célula. Essas enzimas não contêm grupos prostéticos, sendo as

reações de oxidorredução mediadas por uma ou duas cisteínas no centro ativo. Além dessas, as células ainda contam com as tiorredoxinas, que eliminam peróxidos à custa de NADPH na mitocôndria (tiorredoxina 2) e citosol (tiorredoxina 1), utilizado na sua regeneração mediada pela tiorredoxina-redutase, outra enzima dependente de selênio [7]. A ação coordenada dessas enzimas elimina o peróxido de hidrogênio, evitando que o mesmo reaja com íon ferroso, por meio da reação de Fenton, e origine o radical hidroxil, a mais danosa das espécies reativas. As proteínas glutarredoxinas (Grx), também chamadas de tioltransferases, regeneram grupos tiois oxidados, utilizando GSH como substrato, sendo encontradas na mitocôndria e citosol. A GSH, além de ser substrato das enzimas antioxidantes GPx e Grx, também atua como varredora de radicais livres, assim como as vitaminas C e E, entre outras moléculas antioxidantes de baixo peso molecular [6, 7, 10, 17].

1.3. Restrição calórica

A restrição calórica é um fator de simples aplicação quando utilizado como tratamento, e também, uma realidade frequente em populações de baixa renda, contribuindo para o interesse científico na mesma. Já foi demonstrado que a restrição calórica está associada à alteração em processos normais de envelhecimento em modelos animais adultos, por modular a maioria dos declínios associados ao envelhecimento [19]. Experimentos demonstram que a restrição calórica é capaz de frear o desenvolvimento de diversas doenças relacionadas à idade [20]. Dentre elas estão as doenças cardiovasculares [21], diabetes [22], vários tipos de câncer [23] e doenças neurodegenerativas [24, 25]

Diversos estudos demonstraram que a restrição calórica está associada à modulação do estado redox e das defesas antioxidantes [26-28]. Foi demonstrado que a restrição calórica em ratos adultos melhorou o estado redox em relação a animais alimentados com dieta *ad libitum*, aumentando as concentrações de GSH, e também diminuindo a produção de espécies reativas, ambos os parâmetros em córtex cerebral e hipocampo [29]. Resultados também relacionam uma diminuição de 30% no dano ao DNA em hipocampo de ratos adultos induzida pela restrição calórica [29]. Nesse contexto, tem sido sugerido na literatura que a intervenção dietética na forma de restrição calórica no período pós-desmame pode ser a melhor estratégia conhecida para retardar os efeitos degenerativos da idade e promover um aumento da expectativa de vida de mamíferos [30].

Apesar de a restrição calórica ter uma correlação positiva com a expectativa de vida, prevenindo alterações associadas ao processo de envelhecimento, a restrição calórica no período anterior ao desmame pode causar uma redução na expectativa de vida de roedores [31]. A intervenção nutricional na forma de restrição calórica em ratas prenhes é o alvo de estudo do presente trabalho. Nesse contexto, a restrição calórica de fêmeas de camundongo prenhes foi relacionada ao aumento no peso do cérebro dos filhotes [32], entretanto, outros autores haviam demonstrado resultados contrastantes [33]. Partadiredja e colaboradores [34] também evidenciaram um aumento nas defesas antioxidantes não-enzimáticas. Um dos poucos estudos que relaciona marcadores do estado redox e restrição calórica no período fetal e de amamentação sugere que os resultados são contraditórios e que mais parâmetros precisam ser pesquisados, como por exemplo, marcadores de

dano oxidativo, desde que, em seu estudo, foi evidenciada apenas a redução na expressão da enzima SOD, enquanto a CAT e a GPx não foram afetadas [32].

A restrição calórica parece ter um efeito hormético também sobre a biogênese mitocondrial em ratos adultos e há um forte debate no que diz respeito à função mitocondrial em relação a doenças causadas pela idade avançada [35]. Entretanto, não há correlação na literatura entre aumento ou diminuição da biogênese mitocondrial e restrição calórica na vida intrauterina. A sirtuína 1 (SIRT1), que por um mecanismo desconhecido, tem sua expressão aumentada durante a restrição calórica está relacionada com a promoção da biogênese mitocondrial, ativando o coativador-1 α do receptor ativador da proliferação de peroxissomos (PGC1-α) [36], que aumenta a massa mitocondrial e aumenta a expressão, inclusive, de enzimas antioxidantes [37], entretanto, não está descrito na literatura o papel da restrição calórica intrauterina sobre a expressão de SIRT1 e de PGC1-α. O fator de transcrição nuclear eritroide 2 p45-relacionado (Nrf2) é um regulador de resistência celular a elementos oxidantes, estando relacionado com a expressão basal e induzida de diversos elementos antioxidantes [13], entretanto, alterações na sua expressão em relação à restrição calórica não estão presentes na literatura.

Considerando-se os diversos resultados existentes na literatura em relação à restrição calórica, é notável o fato de que o período de aplicação da restrição calórica é de suma importância no que diz respeito ao aumento ou diminuição da progressão de diversas doenças, embora diversos trabalhos demonstrem uma correlação entre restrição calórica e marcadores do estado redox em diversos modelos animais adultos. Os mecanismos pelos quais a restrição

calórica pode acelerar o desenvolvimento de certas doenças quando aplicada na fase fetal e de amamentação, mas diminuir o desenvolvimento das mesmas quando aplicada na fase adulta ainda necessitam ser esclarecidos. Algumas hipóteses incluem adaptações metabólicas, alterações no ciclo celular, modulação apoptótica, biogênese mitocondrial, alteração do perfil neuroendócrino e/ou do estado redox [38-41]. Na literatura são frequentes os estudos que demonstram uma relação positiva entre restrição calórica e a melhora dos parâmetros do estado redox, envolvendo o aumento de expectativa de vida, como demonstrado nos estudos citados acima, entretanto, os resultados em relação a esta estratégia no período de gravidez e lactação ainda não são claros, motivando o presente estudo.

2. HIPÓTESE

A restrição calórica durante a gestação pode ter um efeito metabólico adaptativo em encéfalo de filhotes de ratas que receberam esta intervenção no período de prenhez.

3. OBJETIVOS

3.1. Objetivo geral

Avaliar o efeito da restrição calórica sobre parâmetros de estresse oxidativo no SNC de ratas submetidas à intervenção nutricional durante o período de prenhez e de seus filhotes.

3.2. Objetivos específicos

Avaliar o efeito da restrição calórica sobre a concentração de espécies reativas, a oxidação de lipídeos e proteínas, a atividade das enzimas antioxidantes (SOD, CAT, GPx e Grx) e os níveis de antioxidantes não enzimáticos em amostras encefálicas provenientes de ratas prenhes, após o parto, e seus filhotes submetidos à restrição calórica durante todo o período gestacional.

Realizar a comparação entre os parâmetros avaliados na prole e nas mães de ratos submetidos ao modelo de restrição calórica gestacional.

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5. ARTIGO ORIGINAL

Caloric restriction during pregnancy alters brain's antioxidant network in dams and their offspring

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Caloric restriction during pregnancy alters brain's antioxidant network in dams and their offspring

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Abstract

Caloric restriction has been considered the cornerstone of health, considering its capacity of increasing life span and protecting distinct organisms against a series of diseases, among which, those related to oxidative stress. Interferences in the maternal environment is known to reprogram the offspring metabolism response, impacting in the risk of chronic diseases development in adulthood. Our aim was to assess the effect of 40% caloric restriction on reactive species levels, enzymatic and non-enzymatic antioxidant defenses, besides the oxidative damage parameters in the cerebellum and the total cerebral cortex of pregnant rats and their offspring. Both dams and pups showed an intense oxidative modulation caused by caloric restriction in the cerebellum and cerebral cortex. Dichlorofluorescein oxidation was reduced in the cerebellum of calorie restricted dams and their offspring, while the cerebral cortex was not affected. Decreased mitochondrial superoxide levels were found in the cerebellum and cerebral cortex of pups, while nitric oxide was increased in cortex. Considering that reactive oxygen species were probably altered in brain of diet restricted rats, we measured the activities of the most important antioxidant enzymes responsible by its elimination. In a comprehensive way, superoxide dismutase activity was increased in the cerebellum of dams and in both structures of pups, while it was decreased on dams' cerebral cortex. Both brain structures were strongly affected concerning to catalase, glutathione peroxidase, and glutaredoxin activities, which were decreased in pups and their mothers. Furthermore, non-enzymatic defenses were significantly decreased in pups, despite the fact that dams showed an adaptive pattern in the cerebellum and no alteration in the cerebral cortex. Even though the results suggest

increased oxidative status, lipids and proteins were not oxidatively affected. Our data clarify, at least in oxidative aspects, the effects of poor nutrition on brain metabolism in critical periods of life: fetal development and pregnancy. In view of our results, we believe that caloric restriction during pregnancy increases the susceptibility of dams and their offspring to a future oxidative aggression.

Keywords: Calorie restriction; oxidative stress; antioxidant enzymes; intrauterine; brain; nutrition; metabolic programming.

List of abbreviations

ABAP – 2,2′-azo-bis (2-amidinopropane)

Akt – Protein kinase B

ATP – Adenosine triphosphate

CAT – Catalase

CNS – Central nervous system

CR – Caloric restriction

DAF-FM® - 4-amino-5-methylamino-2′,7′-difluorescein

DCFH – 2',7'-Dichlorofluorescein

DCFH-DA – DCFH diacetate

EDTA – Ethylenediaminetetraacetic acid

EGTA – Ethyleneglycoltetraacetic acid

FoxO – Forkhead transcription factors

GPx – Glutathione peroxidase

GR – Glutathione reductase

Grx – Glutaredoxin

GSH – Reduced glutathione

HED – Hydroxyethyl disulfide

HPLC – High-performance liquid chromatography

MDA – Malondialdehyde

NADPH – Nicotinamide adenine dinucleotide phosphate

PI3K – Phosphoinositide 3-kinase

PBS – Phosphate buffered saline

PGC1-α – Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PMSF – Phenylmethanesulfonyl fluoride

PND0 – Postnatal day 0

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

SIRT – Sirtuin

SOD – Superoxide dismutase

TBARS – Thiobarbituric acid reactive substances

TAR – Total antioxidant reactivity

TRAP – Total radical-trapping antioxidant potential

Introduction

Prenatal exposure to nutrient deprivation followed by plenitude in postnatal life promotes negative metabolic adaptations [1]. The interest in studying the effects of intrauterine caloric restriction (CR) started when the *Hongerwinter* phenomenon was described. In the German invasion to the Netherlands, caloric restricted women's children presented higher occurrence of obesity and cardiovascular diseases after plenitude in adult life [2], while in the Leningrad Siege in 1941, where women were caloric restricted but children did not have access to high caloric intake later, there was no occurrence of these complications in adulthood [3]. Environmental changes during the intrauterine life may affect the developing brain and reprogram an individual's epigenome [4]. When applied in the post weaning phase, insults may produce minor damaging [5]. When submitted to stressful conditions in the uterus, the response to stressors in the adult life may be inappropriate [6].

While CR is a frequent reality in poor populations, it is also applied as a treatment for a series of diseases, drawing attention from the scientific community. CR in adult life is widespread in the literature as a huge protective factor, playing an essential role in life span extension [7-9] and a series of age related diseases, among which, cardiovascular diseases [10], diabetes [11], cancer [12], and neurodegenerative diseases [13, 14]. It is also commonly associated to improved redox status and diminished DNA damage [15, 16]. CR was defined elsewhere as "the most robust way to extend lifespan in most model organisms studied so far, from yeast to primates" [17] and "the gold

standard of aging intervention" [18]. Although CR is considered the cornerstone of good nutrition and health, several authors alert for its negative aspects when applied in the preweaning phase (intra and extra uterine). Among the most common effects of early nutrient deprivation are late onset of obesity and associated diseases [19, 20]. The mechanisms involved thereon are still not clear and might be related to sirtuin (SIRT) expression [21] and impaired hypothalamic response to fed/fasting conditions [22].

Nutritional aspects have important impact on oxidative stress related diseases [23]. Oxidative status in the brain is extensively related to the effects of CR [24, 25]. Calorie restricted models are commonly associated to reduced oxidative stress hallmarks and there is massive data associating the positive effects of CR on extending life span and preventing and/or treating obesity-related comorbidities [26-30]. Notwithstanding, data from literature are sparse concerning to the oxidative effect of calorie restriction during pregnancy on mothers' and their litters' brain. To accomplish this aim, we evaluated reactive species levels, the enzymatic and non-enzymatic antioxidant network, as well as some parameters of protein and lipid damage in cerebellum and total cerebral cortex of dams and their offspring restricted in 40% of calories during pregnancy. Besides, we also compared the data from dams and pups in order to establish a correlation.

Material and Methods

Animals and Reagents

Eighty female adult Wistar rats (60 days-of-age, nulliparous) and forty male adult Wistar rats (90 days-of-age) were obtained from the Central Animal House of Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room. The animals were housed in the proportion of 2 females per male for mating. After conception diagnosis by the presence of sperm in vaginal smears, we housed three pregnant female rats per cage in order to start the treatment. The experiments were approved by local Ethics Commission (Comissão de Ética no Uso de Animais/Universidade Federal do Rio Grande do Sul) under the number 25447, and followed national animal rights regulation (Law 11.794/2008) and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised 1996). We further attest that all efforts were made to minimize the number of animals used and their suffering.

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

Caloric Restriction Protocol

Control rats had free access to water and a 20% (w/w) protein commercial chow. The CR group also had free access to water and received the same commercial chow; however, the amount was reduced by 40% [31]. The diet for CR group was adjusted daily by body weight, using the chow

amount consumed by control animals as standard. The protocol was applied during the 21 days of gestation.

Sample Processing

The animals were divided into four groups: control dams (8 animals), CR dams (8 animals), control pups (64 animals), and CR pups (64 animals). In the delivery day, control and CR dams and their offspring (postnatal day 0 – PND0) were euthanized by decapitation without anesthesia, for the purpose of avoid chemical contamination of the sample that could alter the biochemical parameters evaluated. The pups were euthanized on PND0 to escape the influence of lactation. Brain was dissected; cerebellum and total cerebral cortex were rapidly isolated in a Petry plate on ice. The tissue was homogenized in 10 volumes (1:10, w/v) of phosphate buffered saline (PBS) pH 7.4, added 1 mM ethyleneglycoltetraacetic acid (EGTA) and 1 mM phenylmethanesulfonyl fluoride (PMSF). Homogenates were centrifuged at 1,000 x g for 10 min at 4ºC, to discard nuclei and cell debris. The pellet was discarded and the supernatant was taken to biochemical assays.

Particular interest on the central nervous system (CNS) is explained by its huge oxygen consumption, which is associated to increased reactive species production in the respiratory chain, allied to its high content of polyunsaturated lipids and iron, as well as reduced enzymatic antioxidant protection, implying in a more vulnerable target to oxidative stress [32, 33].

The cerebellum was chosen because of its importance in terms of motor regulation and learning, controlling the voluntary movements and body balance, while the cerebral cortex is responsible by decisions-making, planning, organization and personality.

Biochemical Assays

Dichlorofluorescein oxidation

The reactive species content was assessed through the 2',7'-dichlorofluorescein (DCFH) oxidation method [34]. Briefly, the biological sample was diluted in 40 volumes (1:40, v/v). Sixty µL of the diluted biologic sample was incubated at 37 °C, in the dark, for 30 min, with the addition of 240 µL of DCFH diacetate (DCFH-DA) in a 96-well plate. DCFH-DA is cleaved by cellular esterases and form DCFH, a non fluorescent compound, that is oxidized by reactive species present in the sample, producing a fluorescent compound, DCF. DCFH oxidation was measured fluorimetrically, using a 488 nm excitation and 525 nm emission wavelength. A standard curve, using standard DCF (0.25–10 mM), was performed in parallel with the samples, and the results were expressed as nmol/mg protein.

Flow cytometry assay

Mitochondrial superoxide was measured using the probe MitoSOX® red, and nitric oxide was measured using the probe 4-amino-5-methylamino-2′,7′-difluorescein (DAF-FM®), both purchased from Invitrogen®, in a FACScalibur flow cytometer (BD Biosciences®). The tissue samples (100 mg) were dissociated with 1 mL of PBS pH 7.4 containing 1 mg% of collagenase IV and 0.5 mg% of DNase, filtered and incubated with the probe. One hundred microliter of each sample was incubated at 37ºC during 20 min in the presence of MitoSox® red in a final concentration of 1 µM. One hundred microliter of each sample was incubated at 37ºC during 1 hour in the presence of DAF-FM® in the

final concentration of 10µM. After that, 10,000 cells were evaluated per sample in the flow cytometer. Data were analyzed using the software FlowJo®.

Antioxidant enzymes activity

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was evaluated by quantifying the inhibition superoxide-dependent autoxidation of epinephrine, verifying the absorbance of the samples at 480 nm [35]. Considering the protocol used in sample preparation, we measured total SOD activity, expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit. The data were calculated as units/mg protein.

Catalase (CAT) (EC 1.11.1.6) activity was assayed according to Aebi [36] by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, and 10 mM potassium phosphate buffer, pH 7.0. One CAT unit is defined as 1 µmol of hydrogen peroxide consumed per minute and the specific activity is reported as units/mg protein.

Glutathione peroxidase (GPx) (EC 1.11.1.9) activity was measured according to the method described by Wendel [37] using *tert*-butyl hydroperoxide as substrate. Nicotinamide adenine dinucleotide phosphate (NADPH) disappearance was monitored spectrophotometrically at 340 nm in a medium containing 2 mM reduced glutathione (GSH), 0.15 U/mL glutathione reductase (GR) (EC 1.8.1.7), 0.4 mM azide, 0.5 mM *tert*-butyl hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as 1 µmol of NADPH consumed per minute and the specific activity is represented as units/mg protein.

Glutaredoxin (Grx) (EC1.20.4.1) activity was measured according to the method described by Holmgren and Aslund [38] using hydroxyethyl disulfide (HED) as substrate. NADPH disappearance was monitored

spectrophotometrically at 340 nm in a medium containing 2.5 mM glutathione, 454 U/mL GR (EC 1.8.1.7), 2 mM HED and 5 mM NADPH. One Grx unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as units/mg protein.

Non-enzymatic antioxidants

Total Radical-trapping Antioxidant Potential (TRAP) and Total Antioxidant Reactivity (TAR) were determined by chemiluminescence

TRAP represents the total non-enzymatic antioxidant capacity of the tissue and was determined by measuring the luminol chemiluminescence intensity induced by 2,2′-azo-bis (2-amidinopropane) (ABAP) at room temperature [39]. Two hundred and forty microliters of 10 mM ABAP dissolved in 50 mM sodium phosphate buffer pH 8.6 plus 5.6 mM luminol were added to the microplate and the background chemiluminescence was measured. Ten microliters of 300 μM trolox or supernatant were added and chemiluminescence was measured until it reached the initial levels. The addition of trolox or sample to the incubation medium reduced the chemiluminescence. The time necessary to return to the levels presented before the addition was considered to be the induction time, which is directly proportional to the antioxidant capacity of the tissue and was compared to the induction time of trolox. The results are reported as nmol of trolox/mg of protein.

In the same assay, we measured TAR [40], which represents the quality of the tissue antioxidants. The chemiluminescence value was measured after 1 min after adding ABAP plus luminol. Ten microliters of trolox or brain supernatants, which decrease light intensity, were then added and chemiluminescence was measured after 60 s (final chemiluminescence). The

ratio between the initial and the final chemiluminescence values is used to calculate TAR measurement. TAR values were expressed as nmol trolox/mg of protein.

Reduced glutathione (GSH) concentration

GSH concentration was measured according to Browne and Armstrong [41], where GSH reacts with the fluorophore o-phthalaldehyde. The proteins in supernatant were initially precipitated with meta-phosphoric acid (1:1, v:v), centrifuged at 5.000 x g, for 10 min, at 25ºC. Fifty μL of supernatant was incubated with 15 μL of 7.5 mM o-phthaldialdehyde and 235 μL of 120 mM sodium phosphate buffer pH 8.0, containing 5 mM ethylenediaminetetraacetic acid (EDTA); at room temperature during 15 min. A blank sample was performed in parallel. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was prepared with standard GSH (0.001–1 mM) and the concentrations were calculated as nmol/mg protein.

Vitamin C concentration

Vitamin C levels were measured by High-performance liquid chromatography (HPLC) employing a reverse-phase column (SUPELCOSIL™LC-18-DBHPLC Column; 15 cm × 4.6 mm, 5 μm particle size), using a mobile phase flow rate of 1 mL/min in 30 mmol/L monobasic potassium phosphate (pH 3.6) and methanol (9:1, v/v). Samples were injected in a volume of 25 μL. The absorbance of the column effluent was monitored at 250 nm during a 10 min run time. Under these conditions, the

retention time of vitamin C was 3.0 min. Vitamin C was expressed as mg/g of protein [42].

Biomolecule oxidative parameters

Carbonyl levels

Protein carbonyl content, a marker of protein oxidative damage, was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370nm [43]. Briefly, 1 mg of sample protein was treated with 20% trichloroacetic acid, and centrifuged at 4,000 x g, 4ºC for 5 min. The pellet was dissolved in 0.2 M NaOH, and was added of 10 mM dinitrophenylhydrazine (prepared in 2M HCl). This was kept in the dark during 1h, and vortexed each 15 min. Samples were added of 20% thiobarbituric acid), and centrifuged at 20,000 x g, 4ºC for 5 min. The pellet was washed three times with ethanol:ethyl acetate (1:1, v/v). The supernatant was discarded and the pellet was re-suspended in 8M urea pH 2.3. The sample was vortexed and incubated at 60ºC for 15 min. After that, it was centrifuged at 20,000 x g for 3 min and the absorbance was measured at 370 nm. Protein carbonyl content was expressed as nmol/mg protein.

Thiobarbituric Acid Reactive Substances (TBARS) levels

The lipid peroxidation was assessed using the methodology described by Yagi [44], which measures the thiobarbituric acid reactive substances (TBARS) levels with slight adaptations. Briefly, 200 µL of 10% trichloroacetic acid and 300 µL of 0.67% thiobarbituric acid in 7.1% sodium sulfate were added to 150 µL of tissue supernatants containing 0.3 mg of protein and incubated for 2 h

in a boiling water bath. The mixture was allowed to cool on running tap water for 5 min. The resulting pink-stained complex was extracted with 400 µL of butanol. Fluorescence of the organic phase was read at 515 nm and 553 nm as excitation and emission wavelengths, respectively. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as supernatants. TBARS levels were calculated as nmol/mg protein.

Malondialdehyde (MDA) measure

Concentration of MDA, a product of lipid peroxidation, was measured by High-performance liquid chromatography (HPLC), in the same assay used to quantify vitamin C. Under these conditions, the retention time of MDA was 5.6 min. MDA levels was expressed as mg/g of protein [42].

Protein concentration assay

Protein concentration was measured by the method of Lowry et al. [45], using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by Student's *t* test, using GraphPad Prism 6.0 software. Dams and pups' results were correlated utilizing Pearson's correlation test. Data were considered statistically significant when $p<0.05$.

Results

CR was able to dramatically reduce weight gain in dams and pups, as well as pregnancy rate

The three-week treatment during pregnancy was able to decrease weight gain in CR dams after 8 days of gestation [$p<0.01$] (Figure 1), and litter weight [$t(22)=5.889$; $p<0.0001$] (Table 1). On the other hand, 40% of calorie restriction during pregnancy was not able to reduce litter size [$t(22)=0.5745$; $p=0.5715$]. Additionally, the pregnancy rate in CR dams was also diminished [$t(73)=4.829$; $p<0.0001$] (Table 1).

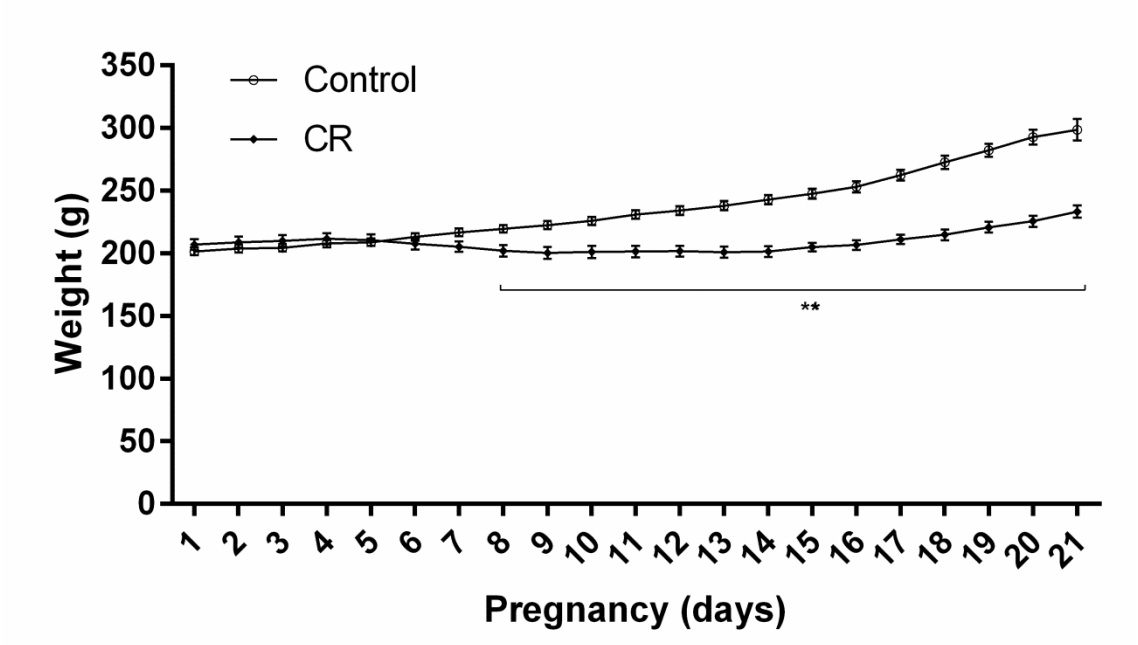

Fig. 1. Effect of caloric restriction (CR) in dam's weight gain during pregnancy. Results are expressed as mean ± S.E.M. for n=8-16. Different for control, **p<0.01 (Multiple *t* tests).

Table 1. Effect of intrauterine caloric restriction on litter's weight and size, and pregnancy rate

	Control	Caloric Restriction	p value
Litter weight (weight/pup)	6.00 ± 0.10	5.00 ± 0.15***	<0.0001
Litter size (number of pups)	9.50 ± 0.65	8.90 ± 0.83 .	0.5715
Pregnancy rate (%)	77.8 ± 11.1	16.7 ± 7.15***	<0.0001

Data was expressed as mean ± S.E.M (Student's *t* test) for n=8-16.

CR altered the reactive species detection in cerebellum of dams and pups

The DCFH oxidation technique encompasses a wide number of reactive oxygen/nitrogen species (ROS/RNS) for detection; meanwhile, it is more sensible to certain species, such as hydrogen peroxide (in combination with peroxidase, cytochrome c, or Fe^{2+}), peroxynitrite, and hydroxyl radicals. The flow cytometry assays performed are able to detect the levels of mitochondrial superoxide and nitric oxide using specific probes.

In the cerebellum of pups, mitochondrial superoxide levels were decreased [t(10)=9.449; p<0.0001)] (Figure 2b) and nitric oxide levels were not altered [t(9)=1.271; p=0.2357)] (Figure 2c). In the cerebral cortex, mitochondrial superoxide levels were decreased [t(18)=2.943; p=0.0087)] (Figure 3b), while nitric oxide levels were increased [t(10)=4.068; p=0.0023)] (Figure 3c).

The DCFH oxidation was decreased in the cerebellum of CR dams [t(14)=2.474; p=0.0268] (Figure 4a) and their offspring [t(10)=2.473; p=0.0329]

(Figure 2a). On the other hand, DCF oxidation in the cerebral cortex remained unaltered, both in CR dams [t(12)=1.007; p=0.3339] (Figure 4b) and pups [t(14)=0.9889; p=0.3395] (Figure 3a).

In order to establish a correlation between mothers and their offspring parameters, we performed Person's analyses. In the cerebellum, dams and pups showed a positive Pearson's correlation [r=0.6665; p=0.0179].

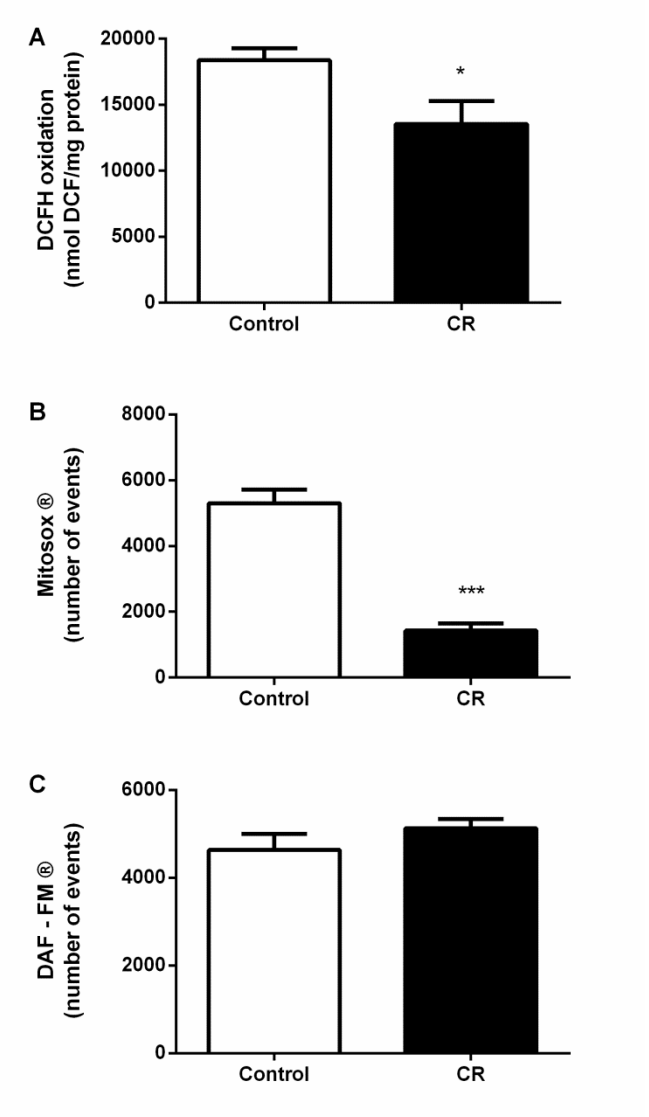

Fig. 2. Effect of intrauterine caloric restriction (CR) on 2'7'-dichlorofluorescein (DCFH) oxidation (A), mitochondrial superoxide (B), and nitric oxide (C) levels

in pups' cerebellum. Results are expressed as mean ± S.E.M. for n=4-8. *p<0.05, ***p<0.001 (Student's *t* test). DAF-FM: 4-amino-5-methylamino-2′,7′-difluorescein.

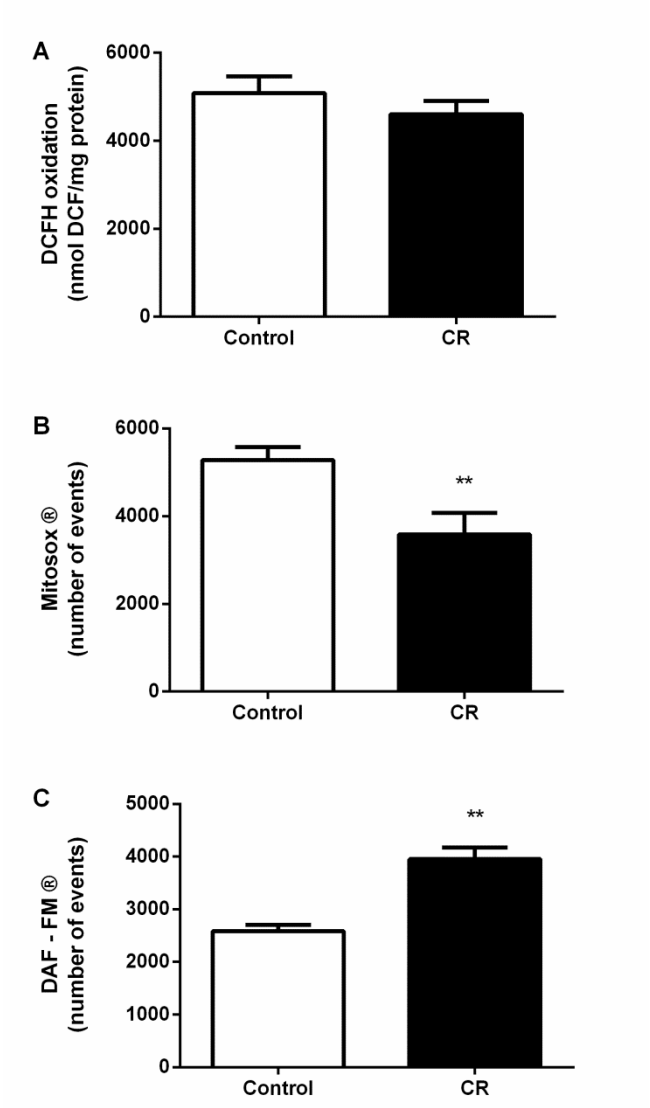

Fig. 3. Effect of intrauterine caloric restriction on 2'7'-dichlorofluorescein (DCFH) oxidation (A), mitochondrial superoxide (B), and nitric oxide (C) levels in pups' cerebral cortex. Results are expressed as mean ± S.E.M. for n=4-8. **p<0.01 (Student's *t* test). DAF-FM: 4-amino-5-methylamino-2′,7′-difluorescein.

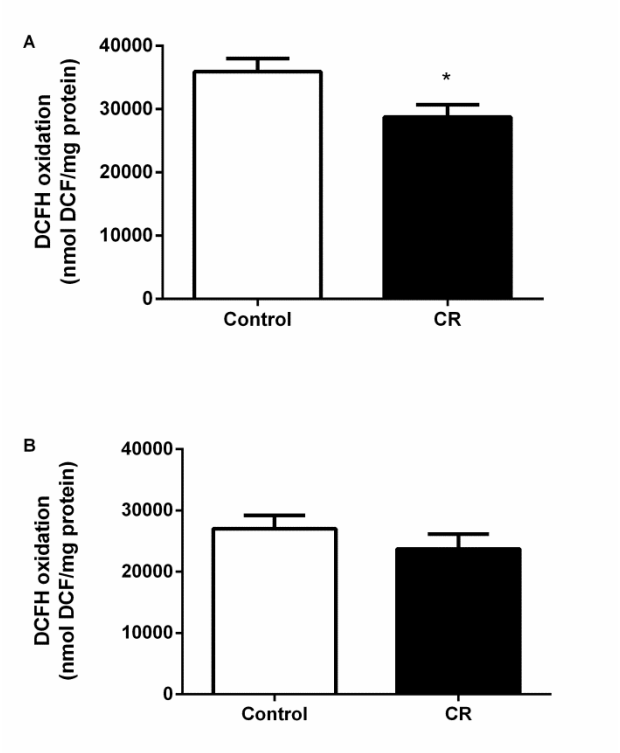

Fig. 4. Effect of caloric restriction during pregnancy on 2'7'-dichlorofluorescein (DCFH) oxidation in cerebellum (A) and cerebral cortex (B) of adult Wistar rats. Results are expressed as mean ± S.E.M. for n=6-8. *p<0.05 (Student's *t* test).

CR significantly affects the enzymatic antioxidant network in dams and pups

SOD, CAT, GPx, and Grx activities were measured in cerebellum and cerebral cortex of adult female rats exposed to nutrient deprivation during pregnancy and their offspring. Results showed that CR acted promoting alterations in all of the assessed enzymes, producing a notable unbalance in the antioxidant system. The CR pups showed increased SOD activity [t(13)=2.441; p=0,0297], allied to decreased activity of CAT [t(14)=4.174; p=0.0009], GPx [t(14)=2.165; p=0.0482], and Grx [t(9)=3.348; p=0.0085] both in the cerebellum (Figures 5a, 5b, 5c and 5d, respectively) and the cerebral cortex

[SOD: t(14)=2.231; p=0.0425, CAT: t(14)=2.181; p=0.0467, GPx: t(14)=2.565; p=0.0225], even though Grx alteration was not statistically significant in the cerebral cortex of pups [t(12)=1.427; p=0.1790] (Figures 6a 6b 6c and 6d, respectively)

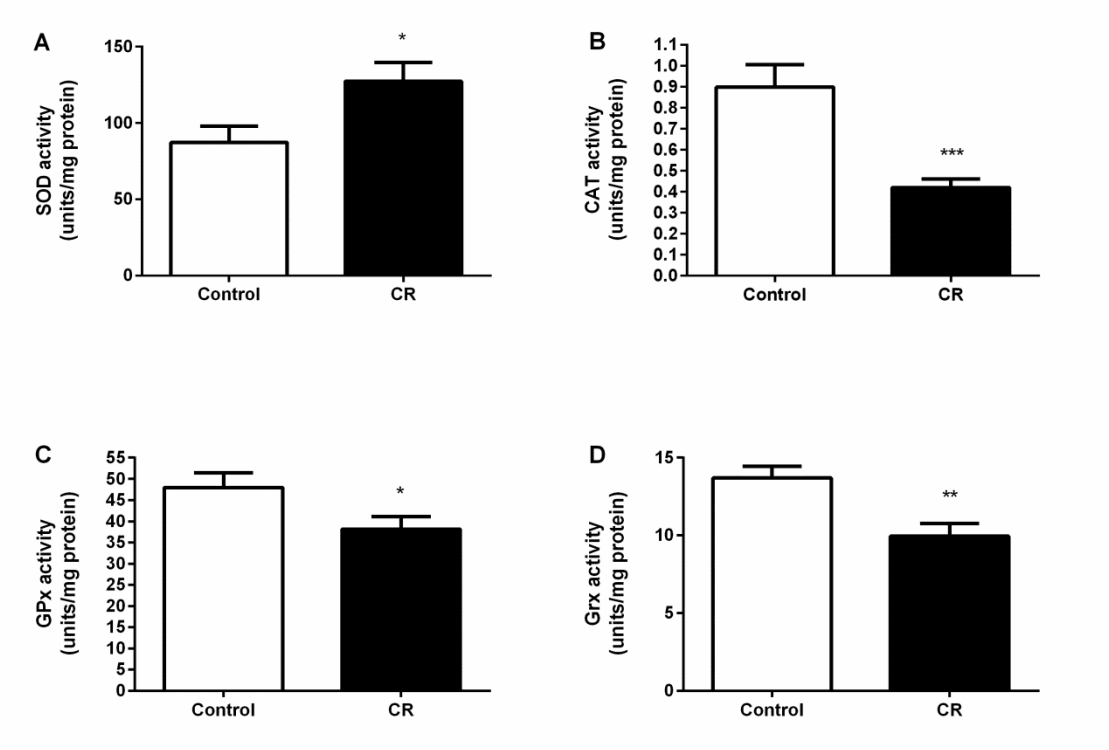

Fig. 5. Effect of intrauterine caloric restriction (CR) on superoxide dismutase (SOD) (A), catalase (CAT) (B), glutathione peroxidase (GPx) (C), and glutaredoxin (Grx) (D) activities in the cerebellum of pups. Results are expressed as mean ± S.E.M. for n=5-8. *p<0.05; **p<0.01; ***p<0.001 (Student's *t* test).

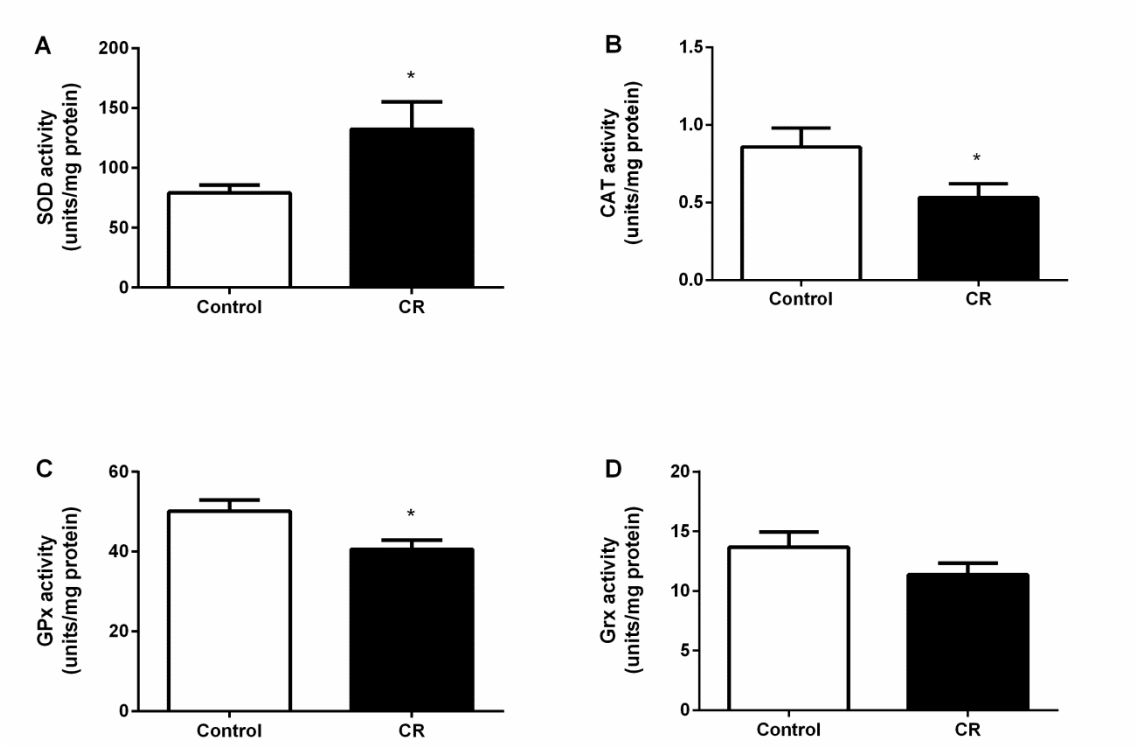

Fig. 6. Effect of intrauterine caloric restriction (CR) on superoxide dismutase (SOD) (A), catalase (CAT) (B), glutathione peroxidase (GPx) (C), and glutaredoxin (Grx) (D) activities in the cerebral cortex of pups. Results are expressed as mean ± S.E.M. for n=7-8. *p<0.05 (Student's *t* test).

Brain antioxidant network was affected in a similar way in dams. In the cerebellum of CR dams, SOD activity was increased [t(13)=2.265; p=0.0412], while CAT [t(14)=2.871; p=0.0123] and Grx [t(13)=2.302; p=0.0385] had their activities diminished (Figures 7a, 7b and 7d). GPx was not affected in dams cerebellum [t(14)=0.2078; p=0.8384] (Figure 7c). In the cerebral cortex of CR dams, SOD [t(11)=3.273; p=0.0074], CAT [t(11)=2.655; p=0.224], and Grx [t(10)=2.584; p=0.0272] had their activities decreased (Figures 8a, 8b and 8c) while GPx remained unaltered [t(12)=1.154; p=0.2711], similarly to cerebellum (Figure 8d).

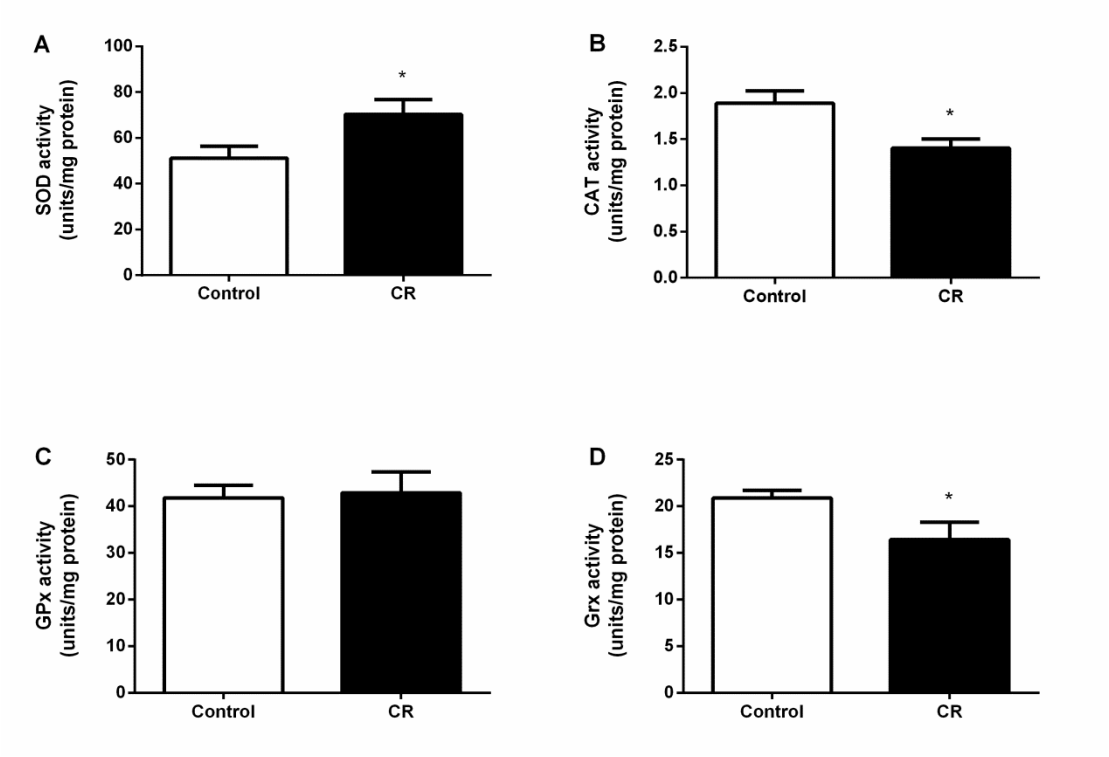

Fig. 7. Effect of caloric restriction (CR) during pregnancy on superoxide dismutase (SOD) (A), catalase (CAT) (B), glutathione peroxidase (GPx) (C), and glutaredoxin (Grx) (D) activities in the cerebellum of dams. Results are expressed as mean ± S.E.M. for n=7-8. *p<0.05 (Student's *t* test).

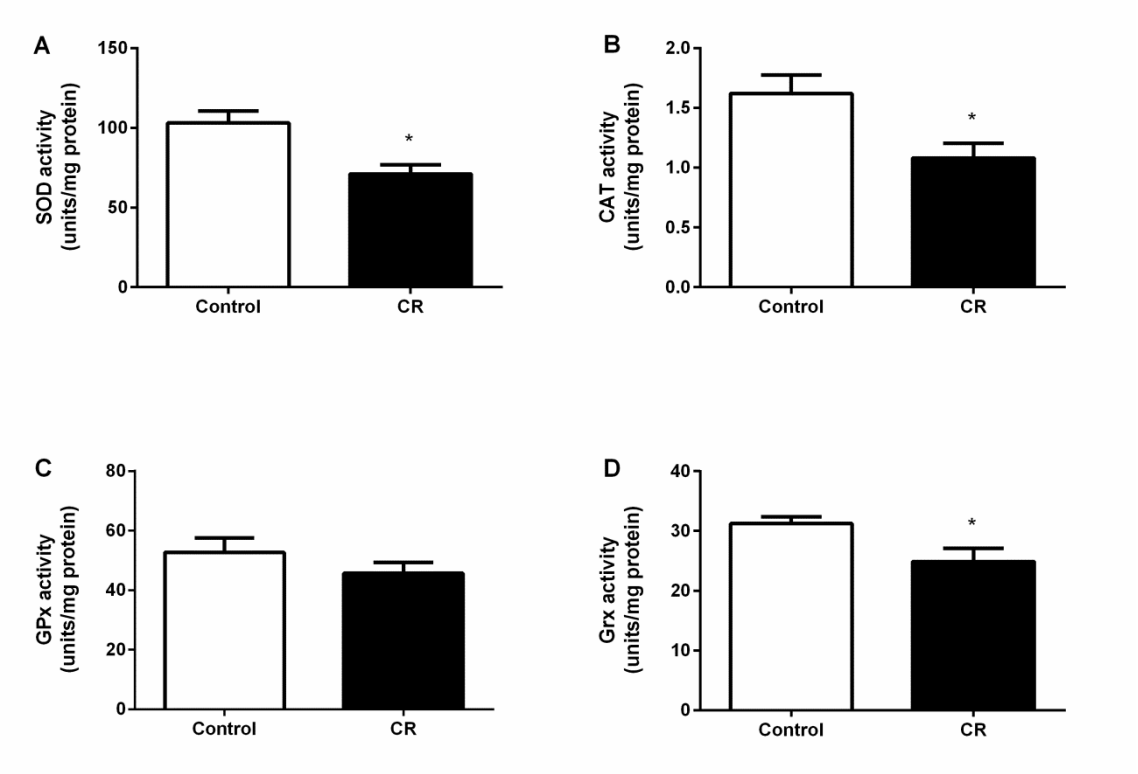

Fig. 8. Effect of caloric restriction (CR) during pregnancy on superoxide dismutase (SOD) (A), catalase (CAT) (B), glutathione peroxidase (GPx) (C), and glutaredoxin (Grx) (D) activities in the cerebral cortex of dams. Results are expressed as mean ± S.E.M. for n=6-7. *p<0.05 (Student's *t* test).

Person's analyses showed a positive correlation between mothers and their offspring, in cerebellar Grx activity [r=0.7856; p=0.0071].

Non-enzymatic antioxidant defenses presented differential modulation in CR pups and dams

CR pups possess decreased non-enzymatic defenses. TRAP [t(14)=2.459; p=0.0276], TAR [t(14)=2.925; p=0.0111], and GSH [t(13)=2.285; p=0.0398] were decreased in the cerebellum, while vitamin C presented a statistical tendency to diminish [t(10)=1.519; p=0.1598] (Figures 9a, 9b, 9c and 9d, respectively). In the cerebral cortex, TRAP was decreased [t(12)=1.851;

p=0.0890], while TAR [t(12)=1.606; p=0.1343] and vitamin C [t(10)=1.264; p=0.2349] tended to diminish (Figures 10a, 10b and 10d). GSH was not altered [t(14)=0.5757; p=0.5739] (Figure 10c). Conversely, in the cerebellum of CR dams, TRAP tended to increase, although Student's *t* test was not significant [t(14)=1.362; p=0.1948], TAR was increased [t(14)=2.218; p=0.0436], and GSH was not altered [t(13)=0.2350; p=0.8179] (Figures 11a, 11b and 11c). In the cerebral cortex of CR dams, none of the non-enzymatic antioxidant parameters evaluated was altered [TRAP: t(12)=0.02313; p=0.9819, TAR: t(12)=0.1078; p=0.9159, GSH: t(12)=0.05573; p=0.9565] (Figures 12a, 12b and 12c).

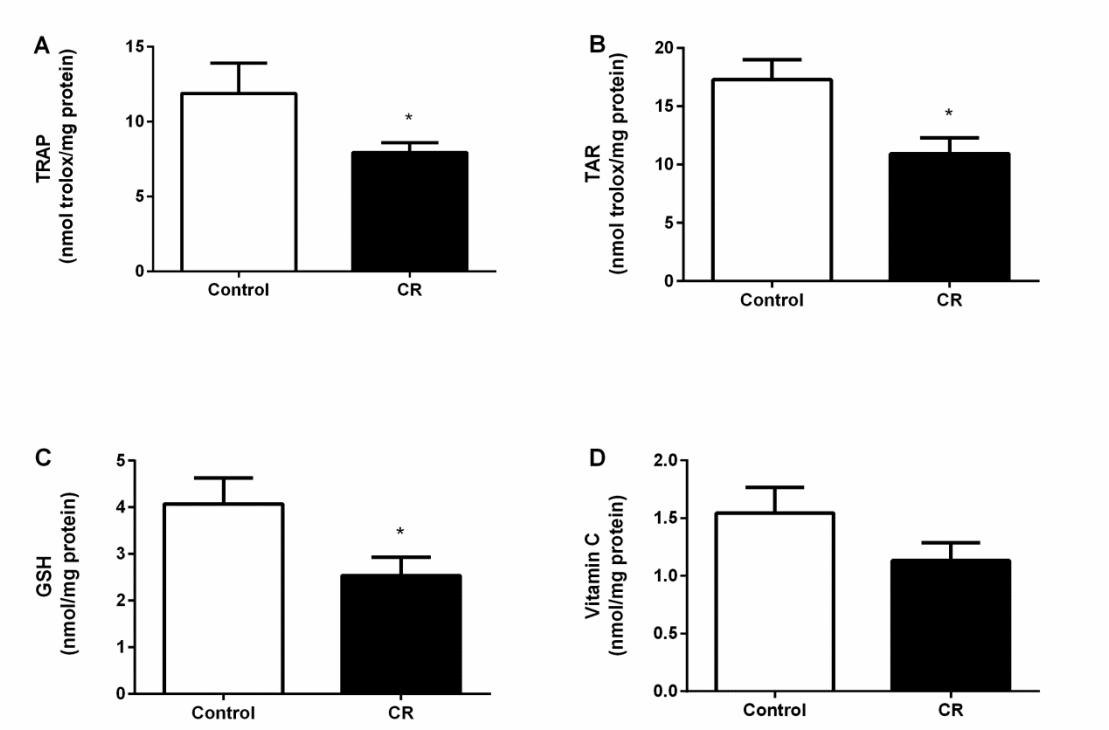

Fig. 9. Effect of intrauterine caloric restriction on total radical-trapping antioxidant potential (TRAP) (A), total antioxidant reactivity (TAR) (B), reduced glutathione (GSH) content (C), and vitamin C levels (D) in the cerebellum of pups. Results are expressed as mean ± S.E.M. for n=7-8. *p<0.05 (Student's *t* test).

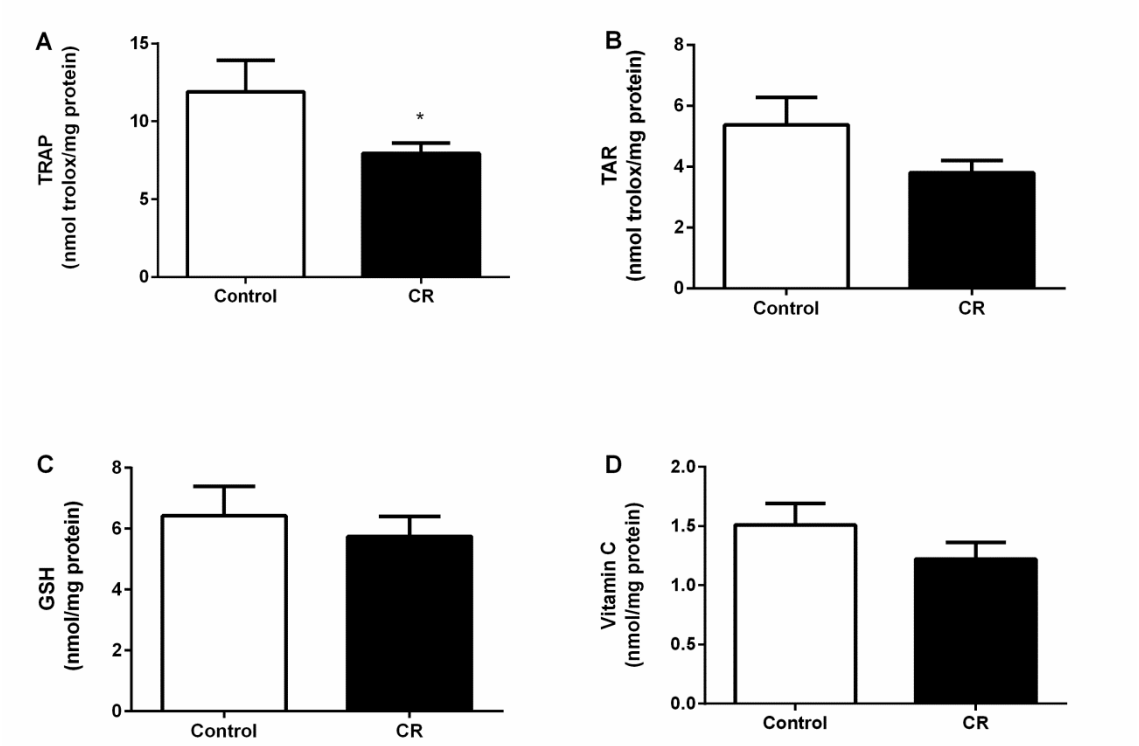

Fig. 10. Effect of intrauterine caloric restriction on total radical-trapping antioxidant potential (TRAP) (A), total antioxidant reactivity (TAR) (B), reduced glutathione (GSH) content (C), and vitamin C levels (D) in the cerebral cortex of pups. Results are expressed as mean ± S.E.M. for n=6-8. *p<0.05 (Student's *t* test).

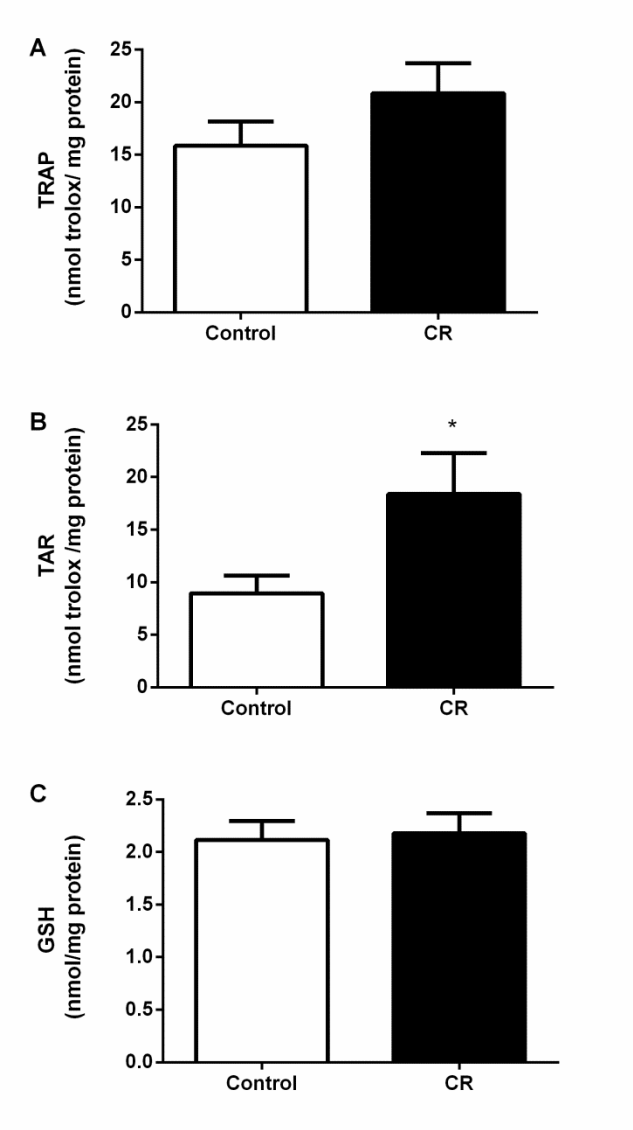

Fig. 11. Effect of caloric restriction during pregnancy on total radical-trapping antioxidant potential (TRAP) (A), total antioxidant reactivity (TAR) (B), and reduced glutathione (GSH) content (C) in the cerebellum of dams. Results are expressed as mean ± S.E.M. for n=7-8. *p<0.05 (Student's *t* test).

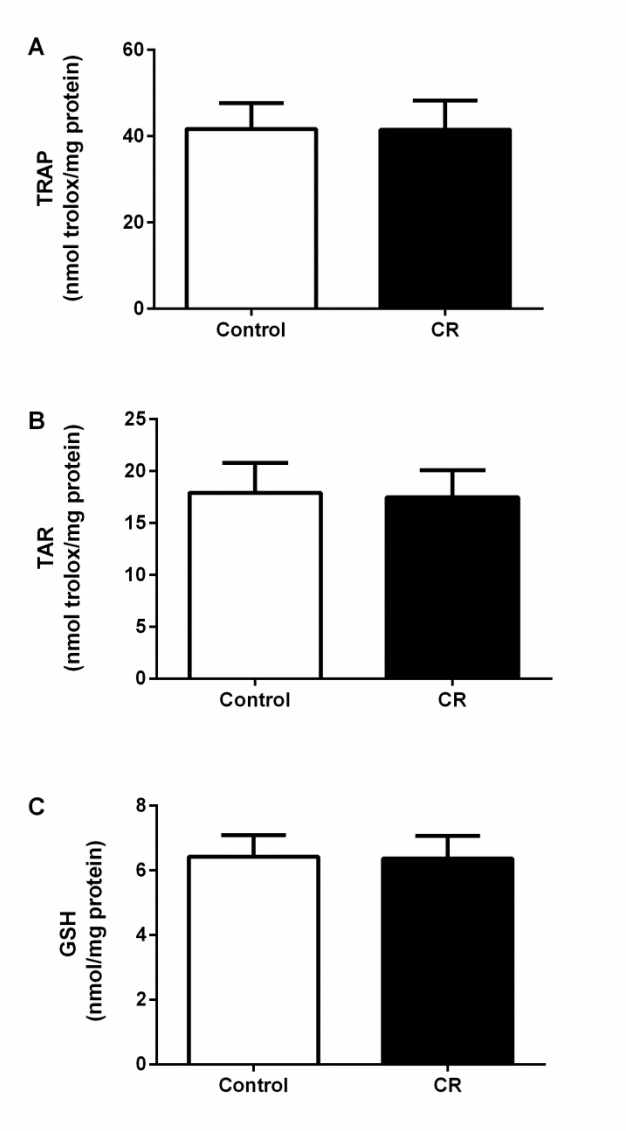

Fig. 12. Effect of caloric restriction during pregnancy on total radical-trapping antioxidant potential (TRAP) (A), total antioxidant reactivity (TAR) (B), and reduced glutathione (GSH) content (C) in the cerebral cortex of dams. Results are expressed as mean ± S.E.M. for n=7-8 (Student's *t* test).

Oxidative damage parameters remained unaltered both in pups' and dams' brain

Carbonyl, TBARS, and MDA content were assessed as indexes of oxidative damage. None of the parameters were altered, neither in pups' brain [cerebellum: carbonyl: $t(12)=0.9122$; $p=0.3796$, TBARS: $t(13)=0.5611$; $p=0.5843$, MDA: $t(11)=0.6487$; $p=0.5298$; cerebral cortex: carbonyl: $t(12)=0.5070$; $p=0.6214$, MDA: $t(10)=1.048$; $p=0.3193$] (Table 2), nor in dams [cerebellum: carbonyl: $t(14)=1.716$; $p=0.1081$, TBARS: $t(12)=0.3713$; $p=0.7169$; cerebral cortex: carbonyl: $t(12)=0.2571$; $p=0.8014$, TBARS: $t(9)=1.439$; $p=0.1839$] (Table 3).

Table 2. Effects of intrauterine caloric restriction on oxidative damaging parameters on cerebellum and cerebral cortex of pups

	Control	Caloric Restriction	p value
Cerebellum			
Carbonyl levels	43.3 ± 4.5	47.7 ± 1.6	0.3796
TBARS	27.8 ± 3.5	30.7 ± 4.0	0.5843
MDA concentration	1.00 ± 0.1	0.90 ± 0.2	0.5298
Cerebral cortex			
Carbonyl levels	36.3 ± 2.9	39.2 ± 4.9	0.6241
MDA concentration	1.00 ± 0.2	0.80 ± 0.1	0.3193

Data was expressed as mean ± S.E.M (Student's *t* test) for n=5-8.

Table 3. Effect of caloric restriction on oxidative damaging parameters on cerebellum and cerebral cortex of pregnant rats

	Control	Caloric Restriction	p value
Cerebellum			
Carbonyl levels	12.0 ± 1.7	11.50 ± 1.6	0.8014
TBARS	24.7 ± 2.6	28.90 ± 1.5	0.1839
Cerebral cortex			
Carbonyl levels	16.7 ± 2.0	15.40 ± 1.5	0.1081
TBARS	22.6 ± 4.0	20.87 ± 1.5	0.7169

Data was expressed as mean ± S.E.M (Student's *t* test) for n=5-8.

Discussion

Although the beneficial effects of CR are some sort of common sense in the literature, a recent review by Sohal and Forster [30] postulated a huge critic to it, revising concepts and pointing issues. Based in a vast amount of evidence, the authors share their concerns about the interpretation of published results, especially (i) CR has inter and even intraspecific particularities [46] and (ii) *ad libitum* controls are not suitable, especially for showing abnormal tendency to present age related diseases [47]. Albeit the focus of the present work is to assess the CR effects under pregnant and neonate rats, it is mandatory to mention that, unfortunately, the *ad libitum* control model utilized in laboratory rodents is increasingly near to the western civilization lifestyle: a sedentary overfed mammal.

Literature presents mostly two intensities of CR: moderate (usually 20-25%) and severe (usually 40%, including this work). One may expect a drastic reduction dams' weight gain and litter weight, which was actually found in this work. Data in the literature suggests that dams may have a decreased weight gain when their energy intake is restricted by 40% during pregnancy; however, litter's size and weight shall not be altered [31]. Our data is in agreement in respect to dams' reduced weight gain and unaltered litter size; however, it is contrasting in respect to litter weight, considering we found a significant reduction.

It has been demonstrated that CR may decrease the production of reactive species, such as superoxide anion and hydrogen peroxide in adult mice [48]. In this work, we found decreased mitochondrial superoxide levels in the cerebellum and cerebral cortex of CR pups. We also found a decreased DCFH oxidation in the cerebellum of both dams and pups, suggesting a reduction of ROS identified in this method, specially hydrogen peroxide, peroxynitrite, and/or hydroxyl radical [34, 49]. The reaction between nitric oxide, which is increased in the cerebral cortex of pups, and superoxide might produce peroxynitrite. We believe that was not the case, considering that decreased DCFH oxidation was found in the cerebellum, and remained unaltered in the cerebral cortex, moreover, lipids and proteins were not oxidized. Corroborating with our data, increased nitric oxide levels had already been found in response to adult CR models elsewhere [50, 51]. Since the enzymatic modulation presented both in dams and pups' brain suggests increased ROS, especially hydrogen peroxide, one may question why the DCFH oxidation was decreased in the cerebellum and unaltered in the cerebral cortex. We believe that

hydrogen peroxide could not be detected by this probe, considering the required presence of peroxidase, iron, or cytochrome c to full reaction, or alternatively, it should be metabolized by other antioxidant defenses, such as peroxiredoxins and thioredoxins, which were not evaluated in our study [52-54]. The lack of protein or lipid oxidation, demonstrated by carbonyl content, TBARS, and MDA levels reinforce this hypothesis. Moreover, we might not discard the conversion of DCFH reactive ROS in types of reactive species unable to oxidize DCFH, resulting in unaltered results as found in cerebral cortex.

Although the reactive species own a negative facet, related to oxidative stress and even to cell death, this chemical molecules are indispensable to physiological cell signaling [33], promoting cellular adaptation to many moderate stress inductors, notably aerobic exercise and dietary pattern [24, 25, 55]. Activation of a plethora of transcription factors by reactive species modulates cell metabolism and function [33, 56], as the expression of antioxidant enzymes [57-59]. Our concern is that the reduction in reactive species levels should affect the cellular antioxidant status in calorie restricted animals [7, 60].

With the purpose to examine the hypothesis that calorie restriction reduces oxidative stress in rodents, Walsh et al. [55] reviewed a great number of different animal models, including organs evaluated, intensity of CR, and period of onset, and concluded that literature presents conflicting data regarding to ROS production, antioxidant enzymes activity, and oxidative damage. Interestingly, brain SOD, CAT, and GPx are not altered in the majority of studies evaluated by Walsh and colleagues; and only 1% of the evaluated studies showed a decreased activity of these enzymes in dietary restricted rodents. Nevertheless, literature is meager concerning to animal models caloric

restricted during pregnancy, and in our knowledge our data is one of the first to evaluate maternal effect of 40% CR on brain oxidative status of dams and their offspring. In our study, we found a meaningful modulation of antioxidant enzymes in dams and their offspring, indicating an antioxidant unbalance responsible by hydrogen peroxide accumulation, as a result of SOD activation, which promotes the conversion of superoxide anion in hydrogen peroxide. The mitochondrial superoxide decreased levels both in cerebellum and cerebral cortex reinforce this hypothesis. Allied to that, we also found inhibition of hydrogen peroxide eliminating enzymes, represented by CAT and GPx [61, 62]. Grx, responsible by disulphide reduction, was also inhibited by calorie restriction, compromising the recovery of oxidized thiols [33, 38] in dams and their litter.

We also assessed the effects of gestational CR on non-enzymatic antioxidant defenses, where we found the largest difference between CR dams and pups. CR dams present a feature of metabolism adaptation in cerebellum, with increased antioxidant potential, GSH-independent. On the other hand, cerebellum and cerebral cortex of pups submitted to intrauterine calorie restricted presented diminished non-enzymatic antioxidant potential and reactivity evaluated by TRAP and TAR, respectively. GSH was statistically reduced in cerebellum, while vitamin C shows a tendency to reduction in both structures. From this set of data emerges a relevant difference between mothers treated during their first pregnancy and the pups delivered from them, emphasizing the high sensitivity of the offspring. Agale et al. [31] found a diminished total antioxidant potential both in dams and male pups applying a similar restriction protocol but using a different detection technique, while

Partadiredja et al. [62] found increased GSH levels in the brain of mice applying a 25% CR in the gestational and breast-feeding periods, analyzing 21 and 61 day-old offspring. The contrasting results may exist considering: (i) different animal models may have different responses to CR [30], (ii) CR intensity may produce different results, and/or (iii) CR was applied in different developmental periods, and the animals may have suffered adaptations during extra uterine life. Albeit mostly results discussed so far suggest a pro-oxidative status in the brain of CR dams and pups, the damage parameters assessed showed no alterations. Agale et al. [31] found decreased MDA content in dams' brain, contrasting to our results, but also did not find any alteration in pups' brain.

The mechanisms by which CR acts attract a huge interest of scientific community. Evidences suggest that CR may have an important role on the phosphoinositide 3-kinase/protein Kinase B (PI3K/Akt) pathway, which promotes the phosphorylation of Forkhead transcription factors (FoxO) family members [18, 63], which affect oxidative stress response [64]. Kim et al. [18] purposed that age-related phosphorylation of FoxO6 by PI3K/Akt activation, which is promoted by aging, negatively affected the expression of MnSOD and CAT, while CR was able to protect rat kidney cells from this effect. FoxO4 has been shown to reduce oxidative stress by directly increasing MnSOD and CAT [65]. FoxO1 regulates genes involved in cell cycle arrest, DNA repair, apoptosis and oxidative stress in the liver [66]. Jian et al. [67] showed that FoxO1 (whose activation is promoted via deacetylation by sirtuin 1 - SIRT1) mediates transcription of MnSOD. It is also proposed that CR-related stress resistance is promoted via SIRT1 pathway [68], which improves bioenergetics via peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α)

pathway activation [7]. SIRT1 overexpression was found in CR rats by Ran et al. [69] and its synthesis, according to their results, may be a protective factor for ischemic injury. As shown above, several pathways may exert effect on CR, in minor or major extension, and we certainly are far away from establishing a consensus. Even less is known about how CR exerts its effects in the intrauterine life. Our results indicate that intrauterine CR exerts a robust antioxidant disruption characterizing more intense effect in pups than in dams, which may be related to the vulnerability of the developing brain [4].

Conclusion

We are able to conclude that CR promotes a huge negative modulation in the brain antioxidant network, allied to reduction in cerebellar reactive species, especially mitochondrial superoxide, while nitric oxide was increased in cerebral cortex from offspring. Although the data present a clear oxidative environment, proteins and lipids appear to be preserved. The results obtained in this study clarify, at least in part, the effect of poor nutrition in brain's oxidative status in two remarkable stages of life. Noticeably, pups appear to be more affected than their mothers, even though a similar oxidative pattern was observed. The mechanisms of action and the impact of the observed metabolic programming in adulthood remain to be elucidated.

Acknowledgements

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Número: 25447
Título: Avaliação do efeito da restrição calórica sobre parâmetros de estado redox no sistema nervoso central de ratos submetidos à intervenção nutricional no período fetal

Pesquisadores:

Equipe UFRGS:

CRISTIANE MATTE - coordenador desde 01/01/2014
THIAGO BELTRAM MARCELINO - pesquisador desde 01/01/2014
KAREN YURIKA KUDO - pesquisador desde 01/01/2014
VINICIUS STONE SILVA - pesquisador desde 01/01/2014
Pauline Maciel August - pesquisador desde 01/01/2014

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 24/02/2014 - SALA I DO GABINETE DO REITOR, PRÉDIO DA REITORIA DA UFRGS, em seus aspectos éticos e metolodológicos, para a utilização de 65 ratos Wistar (50 fêmeas e 15 machos), de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Segunda-Feira, 10 de Março de 2014

STELA MARIS KUZE RATES
Coordenador da comissão de ética

6. ANEXO B – NORMAS DE SUBMISSÃO DO ARTIGO PARA A REVISTA CIENTÍFICA *FREE RADICAL BIOLOGY & MEDICINE*

FREE RADICAL BIOLOGY & MEDICINE
An official Journal of the Society for Free Radical Biology and Medicine
An official Journal of the Society for Free Radical Research-Europe
An Affiliate Journal of the International Society for Free Radical Research (SFRRI)

AUTHOR INFORMATION PACK

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