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EFEITO DA RESTRIÇÃO CALÓRICA DURANTE O PERÍODO GESTACIONAL SOBRE PARÂMETROS BIOQUÍMICOS MITOCONDRIAIS E COMPORTAMENTAIS NA PROLE DE RATOS

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de doutor em Bioquímica.

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Ao partido.

Sumário

Resumo

Alterações no ambiente intrauterino podem levar a consequências que perduram por toda a vida, algumas estão ligadas ao estresse oxidativo e à função mitocondrial, e podem afetar o desenvolvimento físico e motor dos filhotes, além do comportamento alimentar. Nesse contexto, o sobrepeso na gestação é uma preocupação crescente, que pode aumentar o risco de desenvolvimento de doenças crônicas não transmissíveis na vida adulta. Nesse sentido, a restrição calórica (RC) pode exercer um efeito protetor, se bem controlada, já que a desnutrição na gestação também causa efeitos indesejados. Para avaliar o efeito da RC gestacional, dividimos as ratas grávidas entre os grupos controle e RC. O segundo recebeu 20% menos ração, e o consumo de micronutrientes foi equalizado via gavagem. Cerebelo, córtex pré-frontal, hipocampo e hipotálamo dos filhotes foram avaliados nos dias pós-natais (DPN) 0, 7, 21 e 60. A prole também foi submetida a testes comportamentais aos 21 e 60 dias. Observamos um aumento no conteúdo de oxidantes ao nascimento nas regiões encefálicas avaliadas, exceto no cerebelo. Esse efeito foi praticamente abolido nas idades posteriores. As atividades das enzimas antioxidantes estavam, em sua maioria, diminuídas no DPN0, com algumas enzimas ativadas. No DPN7, as enzimas cujas atividades estavam diminuídas passaram a estar inalteradas, e muitas das que não se alteraram ao nascimento estavam ativadas. No DPN60, muitas enzimas foram ativadas, especialmente no córtex pré-frontal e hipocampo. O conteúdo de glutationa reduzida (GSH) diminuiu em três estruturas avaliadas ao nascimento e aumentou no cerebelo e hipotálamo durante o desenvolvimento e no córtex pré-frontal na idade adulta. Os níveis de vitamina C diminuíram nas primeiras idades e aumentaram nas idades posteriores. A lipoperoxidação aumentou no nascimento na maioria das estruturas, e o efeito foi abolido nas idades subsequentes. No córtex pré-frontal, a peroxidação lipídica diminuiu em DPN7, 21 e 60. A oxidação proteica aumentou em DPN0 e diminuiu em DPN60 nessa estrutura. A oxidação proteica também diminuiu no cerebelo na idade adulta. A função mitocondrial, assim como o conteúdo de superóxido, estava diminuída na maioria das estruturas no DPN0, e adaptou-se nas idades posteriores. Na idade adulta a função mitocondrial estava aumentada e o conteúdo de superóxido inalterado ou diminuído. Os complexos do sistema de transporte de elétrons (STEM) apresentaram atividade diminuída em DPN0 e aumentada na idade adulta no cerebelo. A RC não atrasou o desenvolvimento físico e motor dos filhotes e influenciou positivamente no comportamento alimentar dos mesmos na idade adulta. Nossos resultados demonstram que uma RC bem controlada no período gestacional pode melhorar a homeostase redox e a atividade mitocondrial no encéfalo, especialmente na idade adulta, sem promover desnutrição, ao menos em modelo animal em nossas condições experimentais.

Abstract

Alterations in the intrauterine environment can lead to lifelong consequences, some of them are related to oxidative stress and mitochondrial dysfunction and can affect physical and motor development of the pups. Feeding behavior may also be affected. In this context, maternal overweight demands attention, since disease development in adult life may be increased by this condition. In this sense, caloric restriction (CR) may have protective effect if well controlled avoiding maternal undernutrition. In order to evaluate CR effects, we divided pregnant rats into control and CR groups. The second received 20% less chow, and micronutrients consumption was equalized via gavage. Cerebellum, prefrontal cortex, hippocampus, and hypothalamus were evaluated on offspring's postnatal days (PND) 0, 7, 21, and 60. Animals were also submitted to behavioral tests on PND21 and 60. We observed increased oxidant levels in all brain regions evaluated, except for the cerebellum on PND0. This effect was abolished on latter ages. Antioxidant enzyme activities were mostly decreased on PND0. On PND7, affected enzymes were either equal to control or presented increased activity. On PND60, most enzymes were activated, especially in the prefrontal cortex and hippocampus. Reduced glutathione (GSH) levels were decreased in three brain regions evaluated at birth, increased in the cerebellum and hypothalamus during development and in the prefrontal cortex at adult age. Vitamin C levels decreased in the first ages and were increased in the latter ones. Concerning to oxidative markers, lipid peroxidation was increased at birth in most regions and the effect was abolished thereafter. In the prefrontal cortex, lipid peroxidation was decreased on PND7, 21 and 60. Protein oxidation was increased on PND0 and decreased on PND60 in this region. In the cerebellum, protein oxidation was also decreased at adult age. Mitochondrial function, as well as the mitochondrial superoxide content, was decreased in most structures on PND0 and went through adaptation. At adult age, mitochondrial function was increased and superoxide levels were either unaltered or decreased. Mitochondrial electron transport system (METS) complexes showed decreased activity on PND0 and were activated on PND60 in the cerebellum. CR did not delay physical or neuromotor development and positively influenced pups' feeding behavior at adult age. Our results show that maternal CR, if well controlled, may improve redox homeostasis and mitochondrial function in the offspring's brain, especially at adult age, without promoting undernutrition, at least at our experimental conditions.

Lista de abreviaturas

AMPK: Proteína cinase ativada por monofosfato de adenosina

CAT: Catalase

DCFH: Diclorofluoresceína

DOHaD: Origens desenvolvimentistas da saúde e da doença (do inglês, *Developmental origins of health and disease)*

DPN: Dia pós-natal

eNOS: Óxido-nítrico-sintase endotelial

ER: Espécies reativas

GPx: Glutationa-peroxidase

Grx: Glutarredoxina

GSH: Glutationa reduzida

IGF-1: Fator de crescimento semelhante à insulina tipo 1

NO: Óxido nítrico

NOS: Óxido-nítrico-sintase

NRF2: Fator nuclear eritroide 2 relacionado ao fator 2

PGC-1α: Coativador de receptor ativado por proliferador de peroxissomo gama 1 alfa

PPAR: Receptor ativado por proliferador de peroxissomo

POMC: Pró-opiomelanocortina

RA: Restrição alimentar

RC: Restrição calórica

RD: Restrição dietética

SIRT: Sirtuína

SNC: Sistema nervoso central

STEM: Sistema de transporte de elétrons mitocondrial

SOD: Superóxido-dismutase

TOR: Proteína alvo da rapamicina

Trx: Tiorredoxina

TrxR: Tiorredoxina-redutase

1. Introdução

1.1. Origens desenvolvimentistas da saúde e da doença (DOHaD)

Em 1990, o epidemiologista inglês David Barker desenvolveu a hipótese de que a diminuição do aporte nutricional ao feto durante a gestação leva à diminuição do peso ao nascer e desenvolve no mesmo o denominado fenótipo poupador (*thrifty phenotype)* durante o resto da vida, levando a complicações como o ganho de peso aumentado e doenças relacionadas, como hipertensão e diabetes (Barker, 1990).

O avanço científico permitiu notar que não só as intervenções potencialmente negativas exerciam efeito sobre a prole quando aplicadas no período gestacional. Em 2003, David Barker e outros cientistas fundaram uma sociedade dedicada a estudar esses fenômenos, a sociedade para as origens desenvolvimentistas da saúde e da doença, do inglês *Society for Developmental Origins of Health and Disease*. Além de estudar esses fenômenos, outro desafio dessa sociedade é transformar as descobertas científicas em políticas públicas de promoção de saúde nos diversos países (Hanson *et al.*, 2019).

Nosso grupo já demonstrou como diversas intervenções não farmacológicas no período gestacional levam a alterações que perduram pelo resto da vida: restrição alimentar (RA) severa (Stone *et al.*, 2016), restrição calórica (RC) moderada (Stone *et al.*, 2019), dieta hipersódica (Stocher *et al.*, 2018), suplementação com polifenóis (August *et al.*, 2018) e exercício físico de

natação (Marcelino *et al.*, 2013, August *et al.*, 2018, Klein *et al.*, 2018, Klein *et al.*, 2019).

As intervenções sobre o ambiente intrauterino influenciam a atividade genômica. Apesar de não alterar o sequenciamento do DNA, são capazes de produzir alterações fenotípicas importantes, que em alguns casos podem ser transgeracionais, e são conhecidas como alterações epigenéticas (Lee, 2015).

As alterações epigenéticas estão intimamente envolvidas com a reprogramação metabólica fetal. A metilação do DNA, a modificação através de histonas e a expressão de microRNA são submetidas a uma extensa reconfiguração durante o desenvolvimento, afetando genes e vias de sinalização essenciais tanto para o desenvolvimento como para a susceptibilidade de doenças na vida adulta (Balbus *et al.*, 2013, Vickers, 2014).

1.2. Restrição calórica

A RC é definida como a redução no consumo de calorias sem déficit nutricional, sendo essencial que seja levada em consideração a suplementação de micronutrientes. A simples redução da quantidade de alimento é denominada RA, enquanto o termo restrição dietética (RD) é amplamente utilizado na literatura para generalizar qualquer tipo de restrição dentro da dieta (RA, RC, restrição de algum nutriente específico e até jejum intermitente) (McCay *et al.*, 1935, Masoro, 1989, Cerqueira e Kowaltowski, 2010, Gonzalez *et al.*, 2012).

Já foi demonstrado que a RC pode modular o processo normal de envelhecimento em modelos animais adultos (Lopez-Lluch e Navas, 2016, Gensous *et al.*, 2019). Experimentos demonstram que a RC é capaz de frear o desenvolvimento de diversas doenças relacionadas à idade (Gonzalez *et al.*, 2012). Dentre elas estão as doenças cardiovasculares (Mattson e Wan, 2005), diabetes (Anson *et al.*, 2003), vários tipos de câncer (De Lorenzo *et al.*, 2011) e doenças neurodegenerativas (Bishop e Guarente, 2007).

Os mecanismos celulares envolvidos nos efeitos da RC não estão completamente elucidados, sendo objetivo de frequentes estudos. A prevenção do estresse oxidativo e o aumento da atividade mitocondrial estão relacionados aos efeitos positivos, sendo regulados pela inibição do fator de crescimento semelhante à insulina 1 (IGF-1) e da proteína alvo de rapamicina (TOR) e a ativação da proteína cinase ativada por monofosfato de adenosina (AMPK) e sirtuína 1 (SIRT 1), que atuam diretamente sobre o coativador de receptor ativado por proliferador de peroxissomo gama 1 alfa (PGC-1α). (Lopez-Lluch e Navas, 2016).

A restrição alimentar – especialmente a restrição severa e sem prevenção da desnutrição - na vida intrauterina promove adaptações negativas (Barker, 1990, Agale *et al.*, 2010, Schulz, 2010, Stone *et al.*, 2016) principalmente quando o indivíduo tem acesso à plenitude pós-natal, como podemos observar através de estudos que demonstram os efeitos deletérios da mesma em contextos históricos como o cerco a Leningrado em 1941 (Stanner e Yudkin, 2001) e a invasão alemã sobre a Holanda em 1944 (Paneth e Susser, 1995). Estes dados ilustram a hipótese de Barker, que defende que o ambiente

intrauterino prepara o indivíduo para o ambiente externo, e que o estresse gerado neste período pode desencadear uma resposta pós-natal exagerada a agentes estressores (Barker, 1990).

Diversos estudos demonstraram que a RC está associada à melhora da homeostase redox (Lee e Wei, 2012, Ribeiro *et al.*, 2012, Kim *et al.*, 2014). Por outro lado, a restrição dietética quando aplicada no período intrauterino, parece ter uma implicação negativa no que diz respeito ao desenvolvimento de doenças crônicas não transmissíveis na vida adulta e expectativa de vida (Ahmed *et al.*, 1987, Sayer e Cooper, 2002, Partadiredja *et al.*, 2005, Partadiredja *et al.*, 2009, Agale *et al.*, 2010). É importante considerar que nenhum dos trabalhos citados acima levou em consideração a prevenção da desnutrição através da equalização do consumo de micronutrientes entre os grupos.

Se por um lado, o interesse em estudar os efeitos da RC no período gestacional se dá pelos seus efeitos positivos já expostos quando aplicada na idade adulta, por outro, se dá pelo aumento das taxas de sobrepeso e obesidade durante a gestação, relacionados com o acesso facilitado a alimentos hiperpalatáveis e ao comportamento sedentário (Owen *et al.*, 2010, Owen *et al.*, 2010).

O aumento do peso durante a gestação aumenta a ocorrência de diabetes gestacional, hipertensão, pré-eclâmpsia, parto por cesariana e obesidade infantil (Baeten *et al.*, 2001, Whitaker, 2004), afetando 20% das gestantes (Fisher *et al.*, 2013). Além disso, até 2020, dados alarmantes mostram que 60

milhões de crianças devem ser obesas ou apresentar sobrepeso no mundo (de Onis *et al.*, 2010).

É sabido que tanto a desnutrição quanto a superalimentação durante a gestação são danosas para os filhotes. De fato, os efeitos causados por uma ou por outra são semelhantes (Cunha Fda *et al.*, 2015). Entretanto, não é suficiente afirmar que durante a gestação se deve ter uma "dieta balanceada", por isso, utilizamos um modelo animal para melhor entender como a nutrição materna influencia a saúde dos filhotes.

Antes de tudo, é preciso levar em consideração que os animais utilizados hoje na grande maioria dos estudos experimentais refletem uma condição semelhante à de boa parte da população mundial: são superalimentados e sedentários, visto que vivem em uma caixa com espaço limitado e alimentação à vontade. Essa condição leva a alterações metabólicas que aumentam o estresse oxidativo e diminuem a expectativa de vida (Martin *et al.*, 2010). O protocolo aqui utilizado propõe uma restrição moderada (20%) com prevenção da desnutrição em animais com essas características.

1.3. Homeostase redox e restrição calórica

O termo espécie reativa (ER) engloba diversas moléculas reativas, radicais livres ou não, provenientes de oxigênio, nitrogênio, cloro, bromo e enxofre (Halliwell e Gutteridge, 2015), sendo as duas primeiras as mais relevantes. Atualmente, alguns pesquisadores – incluindo nosso grupo – defendem a atualização do termo ER por "oxidante".

Dentre os oxidantes, o óxido nítrico exerce uma ampla gama de papeis no metabolismo fisiológico e patológico, tais como de vasodilatador, neurotransmissor e modulador inflamatório (Siegel, 2006, Song *et al.*, 2014). É sintetizado pela óxido-nítrico-sintase (NOS), em uma reação que converte arginina e oxigênio em citrulina e óxido nítrico (NO). Essa enzima possui diferentes isoformas: neuronal, induzível e endotelial, as quais possuem mecanismos de regulação e localização tecidual diferenciadas (McManus *et al.*, 2014). 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, bem como reagir com outras espécies reativas, tais como superóxido, gerando o radical peroxinitrito (Siegel, 2006, Halliwell e Gutteridge, 2015).

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 (Halliwell e Gutteridge, 2015). 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 (Halliwell, 2007). O radical superóxido pode reagir com outros oxidantes, tais como o NO, produzindo peroxinitrito, ou ser eliminado pela enzima superóxido-dismutase (SOD), presente na matriz mitocondrial na isoforma MnSOD, e no espaço intermembranas e citosol como CuZnSOD (Fridovich, 1995),

produzindo peróxido de hidrogênio. 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 (Fernandes e Holmgren, 2004). Ainda agem sobre o peróxido de hidrogênio e peróxidos orgânicos, as peroxirredoxinas, sendo seis isoformas distribuídas na célula (Halliwell e Gutteridge, 2015). 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 (Trx), que eliminam peróxidos à custa de NADPH na mitocôndria (Trx2) e citosol (Trx1), utilizado na sua regeneração mediada pela tiorredoxina-redutase (TrxR), outra enzima dependente de selênio (Halliwell e Gutteridge, 2015). 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, o mais danoso dos oxidantes. As glutarredoxinas (Grx) regeneram grupos tióis oxidados, utilizando glutationa reduzida (GSH) como substrato, sendo encontradas na mitocôndria e citosol. A GSH, além de ser substrato das enzimas antioxidantes GPx e Grx, também atua como *scavenger* de radicais livres, assim como as vitaminas C e E, entre outras moléculas antioxidantes de baixo peso molecular (Fridovich, 1995, Halliwell, 2006, Halliwell, 2007, Halliwell e Gutteridge, 2015).

Diversos aspectos nutricionais estão relacionados à prevenção ou desencadeamento de doenças que contém em sua fisiopatogenia o estresse oxidativo (Da Costa *et al.*, 2012). O interesse em encontrar estratégias para

diminuir o estresse oxidativo se dá pelo fato de que com um aumento do mesmo, o indivíduo está sujeito a uma série de complicações relacionadas à oxidação de lipídeos de membranas, proteínas e ácidos nucleicos (Esposito *et al.*, 2002).

O sistema nervoso central (SNC), alvo do presente estudo, apresenta um grande consumo de oxigênio, que está relacionado com a formação de espécies reativas. Devido ao alto conteúdo lipídico, o qual é um alvo dos oxidantes formados no sistema de transporte de elétrons mitocondrial (STEM), bem como à quantidade relativamente baixa de enzimas antioxidantes, o SNC é especialmente vulnerável ao estresse oxidativo (Halliwell, 2007, Halliwell e Gutteridge, 2015).

A formação do tubo neural é um ponto crucial no desenvolvimento, ocorre entre os dias gestacionais 10 e 11 em ratos (Semple *et al.*, 2013). Ademais, grande parte da neurogênese em regiões subcorticais inicia no dia gestacional 9 (Babikian *et al.*, 2010), enquanto no hipocampo, há altas taxas de neurogênese tanto antes como depois do nascimento (Diamond, 1990). Para evitar complicações na vida adulta, é essencial prevenir o dano oxidativo durante as principais fases do desenvolvimento do SNC (Semple *et al.*, 2013).

A RC parece ter um efeito hormético, causando um estresse moderado que previne o organismo de futuros danos por agentes estressores (Testa *et al.*, 2014). Este efeito perpassa a manutenção da integridade dos complexos do STEM e a diminuição na produção de oxidantes (Lopez-Lluch e Navas, 2016)

1.4. A mitocôndria e a restrição calórica

A mitocôndria é a maior fonte de oxidantes celulares (Cadenas e Davies, 2000, Lenaz *et al.*, 2002, Sesti *et al.*, 2010), e também é o principal alvo dos mesmos (Ku *et al.*, 1993, Sohal e Weindruch, 1996). A RC (bem como o exercício físico e o resveratrol) aumenta a biogênese (Lopez-Lluch *et al.*, 2006), o volume (Khraiwesh *et al.*, 2014) e a atividade mitocondrial (Khraiwesh *et al.*, 2013). A respiração celular aumentada tem papel importante na promoção da expectativa de vida (Barros *et al.*, 2004), e no envelhecimento, ocorre uma diminuição da função mitocondrial, sendo que este processo está relacionado com diversas alterações fisiológicas, como o declínio da capacidade muscular associado ao envelhecimento (Rooyackers *et al.*, 1996).

A RC previne o vazamento de elétrons dos complexos do sistema de transporte de elétrons mitocondrial (STEM), evitando a formação de superóxido (Martin-Montalvo e de Cabo, 2013). Outro efeito desta intervenção é o aumento da retenção de cálcio na mitocôndria, conferindo proteção contra a excitotoxicidade, e promovendo o aumento da biogênese mitocondrial e da eficiência bioenergética (Amigo *et al.*, 2017).

A diminuição na produção de oxidantes pela mitocôndria está fortemente relacionada com os efeitos positivos da RC em modelos animais adultos sobre o envelhecimento (Sohal e Weindruch, 1996, Masoro, 2000). A produção de superóxido mitocondrial é um processo fisiológico e necessário para uma série de processos celulares, como sinalização, crescimento e apoptose (Fukai e Ushio-Fukai, 2011), entretanto, a disfunção mitocondrial pode gerar aumento

da produção de superóxido mitocondrial e aumentar o risco de doenças metabólicas e neurológicas (Cerqueira *et al.*, 2012).

A biogênese mitocondrial é controlada principalmente pelo PGC-1α, que ativa uma cascata de sinalização que envolve diversas proteínas. Evidências apontam que a regulação do PGC-1α seja dependente da ação do NO (Cerqueira *et al.*, 2012, Ferreira *et al.*, 2016).

A maior parte do ATP existente nas células eucarióticas é gerado na mitocôndria através da fosforilação oxidativa (Sakamuru *et al.*, 2016). A transferência de elétrons que ocorre durante a produção de ATP gera um gradiente eletroquímico, responsável pela ocorrência do potencial de membrana mitocondrial, que é utilizado como um parâmetro de função mitocondrial, além da própria atividade dos complexos do STEM (Sakamuru *et al.*, 2016).

1.5. Aspectos do desenvolvimento físico, motor e comportamental

Diversas intervenções no período gestacional podem afetar o desenvolvimento físico e motor dos filhotes. Por exemplo, a RA severa leva à diminuição do peso ao nascer (Stone *et al.*, 2016), e a exposição à nicotina leva ao atraso no desenvolvimento físico e motor (Schneider *et al.*, 2010). Sabendo que as consequências negativas causadas pela RA estão diretamente ligadas à diminuição do peso ao nascer (Barker, 1990),

consideramos de suma importância fazer uma avaliação aprofundada sobre diversos parâmetros de desenvolvimento para verificar se o protocolo de RC aplicado promoveu algum atraso no desenvolvimento dos filhotes.

O desenvolvimento pós-natal envolve a maturação de diversos sistemas. As intervenções durante a gestação, especialmente a administração de algumas drogas, podem impactar o desenvolvimento encefálico através de alterações epigenéticas, agindo sobre neurotransmissores e sistemas endócrinos (Mirmiran *et al.*, 1985, Mirmiran e Swaab, 1987). As alterações fenotípicas geradas podem ser verificadas através de uma bateria de testes desenvolvida por Fox (Fox, 1965).

O comportamento alimentar também pode ser modulado por intervenções no período gestacional. O consumo de aspartame pela mãe durante a gestação, por exemplo, leva à preferência por alimentos palatáveis na prole (von Poser Toigo *et al.*, 2015). Já a restrição proteica materna leva ao aumento do consumo de alimentos ricos em gordura na idade adulta (Bellinger *et al.*, 2004).

É importante avaliar o comportamento alimentar dos filhotes na idade adulta, visto que as alterações negativas como aumento de peso e doenças relacionadas estão diretamente ligadas à reprogramação dos centros ligados ao comportamento alimentar, principalmente no hipotálamo, que se estabelecem durante o desenvolvimento (Bouret, 2010, Gali Ramamoorthy *et al.*, 2015).

O hipotálamo é responsável pelo controle da ingestão alimentar e do gasto energético, sendo um alvo importante quando se trata da programação

metabólica promovida pela nutrição materna. É sabido que tanto a desnutrição como o excesso de nutrientes na gestação influenciam esse centro regulador (Gali Ramamoorthy *et al.*, 2015). A principal área hipotalâmica responsável por esse controle é o núcleo arqueado, composto por neurônios orexigênicos e anorexigênicos, embora outras regiões tenham importância secundária (Gali Ramamoorthy *et al.*, 2015).

A atividade locomotora e o comportamento do tipo ansioso da prole também são modulados pela RA durante a gestação (Ramirez-Lopez *et al.*, 2016), e quando aplicada no envelhecimento, a RD acentuou a diminuição da atividade locomotora causada pelo envelhecimento e aumentou o comportamento do tipo ansioso em camundongos, entretanto, melhorou o aprendizado espacial e a memória de trabalho (Kuhla *et al.*, 2013).

2. Objetivos

2.1. Objetivo geral

Avaliar o efeito da RC durante a gestação sobre parâmetros de homeostase redox e mitocondriais no encéfalo de filhotes de ratos ao longo do desenvolvimento. Avaliar parâmetros de desenvolvimento físico, motor e comportamental nestes filhotes nas idades iniciais, na adolescência e na idade adulta.

2.2. Objetivos específicos

- Avaliar o efeito da RC durante a gestação no cerebelo, córtex pré-frontal, hipocampo e hipotálamo de filhotes ao longo do desenvolvimento (0, 7, 21 e 60 dias de vida) sobre:

    - os níveis de oxidantes;

    - a capacidade antioxidante enzimática e não enzimática;

    - o dano oxidativo a lipídeos e a proteínas;

    - a função mitocondrial, através de medidas de massa e potencial de membrana;

    - o conteúdo de superóxido mitocondrial;

    - o conteúdo de óxido nítrico;

    - e a atividade dos complexos do sistema de transporte de elétrons mitocondrial (STEM).

- Avaliar o efeito da RC durante a gestação sobre os seguintes parâmetros de desenvolvimento e comportamentais na adolescência (DPN21) e na idade adulta (DPN60):

    - desenvolvimento físico;

    - desenvolvimento neuromotor;

    - comportamento alimentar;

    - atividade locomotora;

    - comportamento do tipo ansioso;

    - e memória espacial.

3. Capítulo I

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Gestational caloric restriction improves redox homeostasis parameters in the brain of Wistar rats: a screening from birth to adulthood

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Abstract

Caloric restriction (CR) improves health and life span in animal models. Although CR effects in adult life are well described, little is known about effects on offspring when applied during gestation. Pregnancy is a remarkable period of life, alterations in this stage lead to lifelong consequences, some of which, associated to redox unbalance. Furthermore, gestational overweight is a growing issue that can lead to detrimental outcomes. To address this issue, we divided pregnant rats into control (ad libitum food) and CR groups, which received 20% less food than control. Micronutrients consumption was equalized between groups by oral gavage. Cerebellum, prefrontal cortex, hippocampus, and hypothalamus were evaluated on post-natal day (PND) 0, 7, 21, and 60. We observed increased oxidants content on PND0 in all brain structures, except for the cerebellum. Key enzymatic antioxidant defenses showed decreased activity on PND0. Interestingly, on PND60, we observed a positive modulation of most antioxidant enzymes, especially on the prefrontal cortex and hippocampus. Non-enzymatic antioxidant defenses were decreased at birth and increased during development and adult age. Lipid peroxidation was increased at birth on most structures, and the effect was abolished thereafter. In the prefrontal cortex, lipid peroxidation was unaltered at birth and diminished thereafter, while protein oxidation was increased on PND0 and decreased on PND60. Protein oxidation was also decreased in the cerebellum at adult age. Our results shown controlled gestational CR to improve antioxidant defenses and protect offspring's brain from oxidative stress, especially in adulthood, as a result of developmental metabolic programming.
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Keywords: Dietary restriction; Intrauterine environment; Redox status; Metabolic programming; Pregnancy

1. Introduction

Increased weight during pregnancy increases the occurrence of gestational diabetes, hypertension, preeclampsia, cesarean delivery, and childhood obesity [1,2], affecting 1 out of 5 pregnant women [3]. Up to 2020, 60 million children shall be either overweight or obese in the world [4]. The growing obesity ratios are related to increased sedentary behavior [5] and facilitated access to food, especially food containing high amounts of calories, from carbohydrates and fats. In rodent studies, the animals used as control are sedentary and overfed, which leads to metabolic alterations that increase oxidative stress and shorten lifespan [6].

On the other hand, there is plenty of evidence to state that caloric restriction (CR) improves health and life span, acting through diverse mechanisms that increase mitochondrial activity and promote redox homeostasis [7–9]. Of note, CR is a protocol which does not involve malnutrition [10,11]. In adult animal models, it was demonstrated that CR increases life and health span, retarding the development of age-related diseases, such as diabetes and cardiovascular diseases [12–14]. CR positive effects on redox homeostasis were also reported elsewhere [15–17]. CR effects during pregnancy are poorly reported, and most protocols in the literature involve malnutrition.

It is well established that the intrauterine life is a key period in the brain development of mammals, including humans. It is known that

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taking care of the woman's health during pregnancy is essential for the development of the child [18], in accordance to the Developmental Origins of Health and Disease (DOHaD) concept. In this context, developing and applying health promotion strategies during pregnancy is a demand of social interest around the world, since there is solid evidence showing the maternal origins of metabolic diseases and how maternal stressors influence pathological outcomes in adult life [19–21].

Oxidative stress plays an important role on metabolic programming; and it occurs through an unbalance between oxidants and antioxidants, in favor of oxidants, being related to several pathological conditions generated in the intrauterine life [22–24]. On the other hand, it is known that a small increase in oxidants content may promote cellular adaptation, leading to increased redox homeostasis through metabolic adaptation in agreement with the concept of hormesis [25]. It was already shown by our group that different gestational interventions influence pups' brain redox homeostasis. If in one hand the exercise training improves antioxidant defenses in different brain regions [26], severe food restriction during pregnancy decreases the activity of different antioxidant enzymes on the brain of newborn rats [27]. Due to high oxygen consumption and low antioxidant levels, the brain is highly susceptible to oxidative stress [28]. Of note, the developing brain is a special target, since neurogenesis starts at gestational day 9 in most cortical and subcortical regions [29,30]. The hippocampus, in turn, present high neurogenesis levels before and after birth [31]. Avoiding oxidative injury during brain development is essential for healthy outcomes in adult life [32].

In this work, the authors show the redox effects of a moderate, well controlled gestational caloric restriction on several brain areas of the offspring since the day of delivery until adulthood, including oxidants content, antioxidant defenses, and oxidative damage parameters.

Different brain regions were explored, in order to provide evidence on what functional alterations may be altered and should be primarily assessed. Cerebellum was assessed because of its role on motor learning and cognitive functions, prefrontal cortex on working memory, hippocampus on long term and spatial memory, and hypothalamus on feeding behavior [33]

2. Methods

2.1. Animals and reagents

Female adult Wistar rats (120 days-of-age, nulliparous, weighing approximately 230 g, 150 animals) 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. Conception diagnosis was performed by the presence of spermatozoids and absence of leucocytes in vaginal smears. Three female rats diagnosed as pregnant were housed per cage in order to start the treatment. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22± 1 °C) colony room. 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 30044, 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 and Invitrogen by Thermo Fischer Scientific, Carlsbad, CA, USA.

2.2. Caloric restriction (CR) protocol

Control rats had free access to water and a 22% (w/w) protein commercial chow. CR group also had free access to water and received the same commercial chow; however, the amount was reduced by 20%. The diet for CR group was adjusted daily by body weight, using the chow amount consumed by control animals as standard. The difference, in grams, between control group mean consumption and CR animal consumption was calculated every day. A formula containing micronutrients in the same proportion of commercial chow was administered to the animals, based on the difference of chow consumption, in order to equilibrate micronutrients consumption between groups (5 mL of formula contains the same micronutrients amount contained in 10 g of chow, Table 1). The micronutrients were solubilized on sucralose 0.1% and methylparaben 0.1% (vehicle). Dams assigned to the control group received the vehicle *via* oral gavage to avoid any unwanted interference.

CR dams were submitted to CR protocol during entire pregnancy. In the day of delivery, the pups were cross-fostered: pups from CR dams were housed with control dams and pups from control dams were housed with another control dam. Cross-fostering was applied in order to abolish the CR intervention after birth. Pups were weaned at PND21, and after weaning, all pups received ad libitum food. In order to avoid litter effect, and increase genetic variability, only one pup per litter was utilized in each biochemical analysis.

2.3. Sample processing

Male pups (400 animals) were euthanized by decapitation without anesthesia (in order to avoid chemical contamination of the sample that could alter the biochemical parameters evaluated) at PND0, PND7, PND21, and PND60. Brain was dissected; cerebellum, prefrontal cortex, hippocampus, and hypothalamus were rapidly isolated in a Petry plate on ice, according to the Atlas of the Developing Rat Brain and stored at −80 °C until utilization [34]. The tissue was homogenized in 10 volumes (1:10, w/v) of phosphate buffered saline (PBS) pH 7.4, containing 1 mM ethyleneglycoltetraacetic acid (EGTA) and 1 mM phenylmethanesulfonyl fluoride (PMSF). Homogenates were centrifuged at 3000 rpm for 10 min at 4 °C, to discard nuclei and cell debris. The pellet was discarded and the supernatant was taken to biochemical assays, except for cytometry and HPLC experiments.

Table 1
Micronutrients content in 10 g of chow or 5 mL of formula

Nutrient	Weight (10 g of chow or 5 mL of formula)
Ca	120 mg
P	80 mg
Vit A (Retinol)	39 μg
Vit D3 (Calcitriol)	0.49 μg
Vit E (Tocoferol)	226 μg
Vit K3 (Phylloquinone)	30 μg
Vit B1 (Thiamin)	50 μg
Vit B2 (Riboflavin)	60 μg
Vit B6 (Pyridoxin)	70 μg
Vit B12 (Cobalamin)	0.22 μg
Vit B3 (Niacin)	600 μg
Vit B5 (Pantothenic acid)	200 μg
Vit B9 (Folic acid)	10 μg
Biotin	0.5 μg
Choline	19 mg
Na	27 mg
Fe	500 μg
Mn	600 μg
Zn	600 μg
Cu	100 μg
Se	0.5 μg
Co	15 μg
F	800 μg

2.4. Biochemical assays

2.4.1. Reactive species content

Reactive species content was analyzed through the 2′,7′-dichloro-fluorescein (DCFH) oxidation in a FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The tissue samples (100 mg) were dissociated with 1 mL of PBS pH 7.4 containing 1 mg% of collagenase IV, and centrifuged at 1500 rpm for 5 min. The supernatant was discarded in order to avoid collagenase toxicity, resuspended in 400 μl PBS, filtered and incubated with the probe. One hundred microliter of each sample was incubated at 37 °C during 30 min in the presence of DCFH in a final concentration of 1 μM.

2.4.1.1. Superoxide dismutase (SOD).
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 one unit. The data were calculated as units/mg protein.

2.4.1.2. Catalase (CAT).
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.

2.4.1.3. Glutathione peroxidase (GPx).
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.

2.4.1.4. Glutaredoxin (Grx).
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.

2.4.1.5. Thioredoxin reductase (TrxR).
TrxR (EC1.8.1.9) activity was measured according to the method described by Arner and Holmgren [39] using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) as substrate. The reduction of 1 mol of DTNB generates 2 mol of TNB. TNB generation was monitored spectrophotometrically at 412 nm in a medium containing 5 mM DTNB and 300 μM NADPH. One TrxR unit is defined as 1 μmol of DTNB reduced per minute and the specific activity is represented as units/mg protein.

2.4.2. Non-enzymatic antioxidants

2.4.2.1. Reduced glutathione (GSH) concentration.
GSH concentration was measured according to Browne and Armstrong [40], where GSH reacts with the fluorophore o-phthaldialdehyde. The proteins in supernatant were initially precipitated with meta-phosphoric acid (1:1, v:v), centrifuged at 6700 rpm, for 10 min, at 25 °C. 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.

2.4.2.2. 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 level was expressed as mg/g of protein [41].

2.4.3. Biomolecule oxidative parameters

2.4.3.1. 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 370 nm [42]. Briefly, 1 mg of sample protein was treated with 20% trichloroacetic acid, and centrifuged at 6200 rpm, 4 °C for 5 min. The pellet was dissolved in 0.2 M NaOH, and was added of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). This was kept in the dark during 1 h, and vortexed each 15 min. Samples were added of 20% thiobarbituric acid), and centrifuged at 14,000 rpm, 4 °C 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 resuspended in 8 M urea pH 2.3. The sample was vortexed and incubated at 60 °C for 15 min. After that, it was centrifuged at 14,000 rpm for 3 min and the absorbance was measured at 370 nm. Protein carbonyl content was expressed as nmol/mg protein.

2.4.3.2. Sulfhydryl content.
Sulfhydryl content essay is based on the reduction of 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) by thiols, which become oxidized (disulfide), generating a yellow derivative (TNB) which is measured spectrophotometrically at 412 nm [43]. Briefly, 30 μl of homogenate were added to 200 μl of PBS pH 7.4 containing 1 mM PMSF and 1 mM EGTA. Then 30 μl of 10 mM DTNB, prepared in a 0,2 M potassium phosphate solution pH 8.0, were added. Subsequently, the samples were incubated for 30 min at room temperature in a dark room. Absorption was measured at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein.

2.4.3.3. 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 [41].

2.4.3.4. Protein concentration assay.
Protein concentration was measured by the method of Lowry et al. [44], using bovine serum albumin as standard.

2.4.4. Statistical analysis

Data were analyzed by multiple Student's t tests, using GraphPad Prism 6.0 software. Data were considered statistically significant when $P<.05$.

3. Results

3.1. Maternal CR decreases weight gain without affecting pregnancy rate

Dams submitted to CR showed decreased weight gain during pregnancy, especially after gestational day 9, when the difference was statistically significant [$P<.05$] (Fig. 1). In the control group, 72.9% of the animals diagnosed as pregnant really gave birth to pups, in the CR group, 70.0% of the animals did, showing that CR did not affect dams' fertility.

3.2. CR effects in the cerebellum

3.2.1. Oxidants content and enzymatic antioxidants

DCFH oxidation, a marker which is altered by the presence of several oxidants (especially hydrogen peroxide and hydroxyl radical) [45], showed no alteration in the cerebellum on PND0 [$t(62) = 0.018$, $P=.9855$], PND7 [$t(62) = 1.7070$, $P=.0928$], PND21 [$t(62) = 0.3818$, $P=.7038$], or PND60 [$t(62) = 0.3041$, $P=.7620$] (Fig. 2A).

SOD is the enzyme responsible for converting the superoxide anion into hydrogen peroxide, and this enzyme's activity was decreased in the cerebellum on PND0 [$t(43) = 2.4329$, $P<.05$]. On PND7 this decrease was abolished [$t(43) = 1.0824$, $P=.2850$] and remained unaltered on PND 21[$t(43) = 0.1575$, $P=.1223$] and PND60 [$t(42) = 0.5265$, $P=.6012$] (Fig. 2B). CAT, one of the enzymes responsible for eliminating hydrogen peroxide, was not altered in the cerebellum on PND0 [$t(44) = 0.8891$, $P=.3787$], PND7 [$t(44) = 0.9783$, $P=.3332$], and PND21 [$t(44) = 0.4141$, $P=.6808$]. On PND60, its activity was increased [$t(44) = 2.237$, $P<.05$] (Fig. 2C). GPx, the most important enzyme responsible for hydrogen peroxide detoxification was also evaluated. On PND0 it was decreased in the cerebellum [$t(47) = 2.8376$, $P<.01$]. On PND 7, this enzyme tended to be decreased [$t(47) = 1.9572$, $P=.056$]. On PND21 the cerebellum remained with decreased GPx activity [$t(47) = 3.1379$, $P<.01$]. The negative effect on GPx activity was abolished in PND60 [$t(47) = 0.1432$, $P=.8867$] (Fig. 2D). Grx is responsible for regeneration of protein thiol groups, including those present in antioxidant enzymes. In the cerebellum, it was unaltered on PND0 [$t(45) = 0.6491$, $P=.5195$]. On PND7, in contrast, its activity was increased [$t(45) = 2.7367$, $P<.01$]. On PND21, the activity returned to control levels [$t(45) = 0.3339$, $P=.7399$] and on PND60, the enzyme was activated [$t(45) = 3.1834$, $P<.01$] (Fig. 2E). TrxR reduces thiol groups in antioxidant enzymes such as thioredoxin and Grx. As Grx, this enzyme is part of the peroxyredoxin system, which is also responsible for detoxifying hydrogen peroxide. In the cerebellum, it was increased on PND0 [$t(55) = 2.4319$, $P<.05$] and unaltered on PND7 [$t(55) = 0.0439$, $P=.9651$], PND21 [$t(55) = 1.6807$, $P=.9848$], and PND60 [$t(55) = 0.4074$, $P=.6852$] (Fig. 2F).

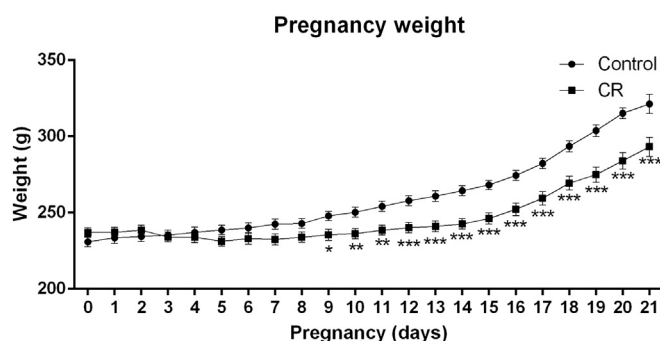### 3.2.2. Non-enzymatic antioxidants

GSH is the main non-enzymatic antioxidant in mammals, and was highly modulated by maternal CR. On PND0, it was decreased in the cerebellum [$t(46) = 2.6644$, $P<.05$]. This effect was reversed on PND7, when GSH levels were increased [$t(46) = 2.5580$, $P<.05$]. On PND21 [$t(46) = 0.0476$, $P=.9621$] and PND60 [$t(46) = 1.6387$, $P=.1081$], GSH levels were unaltered (Fig. 3A). Vitamin C levels were unaltered in the cerebellum on PND0 [$t(58) = 1.4183$, $P=.1615$], PND7 [$t(58) = 0.7860$, $P=.4351$], PND21 [$t(58) = 1.0566$, $P=.2951$] and PND60 [$t(58) = 0.8375$, $P=.4057$] (Fig. 3B).

3.2.3. Oxidative damage

MDA content, which indicates oxidative damage to lipids, was increased on PND0 [$t(58) = 5.6203$, $P<.001$]. On PND7 this effect was abolished [$t(58)=0.3763$, $P=.7081$]. On PND 21 [$t(58) = 1.125$, $P=.2652$] and PND60 [$t(58)=0.0842$, $P=.9332$], MDA levels remained unaltered (Fig. 3C). Sulfhydryl content was not altered in the cerebellum on PND0 [$t(40) = 0.5362$, $P=.5362$], PND7 [$t(40) = 1.4755$, $P=.1479$], and PND21 [$t(40)= 0.1366$, $P=.8920$]. On PND60, it was increased [$t(40) = 3.2833$, $P<.01$], which means decreased protein oxidation at this age (Fig. 3D). The carbonyl content, another indicator of protein oxidation, was unaltered in the cerebellum on PND0 [$t(46)= 0.7350$, $P=.4660$], PND7 [$t(46)= 0.7938$, $P=.4314$], PND21 [$t(46)= 0.9022$, $P= 0.3717$], and PND60 [$t(46) = 1.9559$, $P=.0565$] (Fig. 3E).

3.3. CR effects in the prefrontal cortex

3.3.1. Oxidants content and enzymatic antioxidants

In the prefrontal cortex, DCFH oxidation was increased on PND0 [$t(65) = 3.9333$, $P<.001$]. This alteration was shifted on PND7, when DCFH oxidation was decreased [$t(65) = 4.3959$, $P<.001$]. On PND 21 [$t(65) = 0.5473$, $P=.5860$] and PND 60 [$t(65) = 0.4331$, $P=.6663$] there was no alteration in this parameter (Fig. 4A).

SOD activity was unaltered on PND0 [$t(41) = 0.5122$, $P=.6112$], PND7 [$t(41) = 1.6050$, $P=.1161$], and PND 21 [$t(41) = 0.8177$, $P=.4182$]. On PND60, SOD activity was increased in the prefrontal cortex [$t(41) = 2.2190$, $P<.05$] (Fig. 4B). CAT activity was unaltered on PND0 [$t(40) = 1.0612$, $P=.2949$], while on PND7 it was activated [$t(44) = 2.6921$, $P<.05$]. On PND21 there was no alteration in CAT activity [$t(40) = 0.0396$, $P=.9686$], however, on PND60, the prefrontal cortex presented enhanced CAT activity [$t(40) = 2.8201$, $P<.01$] (Fig. 4C). GPx activity was unaltered on PND0 [$t(41) = 0.7276$, $P=.4709$], remained unaltered on PND7 [$t(41) = 0.7135$, $P=.4795$], PND 21 [$t(41) = 0.7189$, $P=.4762$], and PND60 [$t(41) = 1.0926$, $P=.2809$]. GPx presented lower activity in the prefrontal cortex when compared with other brain areas (Fig. 4D). On PND0, Grx activity in the prefrontal cortex was decreased [$t(42) = 4.2085$, $P<.05$], returning to control levels on PND7 [$t(42) = 1.5702$, $P=.1238$], remaining unaltered on PND21 [$t(42) = 0.0456$, $P=.9637$] and showing an increment on PND60 [$t(42) = 2.4012$, $P<.05$] (Fig. 4E). TrxR activity was increased in the prefrontal cortex on PND0 [$t(52) = 2.0780$, $P<.05$], returned to control levels on PND7 [$t(52) = 0.9689$, $P=.3370$], remained unaltered on PND 21 [$t(52) = 0.9617$, $P=.3407$] and was once again activated on PND60 [$t(52) = 3.3559$, $P<.01$] (Fig. 4F).

3.3.2. Non-enzymatic antioxidants

GSH levels were decreased in the prefrontal cortex on PND0 [$t(42) = 3.0240$, $P<.01$]. This effect was abolished on PND7 [$t(42) = 0.2080$, $P=.8362$] and, on PND21, GSH levels remained unaltered [$t(42) = 0.5464$, $P=.5877$]. On PND60, GSH levels were higher than control [$t(42) = 2.5759$, $P<.05$] (Fig. 5A). Vitamin C levels were unaltered in the prefrontal cortex on PND0 [$t(57) = 0.0289$, 0.9770], PND7 [$t(57) = 1.9459$, $P=.0566$], and PND21 [$t(57) = 0.2038$, $P=$

Pregnancy weight

Fig. 1. Effect of intrauterine caloric restriction (CR) on dam's weight gain during pregnancy. Results are expressed as mean±S.E.M. for $n=20$. *$P<.05$, **$P<.01$, ***$P<.001$ (multiple Student's *t* tests).

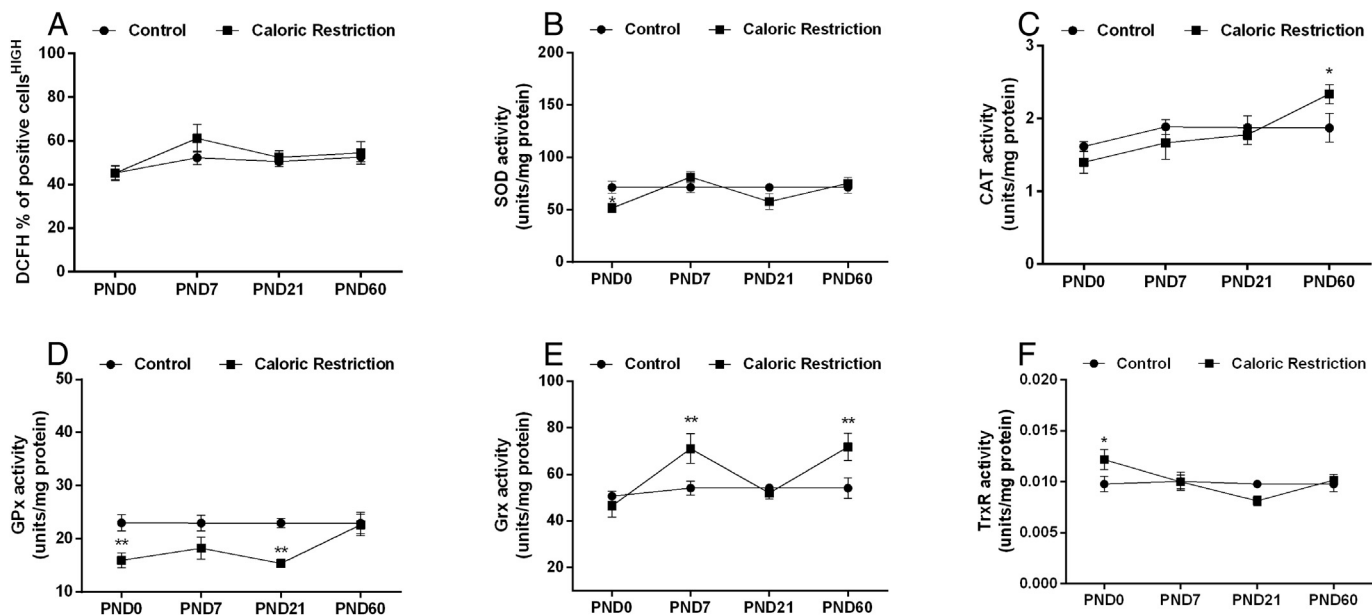V. Stone et al. / Journal of Nutritional Biochemistry 67 (2019) 138–148

Fig. 2. Effect of intrauterine caloric restriction (CR) on 2′7′-dichlorofluorescein (DCFH - A) oxidation, superoxide dismutase (SOD – B), catalase (CAT – C), glutathione peroxidase (GPx – D), glutaredoxin (Grx – E), and thioredoxin reductase (TrxR – F) activities in pups' cerebellum. Results are expressed as mean±S.E.M. for n=5–10. *P<.05, **P<.01 (multiple Student's t tests). PND: post-natal day.

.8392]. On PND60, vitamin C levels were increased [t(57) = 4.7682, P<.001] (Fig. 5B).

3.3.3. Oxidative damage

MDA content in the prefrontal cortex was unaltered on PND0 [t(57) = 0.0754, P=.9401]. On PND7, MDA content was decreased [t(57) = 2.0538, P<.05]. MDA levels remained decreased on PND21 [t(5.1219, P<.001] and PND60 [t(57) = 3.9589, P<.001] (Fig. 5C). There was no alteration on sulfhydryl content in the prefrontal cortex on PND0 [t(42) = 0.3261, P=.7460], PND7 [t(42) = 0.7146, P=.9433], PND21 [t(42) = 0.0551, P=.9563], and PND60 [t(42) = 0.9594, P=.3428] (Fig. 5D). Carbonyl content also showed no alteration on

PND0 [t(49) =1.3840, P=.1726], PND7 [t(49) = 0.0076, P=.9939], PND21 [t(49) =1.4236, P=.1609], and PND60 [t(49) = 0.6870, P=.4952] (Fig. 5E).

3.4. CR effects in the hippocampus

3.4.1. Oxidants content and enzymatic antioxidants

In the hippocampus, DCFH oxidation was increased on PND0 [t(60) = 3.4572, P<.01] and unaltered on PND7 [t(60) = 1.0353, P=.3046] and PND21 [t(60) = 1.4395, P=.1552]. On PND60, it was increased [t(60) = 2.8414, P<.01] (Fig. 6A).
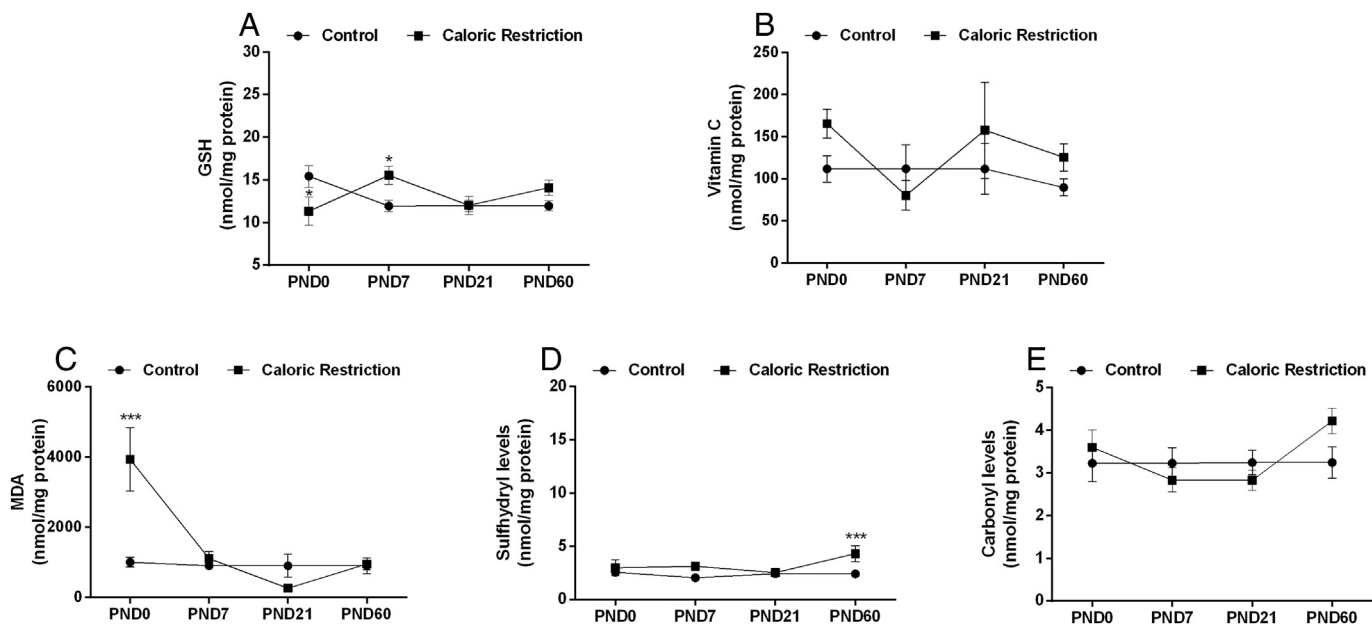

Fig. 3. Effect of intrauterine caloric restriction (CR) on reduced glutathione (GSH – A), vitamin C (B), malondialdehyde (MDA – C), sulfhydryl (D), and carbonyl (E) levels in pup's cerebellum. Results are expressed as mean±S.E.M. for n=5–10. *P<.05, ***P<.001 (multiple Student's t tests). PND: post-natal day.
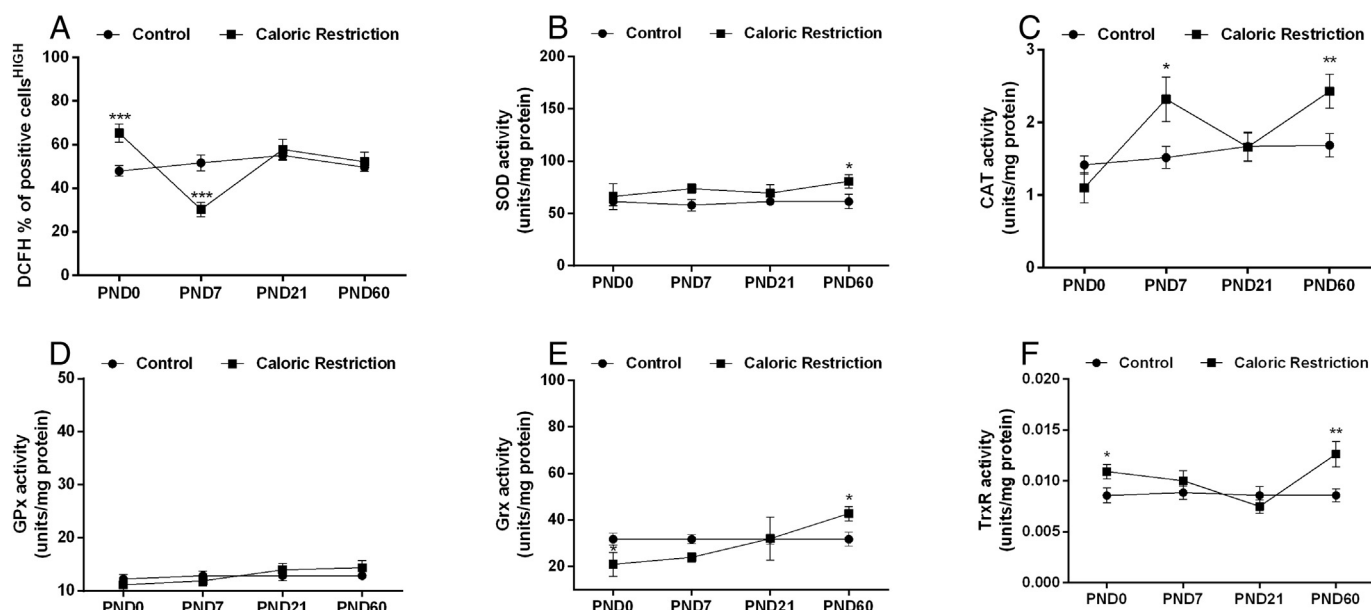
Fig. 4. Effect of intrauterine caloric restriction (CR) on 2′7′-dichlorofluorescein (DCFH - A) oxidation, superoxide dismutase (SOD – B), catalase (CAT – C), glutathione peroxidase (GPx – D), glutaredoxin (Grx – E), and thioredoxin reductase (TrxR – F) activities in pups' prefrontal cortex. Results are expressed as mean±S.E.M. for n=5–12. *P<.05, **P<.01, ***P<.001 (multiple Student's t tests). PND: post-natal day.

SOD hippocampal activity was unaltered on PND0 [t(37) =1.4953, P=.1432], increased on PND7 [t(37) = 2.3580, P<.05] and on PND21 [t(37) = 2.3580, P<.001]. On PND60, the activation was abolished [t(37) = 0.1418, P=.8880] (Fig. 6B). CAT activity was unaltered in the hippocampus on PND0 [t(47) = 0.7163, P=.4773], whereas, on PND7, it was activated [t(47) = 2.7671, P<.01]. On PND21, the activation was eradicated [t(47) = 1.3945, P=.1697] and its activity remained at control levels on PND60 [t(47) = 0.2504, P=.8033] (Fig. 6C). GPx hippocampal activity was unaltered on PND0 [t(43) = 1.1076, P=.2742], and remained unaltered on PND7 [t(43) = 0.8832, P=.3819] and PND 21 [t(43) = 0.4358, P=.665]. On PND60, the enzyme was activated [t(43) = 3.8457, P<.001] (Fig. 6D). Grx hippocampal activity was unaltered on PND0 [t(43) =

0.3265, P=.7456], PND7 [t(43) = 0.6129, P=.5431] and PND21 [t(43) = 0.5540, P=.5824]. On PND 60 the enzyme was activated, showing levels higher than control [t(43) = 3.7267, P<.001] (Fig. 6E). TrxR was not altered in the hippocampus on PND0 [t(53) = 0.8789, P= .3834], PND7 [t(53) = 0.9699, P=.3365], PND21 [t(53) = 0.7779, P= .4401], or PND60 [t(53) = 1.7287, P=.0896] (Fig. 6F).

3.4.2. Non-enzymatic antioxidants

In the hippocampus, GSH showed no alteration on PND0 [t(45) = 1.1299, P=.2645], and PND60 [t(45) = 0.4578, P=.6493] (Fig. 7A). Hippocampal vitamin C levels were decreased on PND0 [t(59) = 2.2155, P<.5] and PND7 [t(59) = 2.3098, P<.05]. On PND21 [t(59) =
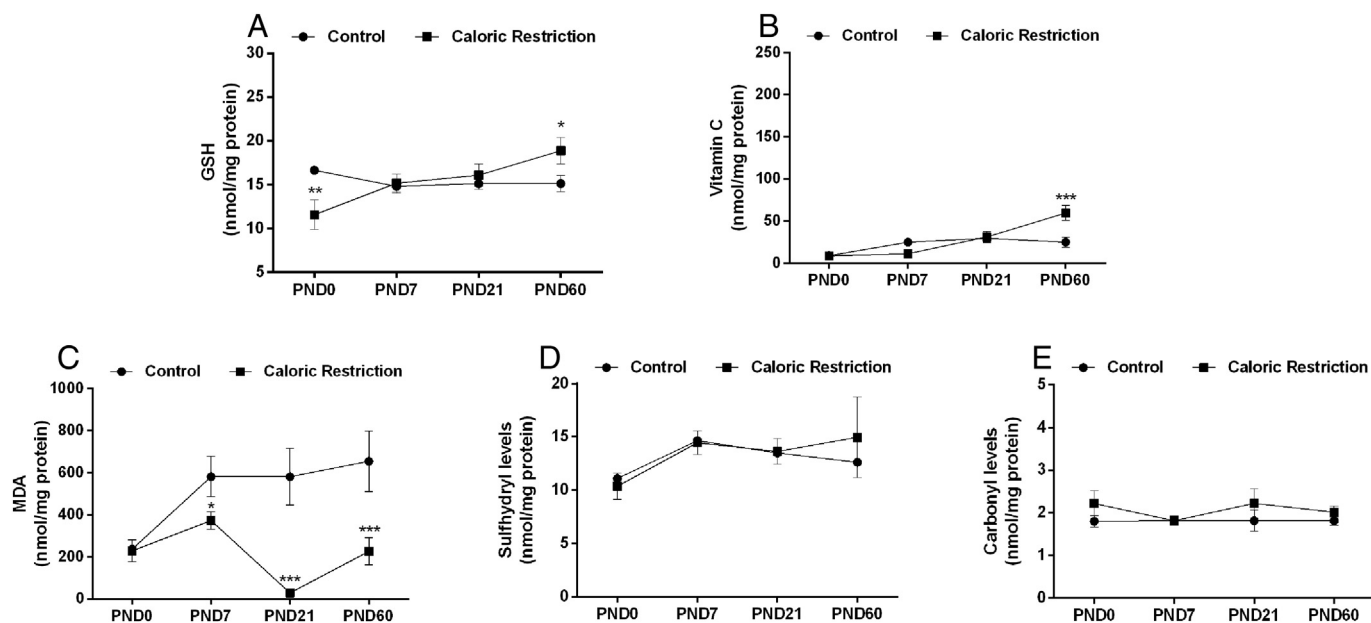

Fig. 5. Effect of intrauterine caloric restriction (CR) on reduced glutathione (GSH – A), vitamin C (B), malondialdehyde (MDA – C), sulfhydryl (D), and carbonyl (E) levels in pup's prefrontal cortex. Results are expressed as mean±S.E.M. for n=5–10. *P<.05, **P<.01, ***P<.001 (multiple Student's t tests). PND: post-natal day.
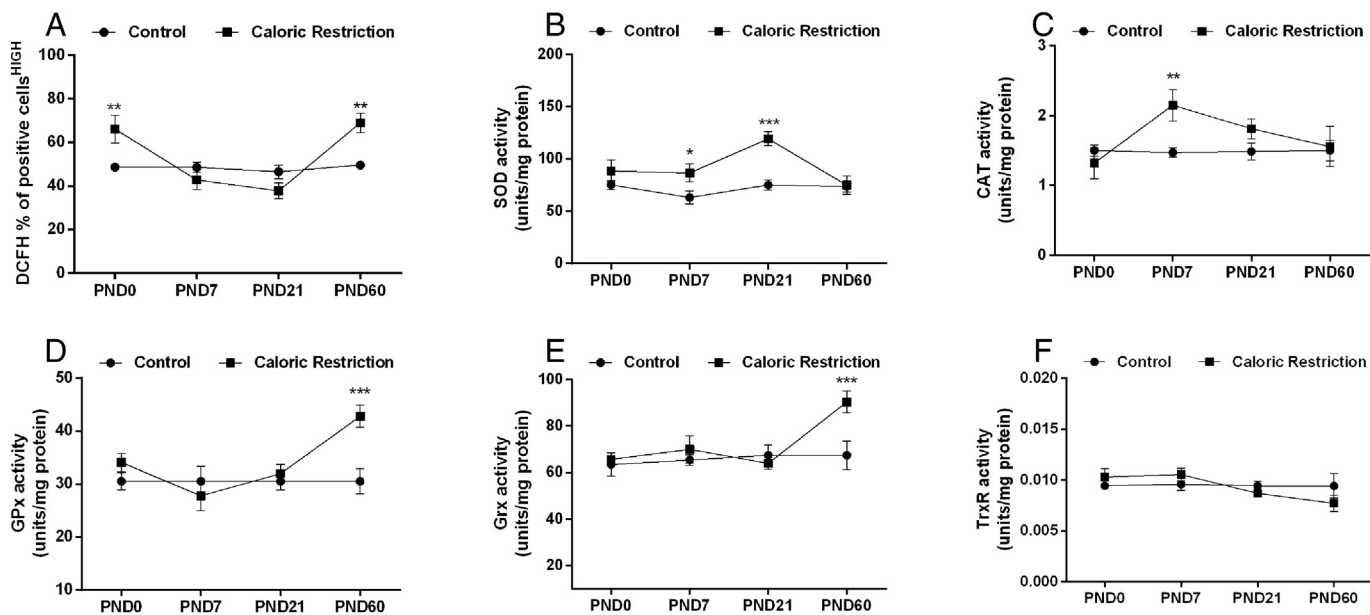
Fig. 6. Effect of intrauterine caloric restriction (CR) on 2′7′-dichlorofluorescein (DCFH – A) oxidation, superoxide dismutase (SOD – B), catalase (CAT – C), glutathione peroxidase (GPx – D), glutaredoxin (Grx – E), and thioredoxin reductase (TrxR – F) activities in pups' hippocampus. Results are expressed as mean±S.E.M. for n=5–11. *P<.05, **P<.01, ***P<.001 (multiple Student's t tests). PND: post-natal day.

1.5388, P=.1292] and PND60 [t(59) = 1.6944, P=.0955] there was no statistical significant alteration (Fig. 7B).

3.4.3. Oxidative damage

MDA content in the hippocampus was increased on PND 0 [t(58) = 10.0886, P<.001]. This effect was abolished on PND7 [t(58) = 0.2364, P=.8139] and remained unaltered on PND21 [t(58) = 0.4235, P= .6735] and PND60 [t(58) = 0.1976, P=.8441] (Fig. 7C). Sulfhydryl content showed no alteration in the hippocampus on PND0 [t(38) = 1.2312, P=.2258], PND7 [t(38) = 1.9070, P=.06410], PND21 [t(38) = 0.2574, P=.7983], and PND60 [t(38) = 0.0628, P=.9502] (Fig. 7D). Carbonyl content was increased on PND0 [t(48) = 2.8212, P= .01], unaltered on PND7 [t(48) = 0.6868, P=.4955] and PND21

[t(48) = 0.9038, P=.3706]; while this effect was reversed on PND60, when protein oxidation, represented by carbonyl content, was decreased [t(48) = 2.3067, P<.05] (Fig. 7E).

3.5. CR effects in the hypothalamus

3.5.1. Oxidants and enzymatic antioxidants

On PND0, DCFH oxidation was increased in the hypothalamus [t (55) = 2.7592, P<.01]. On PND7, oxidants returned to control levels [t (55) = 0.9677, P=.3373] and remained unaltered on PND 21 [(55) = 0.8941, P=.3751] and PND60 [t(55) = 0.8216, P=.4148] (Fig. 8A).
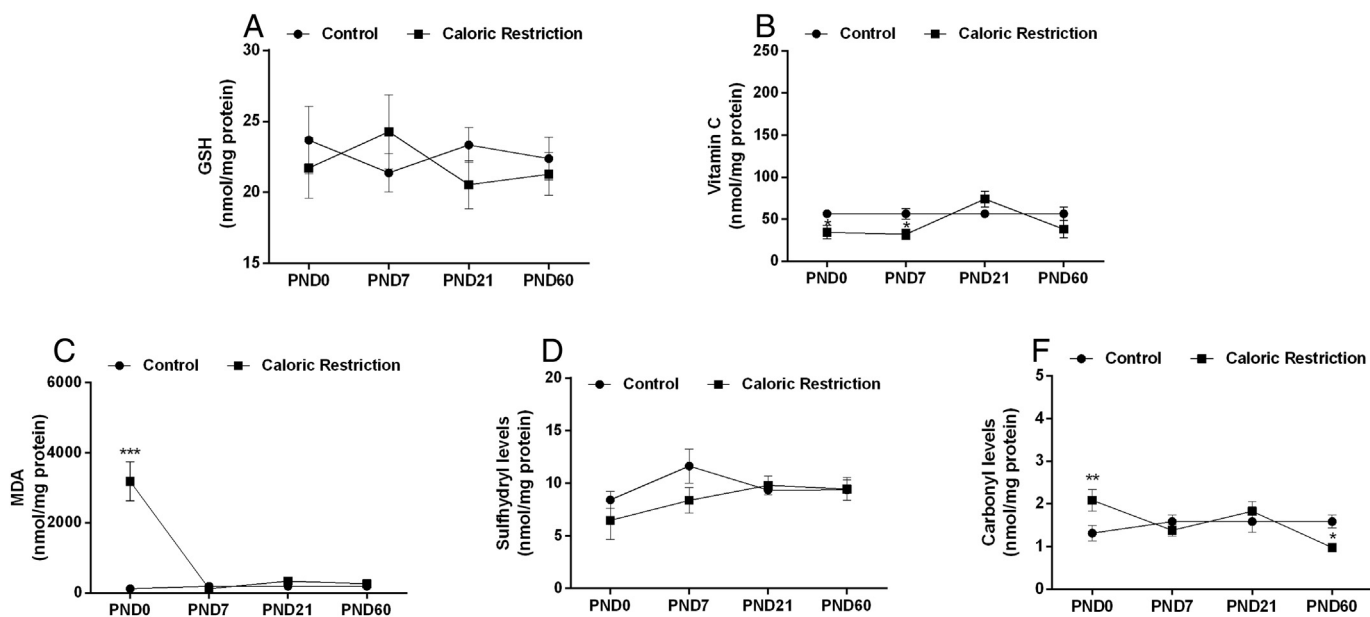

Fig. 7. Effect of intrauterine caloric restriction (CR) on reduced glutathione (GSH – A), vitamin C (B), malondialdehyde (MDA – C), sulfhydryl (D), and carbonyl (E) levels in pup's hippocampus. Results are expressed as mean±S.E.M. for n=4–10. **P<.01, ***P<.001 (multiple Student's t tests). PND: post-natal day.
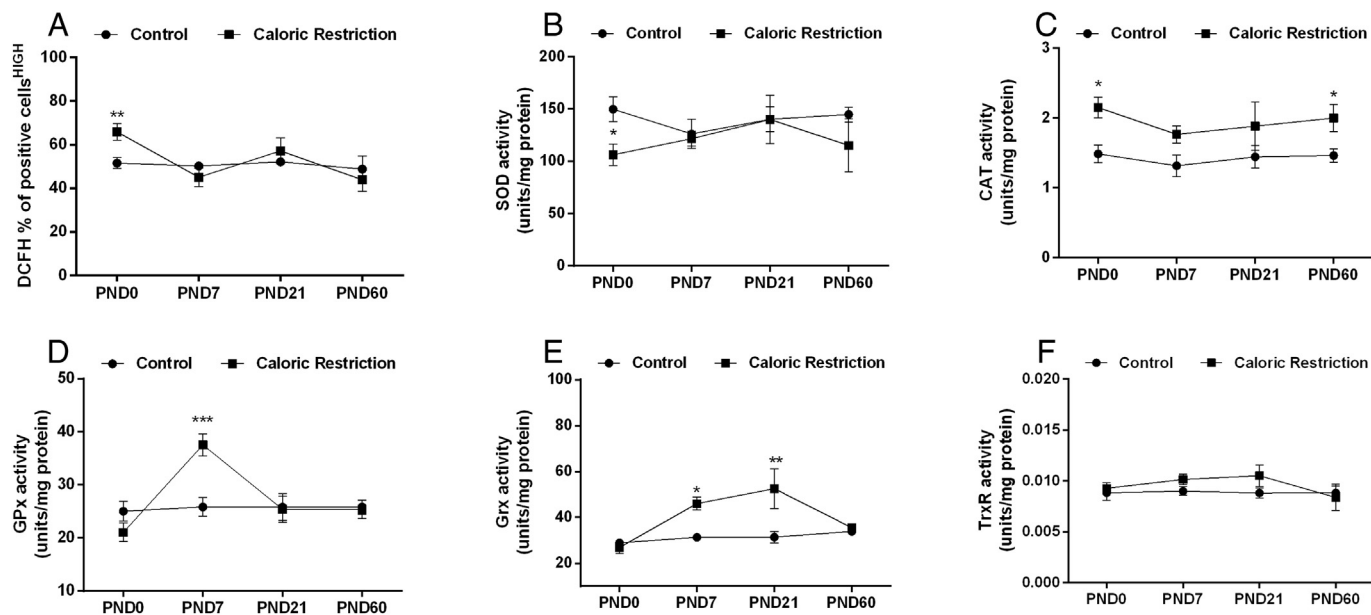
Fig. 8. Effect of intrauterine caloric restriction (CR) on 2′7'-dichlorofluorescein (DCFH - A) oxidation, superoxide dismutase (SOD – B), catalase (CAT – C), glutathione peroxidase (GPx – D), glutaredoxin (Grx – E), and thioredoxin reductase (TrxR – F) activities in pups' hypothalamus. Results are expressed as mean±S.E.M. for n=4–11. *P<.05, **P<.01, ***P<.001 (multiple Student's t tests). PND: post-natal day.

SOD was decreased in the hypothalamus on PND0 [t(38) = 2.0744, P<.05] and went back to control levels on PND7 [t(38) = 0.2252, P=.8230]. Its activity remained unaltered on PND21 [t(38) = 0.0096, P=.9923] and PND60 [t(38) = 1.5350, P=.1331] (Fig. 8B). In the hypothalamus, CAT activity was increased on PND0 [t(42) = 2.3552, P<.05]. On PND7 [t(42) = 1.7321, P=.9059] and PND21 [t(42) = 1.7594, P=.0857], CAT activity was unaltered. CAT was activated on PND60, presenting levels higher than control [t(42) = 2.2117, P<.05] (Fig. 8C). GPx hypothalamic activity was unaltered on PND0 [t(41) = 1.4245, P=.1618] and increased on PND7 [t(41)=4.2288, P<.001]. On PND21 [t(41) = 0.1366, P=.8919] and PND60 [t(41) = 0.1819, P=.8565] this enzyme showed control levels (Fig. 8D). Hypothalamic Grx

activity was unaltered on PND0 [t(38) = 0.3778, P=.7076] and increased on PND7 [t(38) = 2.8744, P<.01]. On PND21, the activation was maintained [t(38) =4.0029, P<.001], returning to control levels on PND60 [t(38) = 0.3142, P=.7550] (Fig. 8E). TrxR activity was unaltered in the hypothalamus on PND0 [t(52) = 0.4234, P=.6737], PND7 [t(52) = 1.0729, P=.2882], PND21 [t(52) = 1.6624, P=.1024], and PND60 [t(52) = 0.3961, P=.6936] (Fig. 8F).

3.5.2. Non-enzymatic antioxidants

Hypothalamic GSH levels were decreased on PND0 [t(42) = 3.1480, P<.01]. This effect was shifted on PND7, when GSH levels were increased [t(42) = 3.2346, P<.01]. On PND21, GSH levels
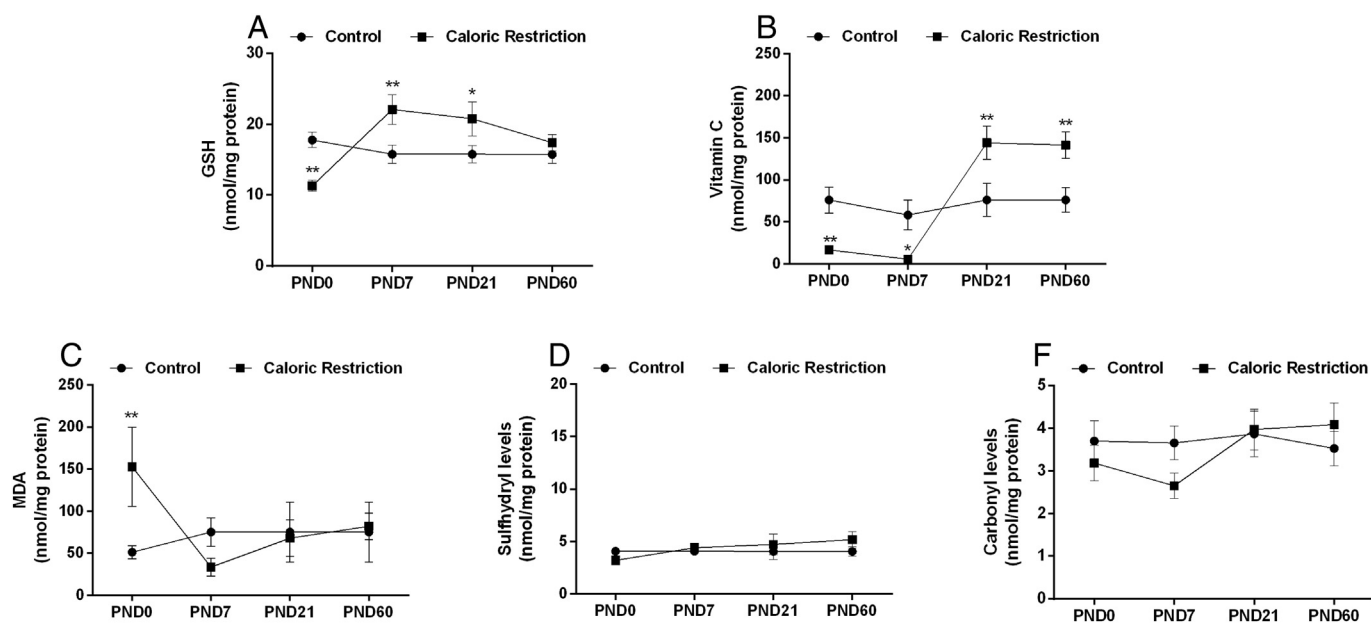

Fig. 9. Effect of intrauterine caloric restriction (CR) on reduced glutathione (GSH – A), vitamin C (B), malondialdehyde (MDA – C), sulfhydryl (D), and carbonyl (E) levels in pup's hypothalamus. Results are expressed as mean±S.E.M. for n= 4–10. *P<.05, **P<.01 (multiple Student's t tests). PND: post-natal day.

remained increased [$t(42) = 2.1272$, $P<.05$]. On PND60, GSH levels went back to control [$t(42) = 0.9508$, $P=.3471$] (Fig. 9A). Vitamin C levels were decreased in the hypothalamus on PND0 [$t(56) = 3.0701$, $P<.01$] and PND7 [$t(56) = 2.3158$, $P<.05$]. On PND21, this effect was shifted and hypothalamic vitamin C levels were increased [$t(56) = 3.2029$, $P<.01$]. On PND60, this vitamin levels remained increased [$t(56) =2.9995$, $P<.01$] (Fig. 9B).

3.5.3. Oxidative damage

MDA content was increased in the hypothalamus on PND0 [$t(58) = 2.7514$, $P<.001$]. Nevertheless, it was unaltered on PND7 [$t(58) = 1.0924$, $P=.2732$], PND21 [$t(58) = 0.1850$, $P=.8540$], and PND60 [$t(58) = 0.1718$, $P=.8642$] (Fig. 9C). Sulfhydryl content was unaltered in the hypothalamus on PND0 [$t(47) = 1.1543$, $P=.2542$], PND7 [$t(47) = 0.4779$, $P=.6349$], PND21 [$t(47) = 0.7221$, $P=.4738$], and PND60 [$t(47) = 1.5171$, $P=.1359$] (Fig. 9D). Carbonyl content was also unaltered on PND0 [$t(45) = 0.7762$, $P=.4417$], PND7 [$t(45) = 1.5702$, $P=.1234$], PND21 [$t(45) = 0.1633$, $P=.8710$], and PND60 [$t(45) = 0.9397$, $P=.3524$] (Fig. 9E).

4. Discussion

Gestational CR appears to modulate oxidants generation, enzymatic and non-enzymatic antioxidant defenses, leading to alterations in biomolecule oxidation in offspring's brains. DCFH oxidation was increased in most structures on PND0, showing increased oxidants content, returning to control or decreased levels in later stages of development. CR in adult mice increased lifespan, especially by decreasing oxidants generation, and boosting antioxidant defenses through different mechanisms [46], the decrease in oxidants content at the later stages of development may be linked to improved antioxidant network, elicited by increased oxidants production in the early stages of life.

Concerning to enzymatic antioxidant network, SOD activity was reduced in most structures evaluated at birth and either returned to control levels or showed increased activity during development; while CAT activity was increased both in the earlier ages and in adulthood. GPx activity was decreased in the cerebellum during development, but returned to control levels at adult age, in other structures it was similar to control. In the hippocampus, this enzyme was activated on PND60. Grx activity was similar to control at birth (except for a decrement in prefrontal cortex) and increased its activity through development, indicating activation throughout life. TrxR was similar to control in all structures, except for the increased activity found in the cerebellum (PND0) and prefrontal cortex (PND0 and PND60). In a general way, our results show an increased antioxidant profile. Data from literature shown 25% food restriction (aiming malnutrition) elicited no alteration in antioxidant enzymes in mice [47], while 40% food restriction promoted a severe redox unbalance [27]. This comparison highlights the importance of malnutrition prevention on CR protocols.

The main non-enzymatic antioxidant, GSH, presented a similar profile, showing lower levels at birth and increased content in the latter ages, except for the hippocampus, which showed similar levels to control at all ages evaluated. CR showed to increase GSH content in the hippocampus of adult rats [48] and in adult pups that went through gestational food deprivation [47]. Vitamin C levels were modulated in all brain structures evaluated, except for the cerebellum. This vitamin showed to be either unaltered or decreased on the early ages. In the later stages of development, including the adult age (PND60), vitamin C had increased levels, except for the hippocampus, where the negative effect of the early ages was abolished, but not reversed. There is no data available in the literature assessing vitamin C levels in the offspring of restricted pregnant rats, except for a work of our group, which found no alteration at all on 40% food restricted PND0 pups' cerebellum and total cerebral cortex [27].

The oxidative damage to lipids was significantly increased in the day of delivery in all brain structures evaluated, except for the prefrontal cortex. This structure showed no alteration at birth and MDA was decreased from PND7 to PND60. In addition, the unbalance in antioxidant defenses in the earlier stages of development was capable to promote oxidative damage to proteins on the hippocampus on PND0. On PND60, however, the effect was reversed. On the cerebellum, we also observed decreased oxidative damage to proteins in the adult age.

Redox homeostasis parameters were shown to be affected by maternal CR, including a previous work performed by our group, which showed 40% food restriction promotes an unbalance in brain redox homeostasis, markedly by decreased activity in the main antioxidant enzymes on the day of delivery [27]. Impaired brain redox homeostasis caused by 40% food restriction was also reported elsewhere [49] and moderate food restriction (25%) was shown to decrease antioxidant enzymes expression but not activity in mice [50]. Although these works evaluated oxidative damage to lipids and protein, these parameters showed no alteration. Indeed, it is important to note that most of the works applying gestational CR, including all references cited here, ignored one part of its concept, which is malnutrition prevention by micronutrient ingestion balance between groups [10,11]. Actually, absence of malnutrition prevention is common in studies utilizing adult animals as well. Despite that, there is plenty of evidence showing the beneficial effects of CR in adult animals, even without malnutrition prevention [9,51,52] although the outcomes, and especially the mechanisms involved are different [7].

On the other hand, literature also shows the detrimental effects of food restriction (when malnutrition is not controlled) during pregnancy on the offspring. Mayeur et al. [53] showed that 30% food restriction during pregnancy promotes mitochondrial abnormalities in rat placenta, increasing biogenesis but reducing bioenergetics efficiency. Ramírez-Lopez et al. showed that 20% maternal food restriction led to increased bodyweight and obesity in adult age, probably by disrupting the endocannabinoid system [54]. Moreover, fetal undernutrition is well stablished as a factor which increases insulin resistance. Kwon showed in a review the metabolic aspects and also epidemiological studies in this way [55].

Altogether, our results suggest that antioxidant enzymes increased during development in response to increased oxidants content in the earlier ages, conferring protection to the brain. It is known that CR in adult animal models upregulates the nuclear factor (erythroid-derived 2)-like 2 (NRF2) pathway, which is responsible for directly increasing enzymatic antioxidant defenses [56]. Moreover, NRF2 also shows relevant role on activating enzymes responsible for GSH metabolism [57]. Since NRF2 acts on enzymatic and non-enzymatic antioxidant defenses, evaluating its immunocontent on gestational CR is a current perspective of work and may clear by which mechanism the adaptation was carried out. However, we could not exclude other cell signaling mechanisms. CR in adult animal models is well stablished as the main strategy to increase life and health span, acting on several pathways and molecular targets, especially energy sensors such as sirtuins (SIRT), markedly SIRT1, and AMP-dependent protein kinase (AMPK). Both SIRT and AMPK activate peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). AMPK also downregulates target of rapamycin (TOR) and activates forkhead box O (FoxO) factors [9]. This signaling modulation contribute to increased redox homeostasis and mitochondrial function, thus, considering positive redox effects found in the present research, investigating the mechanisms involved, including the pathways described above is a direction to be followed in order to clear CR effects during pregnancy.

4.1. Conclusion

CR during pregnancy is commonly associated to negative outcomes, but our data shows that a low intensity CR, associated to

responsible malnutrition prevention by micronutrients supplementation, protects the litter brain against oxidative stress through hormetic adaptation after birth. Whether our data are confirmed in clinical conditions, CR could be applied as a successful and inexpensive neuroprotective strategy.

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Disclosure statement

The authors report no conflict of interest. The authors are exclusively responsible for the content and writing of this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnutbio.2019.02.002.

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4. Capítulo II

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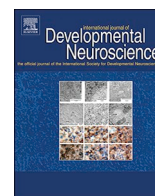
Adaptive effects of gestational caloric restriction in the mitochondria of Wistar rats' brain: A DOHaD approach

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ABSTRACT

Developmental origins of health and disease (DOHaD) is a field of biological science dedicated to investigating how different interventions during development affect an individual's life. Diet is an essential way to interact with the environment, and during pregnancy affects not only the mother but also can impact the next generations. One of these interventions is caloric restriction (CR), which has shown positive redox modulation in rats' offspring when malnutrition is responsibly controlled. Considering that mitochondrial metabolism is determinant for redox status, we investigated parameters related to mitochondrial functionality and reactive species levels in offspring's brain from rats delivered to pregnant caloric restricted dams. Therefore, pregnant rats were divided between control (*ad libitum* food) and CR (20% food restriction plus micronutrients supplementation) groups, and offspring's brain was analyzed on post-natal days (PND) 0, 7, 21, and 60. Mitochondrial function, as well as superoxide content, were decreased in most brain areas on PND0 and went through adaptation, showing increased mass and membrane potential in adulthood. Concerning mitochondrial electron transport system (METS), the most affected area was the cerebellum, which was impaired at birth and activated at adulthood. In conclusion, our results show that gestational CR promotes adaptation from impaired mitochondrial parameters at birth, improving mitochondrial function when compared to control, without increasing superoxide generation, at adult age. More studies are necessary in order to support the use of CR as a clinical approach.

1. Background

In the past few years, developmental origins of health and disease (DOHaD) became a very debated theme in biological science, it occurs because of the importance that lies in taking care of an individual since conception. DOHaD studies consider that the first 1000 days of development (since conception up to 2 years of age) are the main window of vulnerability or opportunity in humans to modulate the risk of several non-transmissible chronic diseases development (Wadhwa et al., 2009). This concept evolved from Barker's studies on the thrifty phenotype, or Barker's hypothesis (Barker et al., 1989). Our group already showed how several different interventions may influence the offspring health

when applied during pregnancy, such as Caloric Restriction (CR) (Stone et al., 2019), food restriction (FR) (Stone et al., 2016), high-salt diet (Stocher et al., 2018), polyphenol supplementation (August et al., 2018), and swimming exercise (August et al., 2018; Klein et al., 2018, 2019; Marcelino et al., 2013).

Caloric restriction has already been proved to increase health and life span in several animal models, from yeast to primates, and the mechanism often involves pathways that modulate redox homeostasis and mitochondrial activity. It is well established that mitochondria are the powerhouse of the cell, the organelle responsible to generate ATP through oxidative phosphorylation. However, during energy generation, mitochondria also generate oxidants, especially superoxide anion,

Table 1
Micronutrients content in 10 g of chow or 5 mL of formula.

Nutrient	Weight (10 g of chow or 5 mL of formula)
Ca	120 mg
P	80 mg
Vit A (Retinol)	39 µg
Vit D3 (Calcitriol)	0.49 µg
Vit E (Tocoferol)	226 µg
Vit K3 (Phylloquinone)	30 µg
Vit B1 (Thiamin)	50 µg
Vit B2 (Riboflavin)	60 µg
Vit B6 (Pyridoxin)	70 µg
Vit B12 (Cobalamin)	0.22 µg
Vit B3 (Niacin)	600 µg
Vit B5 (Pantothenic acid)	200 µg
Vit B9 (Folic acid)	10 µg
Biotin	0.5 µg
Choline	19 mg
Na	27 mg
Fe	500 µg
Mn	600 µg
Zn	600 µg
Cu	100 µg
Se	0.5 µg
Co	15 µg
F	800 µg

which may cause cellular damage (Morgan et al., 2017). Effects of CR on mitochondrial metabolism include increased calcium retention capacity, which confers protection against excitotoxicity, increased mitochondrial biogenesis, and bioenergetics efficiency (Amigo et al., 2017; Amigo and Kowaltowski, 2014; Lopez-Lluch et al., 2006; Lopez-Lluch and Navas, 2016; Ruetenik and Barrientos, 2015).

Beyond the proved effects of CR on health and life span, and also the importance of promoting nutritional interventions during the development period, we performed this study considering that laboratory animals used as control are sedentary and overfed (Martin et al., 2010). Lamentably, this condition is similar to the general human population and also to pregnant women around the world, taking into account that obesity ratio between pregnant women is growing (Fisher et al., 2013) along with obese children population (de Onis et al., 2010). It is always important to state that CR protocol should not promote malnutrition, thus, considering equal or similar micronutrients consumption when comparing to control animals (Stone et al., 2019; McCay et al., 1935; Cerqueira and Kowaltowski, 2010).

Considering presented evidence on how CR acts upon mitochondrial metabolism and the principles of the DOHaD concept, we aimed to assess how mitochondria react to CR during gestation in different stages of pups' life. Moreover, our aim in this study was to perform a screening on mitochondrial function, METS complexes activity and oxidants generation from birth to adulthood.

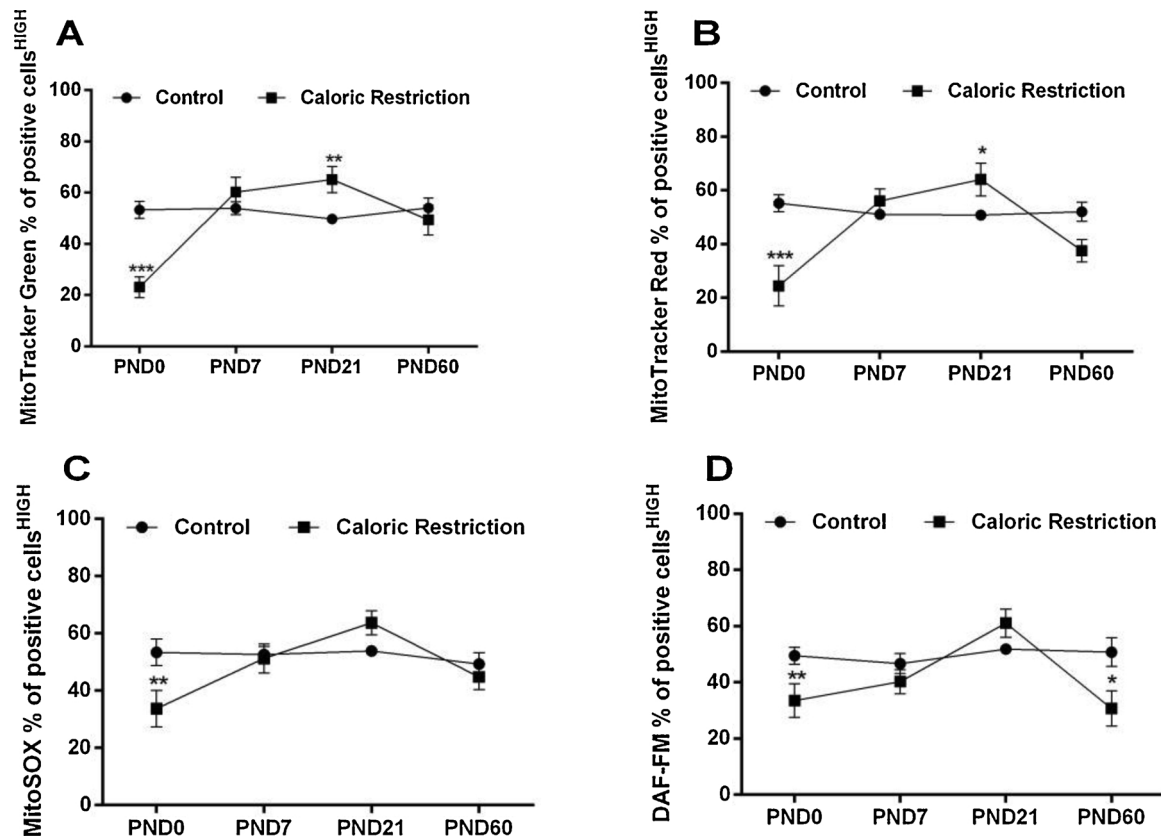**Fig. 1.** Effect of intrauterine caloric restriction (CR) on MitoTracker Green ® (A); MitoTracker Red ® (B); MitoSOX ® (C) and DAF-FM ® (D) % of positive cells in pups' cerebellum. Results are expressed as mean ± S.E.M. for n = 5-12. *p < 0.05, **p < 0.01. ***p < 0.001 (multiple Student's *t* tests). PND: post-natal day. Representative histograms are presented on Supplementary Figs. 1–4.

2. Methods

2.1. Animals and reagents

Female adult Wistar rats (120 days-of-age, nulliparous, weighing approximately 230 g, 50 animals) 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. Conception diagnosis was performed by the presence of spermatozoids and absence of leucocytes in vaginal smears. Three female rats diagnosed as pregnant were housed per cage in order to start the treatment. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. 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 30044, 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, and Invitrogen by Thermo Fischer Scientific, Carlsbad, CA, USA.

2.2. Caloric restriction (CR) protocol

CR group had free access to water and received the same commercial chow as the control group; however, the amount was reduced by 20%. The diet for CR group was adjusted daily by body weight, using the chow amount consumed by control animals as standard. The difference, in grams, between control group mean consumption and CR animal consumption was calculated every day. A formula containing micronutrients in the same proportion of commercial chow was administered to the animals, based on the difference of chow consumption, in order to equilibrate micronutrients consumption between groups (5 mL of formula contains the same micronutrients amount contained in 10 g of chow, Table 1). The micronutrients were solubilized on sucralose 0.1% and methylparaben 0.1% (vehicle). Control rats had free access to water and 22% (w/w) protein commercial chow. Dams assigned to the control group received the vehicle of micronutrients *via* oral gavage to avoid any unwanted interference.

CR dams were submitted to the CR protocol during the entire pregnancy. In the day of delivery, the pups were cross-fostered: pups from CR dams were housed with control dams and pups from control dams were housed with another control dam. Cross-fostering was applied in order to abolish the CR intervention after birth. Pups were weaned at PND21, and after weaning, all pups received *ad libitum* food. In order to avoid litter effect, and increase genetic variability, only one pup per litter was used in each biochemical analysis.

Diet and micronutrient mix composition are published elsewhere (Stone et al., 2019).

2.3. Sample obtaining

Male pups (130 animals) were euthanized by decapitation without anesthesia (in order to avoid chemical contamination of the sample that could alter the biochemical parameters evaluated) at PND0, PND7,
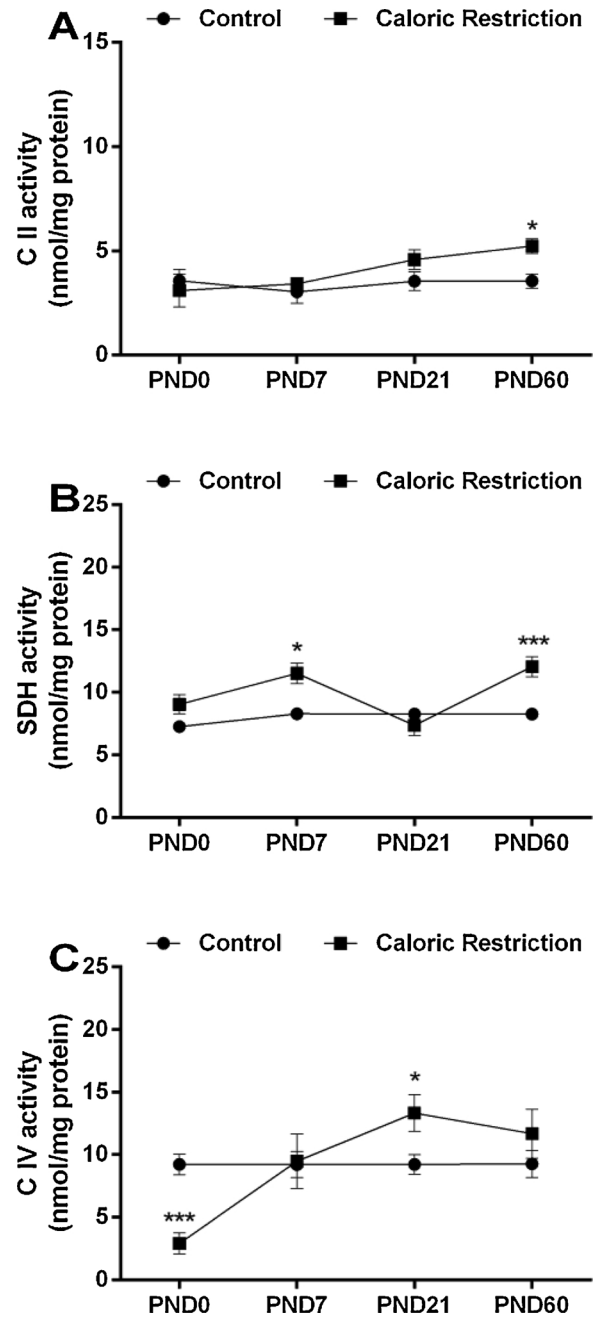
Fig. 2. Effect of intrauterine caloric restriction (CR) on complex II (A); succinate dehydrogenase (B) and complex IV (C) activities in pups' cerebellum. Results are expressed as mean ± S.E.M. for n = 4-8. *p < 0.05, ***p < 0.001 (multiple Student's *t* tests). PND: post-natal day.

PND21, and PND60. The brain was dissected; cerebellum, prefrontal cortex, hippocampus, and hypothalamus were rapidly isolated on a Petry dish on ice, according to the Atlas of the Developing Rat Brain (Paxinos, 1991). First, the cerebellum was separated from the cerebrum

by a transverse section. Pons and medulla were gently removed thereafter. The prefrontal cortex was considered as the third part of the cortex and separated by a transverse section. To isolate the prefrontal cortex, the olfactory lobe was gently removed, as well as all the internal content, including striatum. After, the hippocampus was separated completely, including all CA regions and the dentate gyrus. The hypothalamus was also completely separated, including the ventromedial and lateral hypothalamic regions, using the anterior commissure as horizontal reference.

All brain areas were stored at -80 °C for mitochondrial complexes evaluation. For flow cytometry assays, tissues were used fresh immediately after dissected.

2.4. Biochemical assays

2.4.1. Mitochondrial function parameters
2.4.1.1. Mitochondrial mass and membrane potential. Mitochondrial mass was analyzed using the probe MitoTracker® green, while mitochondrial membrane potential was measured using the probe MitoTracker® red, both purchased from Invitrogen®. Both analyzes above were performed in a FACScalibur flow cytometer (BD Biosciences®). The tissue samples (100 mg) were dissociated with 1 mL of phosphate saline buffer (PBS), pH 7.4, containing 1 mg% of collagenase IV and centrifuged at 1500 rpm for 5 min. The supernatant was discarded in order to avoid collagenase toxicity, resuspended in 400 μL PBS, filtered and incubated with the probe. One hundred microliters of each sample was incubated at 37 °C during 45 min in the presence of MitoTracker® green and red in a final concentration of 1 μM each. After that, 10,000 cells were evaluated per sample in the flow cytometer. Negative control was evaluated without probe addition, and the fluorescence was discounted from the samples. Data were analyzed using the software FlowJo®.

2.4.1.2. Oxidants production. Mitochondrial superoxide content 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®.

One hundred microliters of each sample was incubated at 37 °C during 20 min in the presence of MitoSox® red in a final concentration of 1 μM. One hundred microliters of each sample was incubated at 37 °C during 1 h in the presence of DAF-FM® in the final concentration of 10 μM. After that, 10,000 cells were evaluated per sample in the flow cytometer. Negative control was evaluated without probe addition, and the fluorescence was discounted from the samples. Data were analyzed using the software FlowJo®.

2.4.1.3. Mitochondrial electron transport system (METS) enzymes. METS enzymes activities, namely complex II, succinate dehydrogenase (SDH) and complex IV, were evaluated spectrophotometrically. The brain structures were homogenized (1:20w/v) in SETH buffer, pH 7.4, containing 250 mM sucrose, 2.0 mM EDTA and 10 mM Trizma. The homogenates were centrifuged at 800 × g for 10 min at 4 °C. The supernatant was collected and went through three freeze-thawing procedures.

Complex II (succinate: 2,6-dichloroindophenol oxireductase) and succinate dehydrogenase (EC 1.3.5.1) activities were measured according to Fischer et al. (Fischer et al., 1985) with some modifications. Complex II activity was measured based on the reduction of DCIP (8.3 μM) at 600 nm. Forty mM potassium phosphate buffer, pH7.4), 16 mM sodium succinate, 4 mM sodium azide, 7 μM were also added to the reaction media (approximately 40 μg of protein). Phenazine methasulfate (1 mM) was added to the media and the reaction was monitored at 600 nm in order to obtain the succinate dehydrogenase activity.

Complex IV (cytochrome c oxidase) activity was measured according to Rustin et al. (Rustin et al., 1994) with some modifications, based on the oxidation of cytochrome *c* (75 μM) at 550 nm. Potassium

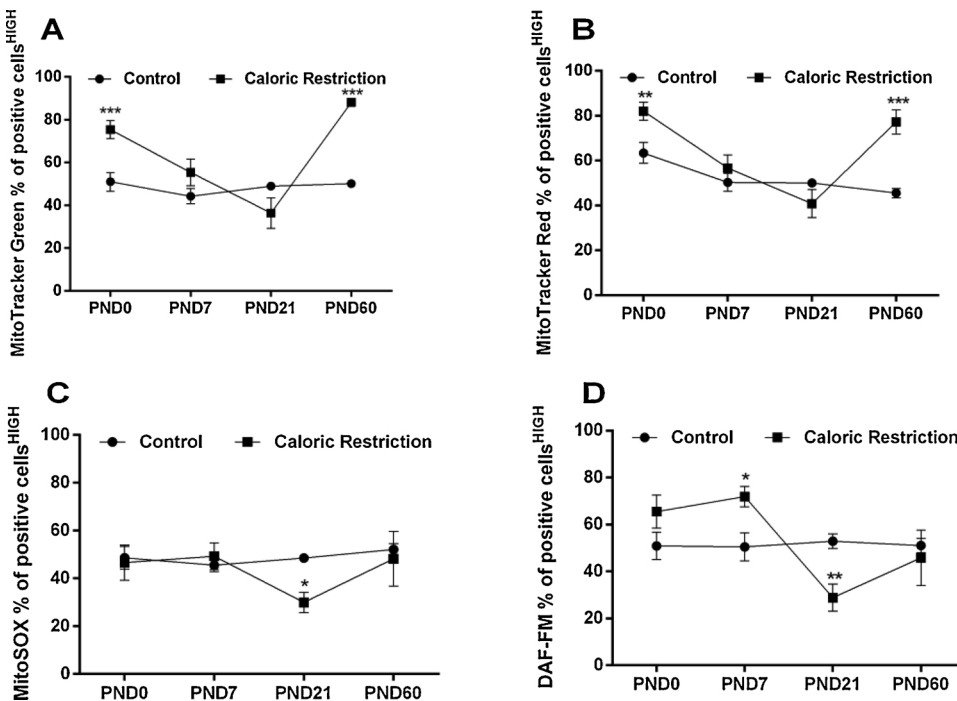**Fig. 3.** Effect of intrauterine caloric restriction (CR) on MitoTracker Green ® (A); MitoTracker Red ® (B); MitoSOX ® (C) and DAF-FM ® (D) % of positive cells in pups' prefrontal cortex. Results are expressed as mean ± S.E.M. for n = 4-12. *p < 0.05, **p < 0.01. ***p < 0.001 (multiple Student's *t* tests). PND: post-natal day. Representative histograms are presented on Supplementary Figs. 5–8.

phosphate buffer (10 mM), dodecylmaltoside (0.6 mM) and supernatant (approximately 1.5 µg of protein) were added to the incubation media. Results were expressed as nmol/min/mg protein.

2.4.2. Protein concentration assay

Protein concentration was measured by the method of Lowry et al. (Lowry et al., 1951), using bovine serum albumin as standard for the spectrophotometrical analysis.

2.4.3. Statistical analysis

Data were analyzed by multiple Student's t tests, using GraphPad Prism 6.0 software. Data were considered statistically significant when $p < 0.05$.

3. Results

3.1. Maternal CR effects in the offspring's cerebellum

On PND0, mitochondrial function was decreased in the cerebellum, evidenced by reduced mitochondrial mass [$t(54) = 5.5949$, $p < 0.0001$] (Fig. 1A) and membrane potential [$t(57) = 5.1992$, $p < 0.0001$] (Fig. 1B). On PND7, both, mass [$t(54) = 1.1409$, $p = 0.2589$] and membrane potential [$t(57) = 0.8458$, $p = 0.4012$], returned to control levels, while on PND 21 were higher than control [$t(54) = 2.7062$, $p < 0.01$; $t(57) = 2.1166$, $p < 0.05$]. Interestingly, on PND60 were at control levels again [$t(54) = 0.6679$, $p = 0.5070$; $t(57) = 1.7819$, $p = 0.0801$].

Regarding oxidants production, both mitochondrial superoxide [$t(64) = 3.3547$, $p < 0.01$] (Fig. 1C) and nitric oxide [$t(57) = 2.7943$, $p < 0.01$] (Fig. 1D) levels were decreased on PND0. Mitochondrial superoxide was comparable to control levels on PND7 [$t(64) = 0.2031$, $p = 0.8397$], PND21 [$t(64) = 1.5977$, $p = 0.1150$], and PND60 [$t(64) = 0.5928$, $p = 0.5554$]. Nitric oxide was similar to control at PND7 [$t(57) = 0.9309$, $p = 0.3558$] and PND21 [$t(57) = 1.4513$, $p = 0.1522$]. On PND60, it was decreased [$t(57) = 2.6528$, $p < 0.05$].

Complex II activity was unaltered on PND0 [$t(48) = 0.6480$, $p = 0.5201$], PND7 [$t(48) = 0.5571$, $p = 0.5800$], and PND21 [$t(48) = 1.5873$, $p = 0.1190$]. On PND60, complex II was increased [$t(48) = 2.5904$, $p < 0.05$] (Fig. 2A).

SDH activity was unaltered on PND0 [$t(48) = 1.3219$, $p = 0.1925$] and PND21 [$t(48) = 0.7276$, $p = 0.4704$], while it was increased on PND7 [$t(48) = 2.4431$, $p < 0.05$] and PND60 [$t(48) = 3.0752$, $p < 0.01$] (Fig. 2B).

Complex IV activity was decreased on PND0 [$t(47) = 3.0508$, $p < 0.01$], similar to control on PND7 [$t(47) = 0.1321$, $p = 0.8955$], increased on PND21 [$t(47) = 2.1171$, $p < 0.05$] and returned to control levels on PND60 [$t(47) = 1.2872$, $p = 0.2043$] (Fig. 2C).

3.2. Maternal CR effects in the offspring's prefrontal cortex

The prefrontal cortex was the only structure showing increased mitochondrial function on PND0, evidenced by mitochondrial mass [$t(52) = 4.1216$, $p < 0.001$] (Fig. 3A) and membrane potential [$t(53) = 3.4057$, $p < 0.01$] [(Fig. 3B). On PND7, both mass [$t(52) = 1.6977$, $p = 0.9555$] and membrane potential [$t(53) = 0.9597$, $p = 0.3415$] were unaltered. The same profile was identified on PND 21 [mitochondrial mass: $t(52) = 1.6706$, $p = 0.1009$; mitochondrial membrane potential: $t(53) = 1.3856$, $p = 0.1717$], while on PND60
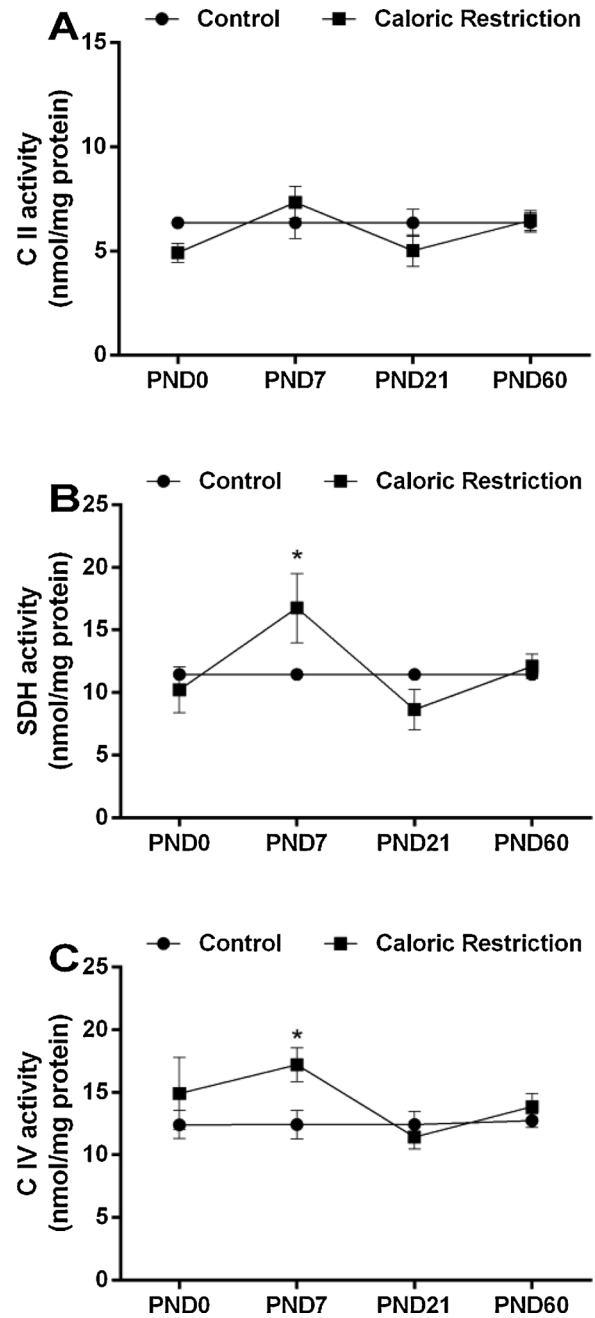

Fig. 4. Effect of intrauterine caloric restriction (CR) on complex II (A); succinate dehydrogenase (B) and complex IV (C) activities in pups' prefrontal cortex. Results are expressed as mean ± S.E.M. for n = 4-8. *$p < 0.05$, ***$p < 0.001$ (multiple Student's t tests). PND: post-natal day.

both mass [$t(52) = 4.1882$, $p < 0.001$] and membrane potential [$t(53) = 3.6870$, $p < 0.001$] were increased.

Mitochondrial superoxide content was decreased on PND21 [$t(55) = 2.0721$, $p < 0.05$], while on PND0 [$t(55) = 0.2544$, $p = 0.8002$], PND7 [$t(55) = 0.4542$, $p = 0.6515$], and on PND60 [t

(55) = 0.3807, p = 0.7049] there was no alteration (Fig. 3C).

Nitric oxide content was unaltered on PND0 [t(50) = 1.6994, p = 0.9546], increased on PND7 [t(50) = 2.5725, p < 0.05], decreased on PND21 [t(50) = 2.8023, p < 0.01], and returned to control levels on PND60 [t(50) = 0.5171, p = 0.6074] (Fig. 3D).

There was no alteration on Complex II activity on PND0 [t(46) = 1.4553, p = 0.1524], PND7 [t(46) = 1.1041, p = 0.2753], PND21 [t(46) = 1.6278, p = 0.1104] or PND60 [t(46) = 1.2723, p = 0.8993] (Fig. 4A).

SDH was increased on PND7 [t(48) = 2.6256, p < 0.05], while on PND0 [t(48) = 0.5873, p = 0.5597], PND21 [t(48) = 1.4932, p = 0.1419], and PND60 [t(48) = 0.3432, p = 0.7330] no alteration was verified (Fig. 4B).

Complex IV was also increased on PND7 [t(47) = 2.6282, p < 0.05] and showed no alteration on PND0 [t(47) = 1.3364, p = 1.1879], PND 21 [t(47) = 0.5567, p = 0.5804], and PND60 [t(47) = 0.6504, p = 0.5186] (Fig. 4C).

3.3. Maternal CR effects in the offspring's hippocampus

On PND0, mitochondrial function was impaired in the hippocampus, evidenced by mitochondrial mass [t(56) = 2.5010, p < 0.05] (Fig. 5A) and membrane potential [t(59) = 2.0017, p < 0.05] (Fig. 5B) decreased. On PND7, both mitochondrial mass [t(56) = 2.9536, p = 0.5575] and membrane potential returned to control levels [t(59) = 1.3231, p = 0.1909]. Mitochondrial mass [t(56) = 1.5454, p = 0.1279] and membrane potential [t(59) = 1.6978, p = 0.0948] remained unaltered on PND21, while on PND60 there was an increase for both [mass: t(56) = 3.4120, p < 0.01; membrane potential: t(59) = 2.9025, p < 0.01].

Mitochondrial superoxide content was unaltered on PND0 [t(60) = 0.9887, p = 0.3279] and PND21 [t(60) = 1.4969, p = 0.1396],

while was decreased on PND7 [t(60) = 2.2999, p < 0.05] and PND60 [t(60) = 5.1357, p < 0.0001] (Fig. 5C).

Nitric oxide content was increased on PND0 [t(55) = 2.1328, p < 0.05] and PND21 [t(55) = 2.3069, p < 0.05], decreased on PND7 [t(55) = 3.7749, p < 0.001] and showed no alteration on PND60 [t(55) = 1.4584, p = 0.1273] (Fig. 5D).

There was no alteration for complex II activity in this structure in neither of the ages evaluated: PND0 [t(47) = 0.1979, p = 0.8439], PND7 [t(47) = 1.0643, p = 0.2926], PND 21 [t(47) = 1.4964, p = 0.1412], and PND60 [t(47) = 0.4408, p = 0.6614] (Fig. 6A).

SDH was unaltered on PND0 [t(47) = 1.0148, p = 0.3154], PND21 [t(47) = 0.7413, p = 0.404], and PND60 [t(47) = 0.9019, p = 0.3717]. On PND7 it was increased [t(47) = 3.4718, p < 0.01] (Fig. 6B).

For complex IV also there was no alteration on PND0 [t(45) = 0.0250, p = 0.9801], PND7 [t(45) = 1.7974, p = 0.0790], PND21 [t(45) = 0.0275, p = 0.9781], or PND60 [t(45) = 0.2812, p = 0.7798] (Fig. 6C).

3.4. Maternal CR effects in the offspring's hypothalamus

In this structure, we also observed decreased mitochondrial mass [t(44) = 2.4758, p < 0.05] (Fig. 7A) and membrane potential [t(47) = 2.4572, p < 0.05] (Fig. 7B) on PND0. On PND7 mitochondrial mass returned to control levels [t(44) = 0.8172, p = 0.4182] while membrane potential remained decreased [t(47) = 2.5977, p < 0.05]. On PND21 mitochondrial mass was decreased [t(44) = 3.1797, p < 0.01] while membrane potential was unaltered [t(47) = 0.7450, p = 0.4599], while on PND60 both mitochondrial mass [t(44) = 3.2549, p > 0.01], and membrane potential [t(47) = 3.1126, p < 0.01] were higher than control.

Mitochondrial superoxide was decreased on PND0 [t
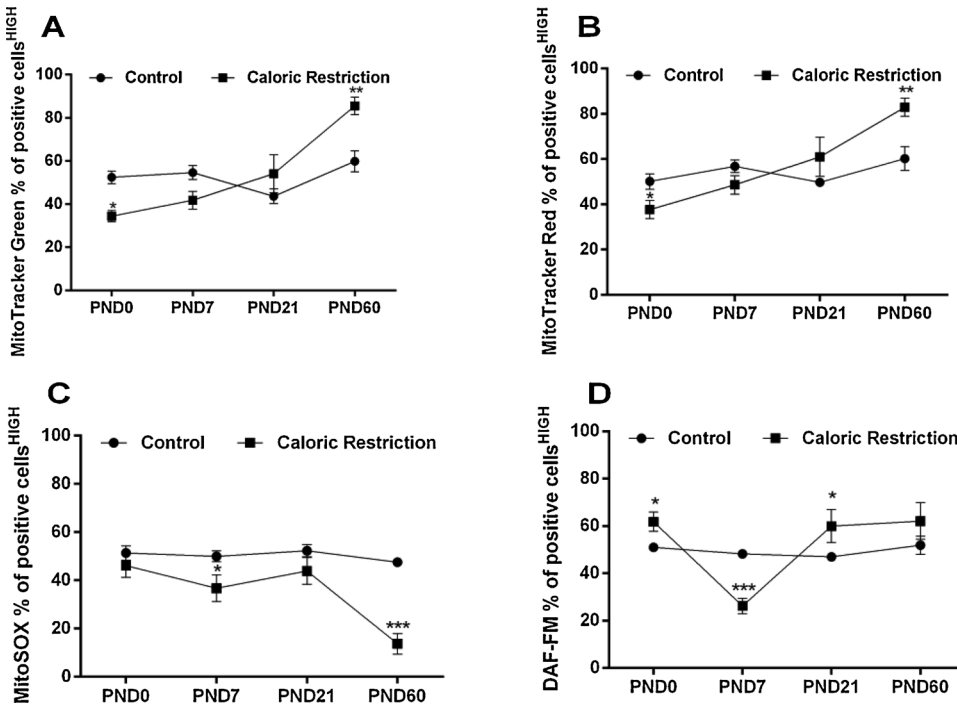

Fig. 5. Effect of intrauterine caloric restriction (CR) on MitoTracker Green ® (A); MitoTracker Red ® (B); MitoSOX ® (C) and DAF-FM ® (D) % of positive cells in pups' hippocampus. Results are expressed as mean ± S.E.M. for n = 5-11. *p < 0.05, **p < 0.01. ***p < 0.001 (multiple Student's *t* tests). PND: post-natal day. Representative histograms are presented on Supplementary Figs. 9–12.
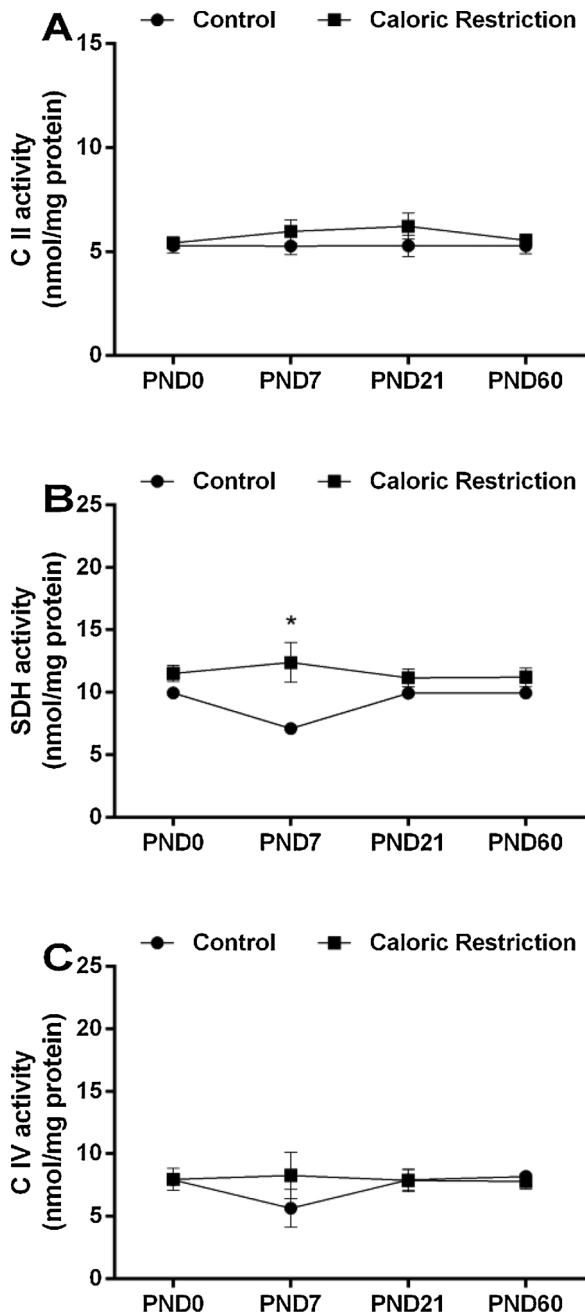
Fig. 6. Effect of intrauterine caloric restriction (CR) on complex II (A); succinate dehydrogenase (B) and complex IV (C) activities in pups' hippocampus. Results are expressed as mean ± S.E.M. for n = 5-8. *p < 0.05 (multiple Student's *t* tests). PND: post-natal day.

(51) = 2.6952,p < 0.01] and unaltered on PND7 [t(51) = 0.4724, p = 0.6387], PND21 [t(51) = 0.3163, p = 0.7531] and PND60 [t (51) = 0.6687, p = 0.5067] (Fig. 7C).

Nitric oxide was unaltered on PND0 [t(58) = 0.1095, p = 0.9132], PND7 [t(58) = 1.0915, p = 0.2796], and PND21 [t(58) = 1.5389,

p = 0.1293]. However, it was increased on PND60 [t(58) = 2.3699, p < 0.05] (Fig. 7D).

Complex II activity was unaltered on PND0 [t(42) = 1.3527, p = 0.1834], PND7 [t(42) = 0.7460, p = 0.4598], PND21 [t (42) = 0.9559, p = 0.3446], and PND60 [t(42) = 0.3546, p = 0.7314] (Fig. 8A). The same profile was verified to SDH activity: PND0 [t (43) = 1.5402, p = 0.1308], PND7 [t(43) = 0.4786, p = 0.6346], PND21 [t(43) = 0.3030, p = 0.7633], and PND60 [t(43) = 1.8492, p = 0.0713] (Fig. 8B).

Complex IV activity was increased on PND7 [t(43) = 2.2281, p < 0.05] and unaltered on PND0 [t(43) = 0.4552, p = 0.6512], PND21 [t(43) = 0.0683, p = 0.9458], and PND60 [t(43) = 0.1393, p = 0.8898] (Fig. 8C).

4. Discussion

Our results clearly show that pups are born with impaired mitochondrial function, evidenced by decreased mass and membrane potential in most of the brain areas evaluated, and recover the function through life, improving mitochondrial activity at adult age. Moreover, evidence shows that long term caloric restriction in adult animals preserves mitochondrial function when the organelle is isolated for evaluation. The preserved function may be associated with decreased oxidants production (Lopez-Lluch et al., 2006; Lanza et al., 2012).

In adult animals, enhanced mitochondrial function is associated with preserved neuronal activity in aging (Lin et al., 2014), thus, elucidating its effects when applied during pregnancy is of importance, since the 1000 days after conception are the main window of opportunity (and vulnerability) in humans and may affect the individual's whole life (Garmendia et al., 2014). It was not an objective of our study to evaluate the life span, however, one of the most important and described effects of CR is life extension. We might hypothesize that gestational CR could increase the offspring's life span by preserving mitochondrial function. This assumption is made upon the mitohormesis theory. The sublethal stress promoted by gestational CR and consequent improved mitochondrial function observed in latter ages may lead to life span extension and global health improvement (Musci et al., 2019).

In our work, we observed that alterations in mitochondrial function were often accompanied by mitochondrial superoxide content modulation, except for the adult age, when mitochondrial function increment was not accompanied by increased levels of mitochondrial superoxide. In the hippocampus, even with increased mitochondrial function, superoxide was reduced. In a previous study, we had already observed similar modulation in concern to redox homeostasis, including DCFH oxidation, antioxidants modulation, and biomolecule damage; evidenced by impaired redox homeostasis at birth followed by adaptation throughout life, culminating in improved redox homeostasis in adulthood (Stone et al., 2019).

A relation between nitric oxide content and mitochondrial function was also observed in the present study. In agreement, Kowaltowski showed that CR is capable of increasing nitric oxide production *in vitro*, by different pathways activation, and that increased nitric oxide production affects mitochondrial biogenesis (Cerqueira et al., 2012). Our results indicate that gestational CR effects may be related to nitric oxide signaling. Nitric oxide regulates PGC-1α activation, which in turn, controls mitochondrial biogenesis (Cerqueira et al., 2012; Le Gouill et al., 2007). Indeed, endothelial nitric oxide synthase (eNOS) is essential for the CR effects on mitochondrial biogenesis (Pani, 2015).
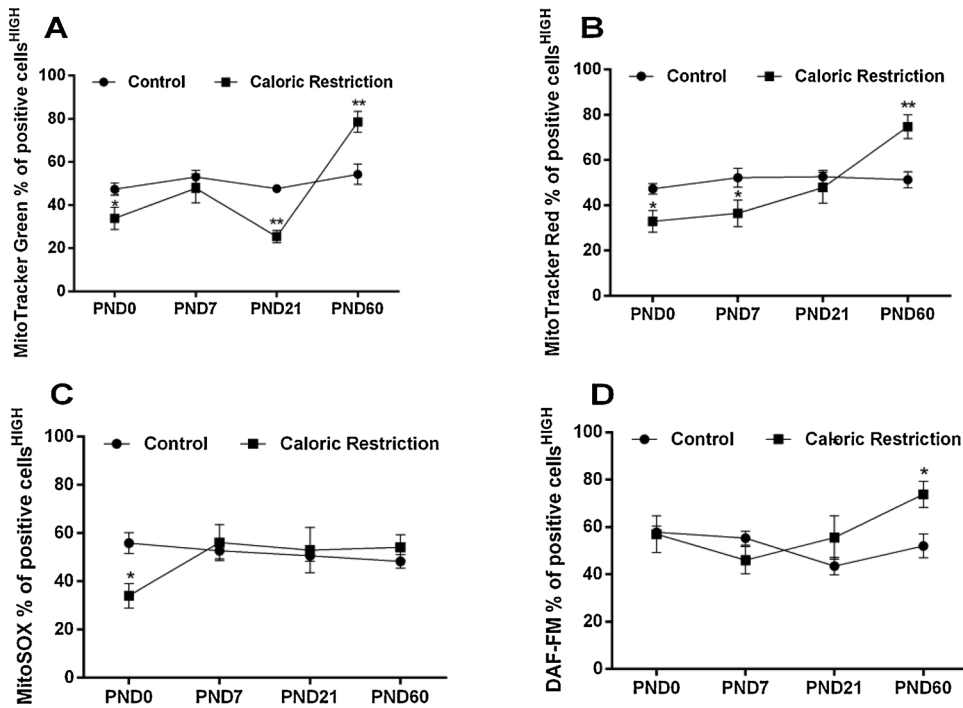
Fig. 7. Effect of intrauterine caloric restriction (CR) on MitoTracker Green ® (A); MitoTracker Red ® (B); MitoSOX ® (C) and DAF-FM ® (D) % of positive cells in pups' hypothalamus. Results are expressed as mean ± S.E.M. for n = 4-10. *p < 0.05, **p < 0.01. (multiple Student's *t* tests). PND: post-natal day. Representative histograms are presented on Supplementary Figs. 13–16.

Actually, CR effects on mitochondrial biogenesis are reduced in eNOS knock out animals (Nisoli et al., 2005).

Additionally, nuclear sirtuin (SIRT1) is involved in redox modulation in several animal models; and SIRT1 deacetylation of PGC-1α has a crucial role in mitochondrial biogenesis (Tang, 2016). Evaluating the mitochondrial sirtuin (SIRT3) is also a perspective. SIRT3 overexpression is known to promote mitochondrial biogenesis, especially under caloric restriction (Gesing et al., 2011; Civitarese et al., 2007). There is also evidence showing SIRT3 has an important role in METS complexes activation (Finley et al., 2011; Ahn et al., 2008), which should be explored since we observed an important modulation on METS complexes in the cerebellum.

On the other hand, it has been shown elsewhere by Mayeur et al. that placental mitochondrial biogenesis was increased by maternal caloric restriction, while ATP production was reduced, depicting placental mitochondrial abnormalities (Mayeur et al., 2013). It is important to note that in Mayeur's study, no micronutrients malnutrition prevention was performed and the animals were submitted to a more severe restriction protocol (30%). If on one hand, our group showed positive redox modulation on pups born from 20% caloric restricted dams (concerning micronutrients malnutrition prevention) (Stone et al., 2019), on the other hand, 40% food restriction (without preventing malnutrition) caused several disruption in pups' and dams' redox homeostasis (Stone et al., 2016).

Although we found evidence for mitochondrial biogenesis modulation in all periods of life, METS complexes activity were modulated especially on PND7 in the maternal CR offspring's cerebrum structures. In the cerebellum, we observed an important modulation, supported by diminished mitochondrial activity at the first ages and activation at adult age. In agreement, data from literature show enhanced electron transport capacity promoted by CR in adult animal model (Amigo et al., 2017). It seems that this effect in our gestational CR model affected mainly the cerebellum. This effect may be related to cerebellar development, which occurs mainly in the last days of gestation and first days after birth (Leto et al., 2016). For instance, the external granular layer of the cerebellum starts at embriony day 15, while the internal granular layer is completely formed only on PND20 (Espinosa and Luo, 2008). There is very few evidence in the literature reporting CR effects on METS complexes activity, when it comes to gestational CR there is no evidence at all, supporting the relevance of our study as the first article addressing this issue.

5. Conclusion

Most of the literature aims to address the negative outcomes related to intrauterine growth restriction, here we reaffirm the possibility of positive outcomes to offspring coming from gestational CR associated with responsible malnutrition prevention by micronutrients supplementation. The litter brain hormetically adapts to the negative effects at birth and present positive mitochondrial modulation in adult life. Whether these effects can contribute to increasing life span is still a question to be addressed in future studies.
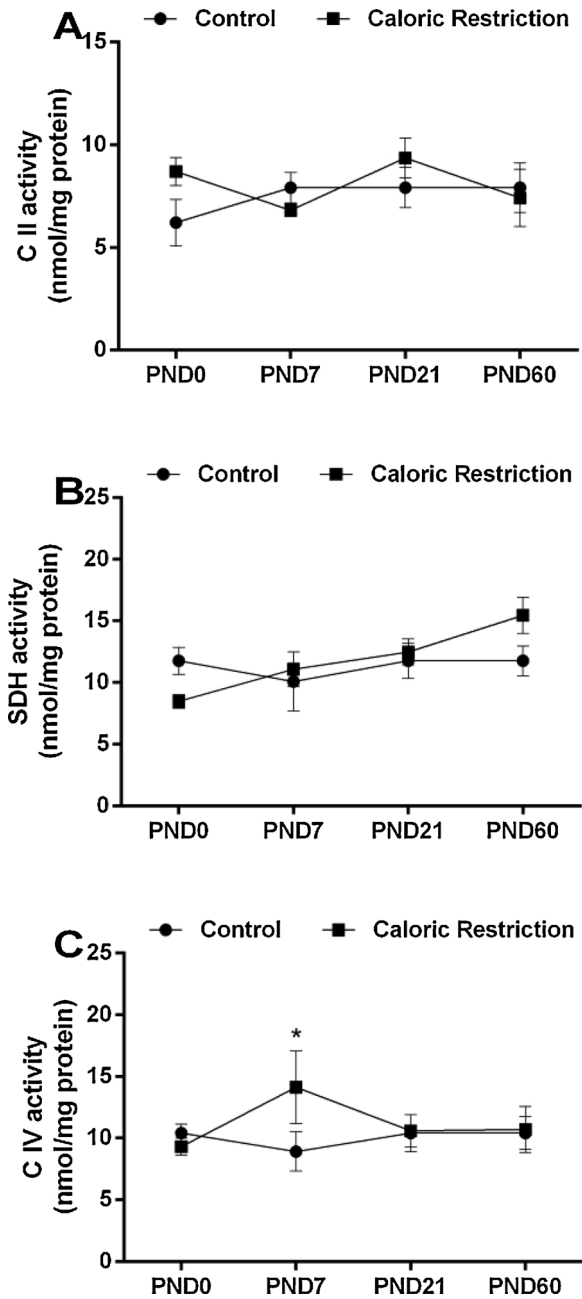
Fig. 8. Effect of intrauterine caloric restriction (CR) on complex II (A); succinate dehydrogenase (B) and complex IV (C) activities in pups' hypothalamus. Results are expressed as mean ± S.E.M. for n = 4-8. *$p < 0.05$ (multiple Student's *t* tests). PND: post-natal day.

Declaration of Competing Interest

The authors report no conflict of interest. The authors are exclusively responsible for the content and writing of this study.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijdevneu.2019.09.004.

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5. Capítulo III

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Gestational caloric restriction with micronutrients supplementation does not delay development and promotes feeding behavior benefits

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ABSTRACT

Introduction: Caloric restriction (CR) has been proven to promote a series of health benefits from yeast to primates. Nowadays, increasing rates of obesity certainly encourage researchers to evaluate CR effects and establish it as a therapeutic approach. Maternal obesity is also a concern, and studies in the developmental origins of health and disease (DOHaD) have shown the importance of interventions during pregnancy, especially those involving maternal nutrition. On the other hand, undernutrition during pregnancy leads to increased weight gain, disturbed feeding behavior and dysfunctional metabolism in adulthood.

Methods: In this way, we utilized moderate CR (20% compared to control consumption) in pregnant Wistar rats as intervention, with malnutrition control by micronutrients supplementation. We assessed CR effects on offspring's developmental milestones, feeding behavior, exploratory behavior, and memory on adolescence (PND21) and adulthood (PND60).

Results: We did not find alterations on litter size or birth weight, although CR pups were leaner at adult ages. Importantly, no delay in development was observed. Besides, female pups showed earlier suction reflex and male pups showed earlier response to the negative geotaxis. CR pups also showed less preference for palatable food (Froot Loops®) at adult age, which could be decisive on obesity tendency. Locomotor activity was increased by CR on PND60 and there was no effect on memory at all.

Discussion: Our results on development and behavior demonstrate that gestational CR may be a helpful health strategy if malnutrition is well controlled, with potential clinical impact.

1. Introduction

Caloric restriction (CR) in adult animal models is well described in the literature as the best strategy to counteract aging effects and associated outcomes, acting through diverse mechanisms, such as redox status improvement and mitochondrial dynamics modulation [1–4]. Not surprisingly, the interest of the research community in this strategy has increased in the last decade, probably as a reaction facing the rising overweight and obesity rates in the general population [5].

Considering that rates of obesity are growing between the general population, pregnant women are also affected [6]. On the other hand, the effects of maternal undernutrition are well described in the literature [7–9] and any intervention in this period should be meticulously planned by qualified professionals. Besides, it is described that either undernutrition and overnutrition during pregnancy may lead to negative outcomes in the offspring [10].

Interventions during the perinatal period may influence an individual's health throughout life. It is urgent to transform scientific evidence in this area of knowledge into public policies [11]. As an example, maternal exercise is considered a safe intervention and is recommended for pregnant women [12]. The mechanisms of effect through which maternal exercise influences maternal and infant health are yet to be clearly described from experimental models [13–15].

The maternal diet also influences offspring feeding behavior. It was demonstrated elsewhere that aspartame consumption during pregnancy programs the brain circuitry and increases palatable food preference when the pups reach adult age in rats [16]. Furthermore, maternal

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protein restriction increases the preference for high-fat foods in adulthood [17].

Bearing in mind that both undernutrition and overnutrition during pregnancy may exert deleterious effects, we believe that healthcare professionals should be dedicated to clarifying what is a healthy diet for pregnant women. In this way, we utilized a rodent model to better understand how maternal nutrition influences offspring health.

We have already demonstrated how gestational CR may promote beneficial effects in the offspring by improving redox homeostasis [18] when malnutrition is responsibly prevented. On the other hand, we have also showed how severe food restriction without malnutrition prevention impairs redox homeostasis, harshly disrupting the antioxidant system in the brain of neonate pups [8].

In this article we aim to demonstrate how biochemical effects are accompanied by developmental and feeding behavior effects, and also demonstrate how malnutrition prevention, in fact, was achieved in this protocol, calling attention for its importance on CR protocols, especially when applied during pregnancy.

2. Material and methods

2.1. Animals

Sixty female adult Wistar rats (120 days-of-age, nulliparous, weighing approximately 230 g, 60 animals) 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. Conception diagnosis was performed by the presence of spermatozoids and the absence of leucocytes in vaginal smears.

Three female rats diagnosed as pregnant were housed per cage in order to start the treatment. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature ($22 \pm 1°C$) colony room.

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 30044, 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.

2.2. CR protocol

CR group had free access to water and received the same 22% protein (w/w) commercial chow as the control group (Nuvilab ®); however, the amount was reduced by 20%. The diet for CR group was adjusted daily by body weight, using the chow amount consumed by control animals as standard. The difference, in grams, between the control group means consumption and CR animal consumption were calculated every day.

A formula containing micronutrients in the same proportion of commercial chow was administered to the animals, based on the difference of chow consumption, in order to equilibrate micronutrients consumption between groups (5 mL of formula contains the same micronutrients amount contained in 10 g of chow). The micronutrients were solubilized on sucralose 0.1% and methylparaben 0.1% (vehicle). Dams assigned to the control group received the vehicle of micronutrients via oral gavage to avoid any unwanted interference.

CR dams were submitted to the CR protocol during the entire pregnancy. On the day of delivery, the pups were cross-fostered: pups from CR dams were housed with control dams and pups from control dams were housed with another control dam. Cross-fostering was applied to abolish the CR intervention after birth.

Pups were weaned at PND21, and after weaning, all pups received *ad libitum* food. In order to avoid litter effect, and increase genetic variability, only one pup per litter was used in each developmental or behavioral test.

Diet and micronutrient formula nutritional content were already described by our group elsewhere [18].

2.3. Developmental and behavioral tests

Different parameters were assessed from birth to adulthood. Developmental milestones were evaluated from PND 1 to PND 15. Open field task was performed on PND 21 and 60. Feeding behavior tests also started at these ages. Morris water maze task was performed on PND 35 and PND 74 (Figure 1). Male and female pups were analyzed separately for the developmental milestones. In the rest of the behavior tests, only male pups were used.

2.3.1. Litter size and pups' weight gain throughout life

The number of pups born in each litter was counted and compared between groups. Pups were individually weighed weekly since the day of delivery until PND60 to build a weight gain curve from birth to adulthood in a balance with 0.1 g readability.

2.3.2. Developmental milestones

We verified the developmental milestones of the pups, using the protocol described by Fox et al. [19] and later used by Chen et al. [20] and Calvino-Nunez et al. [21].
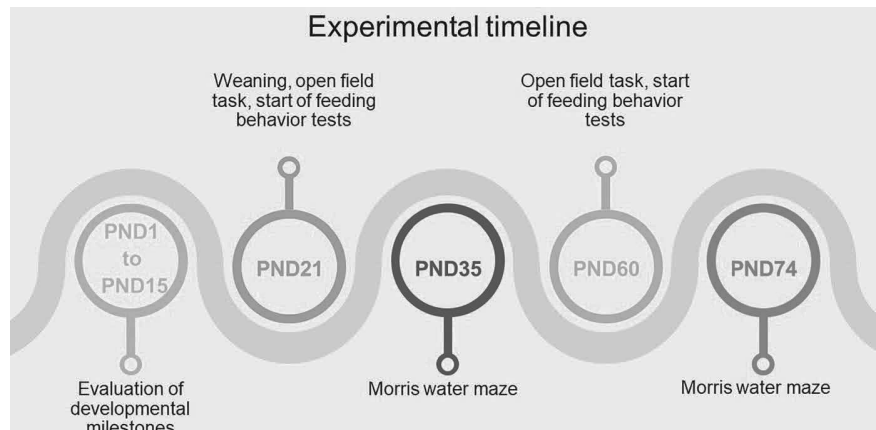
Figure 1. Offspring experimental timeline. PND: postnatal day.

The tests were performed at 4pm in a quiet room at the same temperature as the colony room (22 ± 1°C). After observation or test performance, the pups were returned to the home cage. An effort was made to return the pups to the home cage as soon as possible after testing.

2.3.2.1. Observational tests. We observed the pups daily since PND1 for the following parameters: teeth appearance (considered complete in the day both teeth were apparent); eye opening (considered complete in the day both eyes were open); and fur appearance (considered complete when pups' body was fully covered by fur).

2.3.2.2. Neuromotor behavior tests. We tested the pups daily since PND1 in order to verify the day when they achieved different neuromotor behavior milestones, namely: rooting (response for bilateral stimulation of the face region); suckling reflex (jaw opening in response to perioral stimulus for 30 s); rightening reflex (response from the pup to the normal position after being placed on its side); cliff avoidance (response to escape when placed on the edge of a clifftop); and negative geotaxis (response to return to the resting position when placed with the heading pointing down on a 45° angle slope).

2.3.3. Feeding behavior tests
Feeding behavior tests, as well as memory tasks, were carried out during the daytime in properly acclimatized rooms with constant temperature of 21 ± 1 °C and controlled lighting (30 lux). Animals were habituated in the behavioral room for 1 h before each experiment. The tests started on PND21 for juvenile rats and PND60 for adult rats.

2.3.3.1. Motivation to seek palatable food. To assess motivation for seeking out palatable food, Froot Loops®

(Kellogg's® – pellets of wheat, corn starch, and sucrose) were offered. Animals were placed in one extremity of a rectangular box (40 × 15 × 20 cm) with floor and side walls made of wood and a glass ceiling. Ten Froot Loops® units were placed in a bow at the other extremity of the box. The animals were habituated to this environment for 5 days, 3 min each day, under food restriction (80% of habitual ingestion). After the last training session, the animals were fed *ad libitum* and were exposed to a 3 min test session, 24 h later. Time spent to reach the food and until the initiation of eating, as well as the total number of ingested palatable foods were measured in each trial and in the test session. The test was performed both on PND21 and PND60.

2.3.3.2. Food preference test. In order to assess the preference of the animals for standard chow, sweet palatable food (Froot Loops®), and salty and fat palatable food (Cheetos®); the animals were placed for 20 min in the open field arena (40 cm of width, 50 cm of length and 60 cm of depth) for 4 days as follows. All animals were adapted to the arena for 3 days before starting the tests. In the first day of testing, we provided a bowl containing 20 g of standard chow, in the second day, 20 g of Froot Loops®, in the third day, 20 g of Cheetos® and in the fourth day the animals had access to 3 bowls at the same time, each one containing one of the foods available in the first three days of testing. The test was performed both on PND21 and PND60.

2.3.4. Exploratory behavior and memory tasks
Exploratory behavior and memory tasks were performed in the same conditions of feeding behavior tests.

2.3.4.1. Open field test. Pups were submitted to the open field task on PND21 and PND60 in order to evaluate their motor performance. The test was conducted in

the same apparatus described for the food preference test and adapted from Netto et al. [22], a 40 cm wide, 50 cm length and 60 cm deep box, with the background virtually divided into 12 equal quadrants. Distance traveled and time spent in the central area were analyzed for five minutes. The rats were positioned in the same starting position and were allowed to freely explore the apparatus. After testing, the animal was removed and returned to the housing box, the apparatus was cleaned with ethanol and the procedure was repeated with another animal.

2.3.4.2. Morris water maze reference memory task.

Weaned and young adult pups were submitted to the Morris water maze on PND21 and PND60. The test was conducted in a round black tank, 200 cm in diameter and 100 cm high, filled to a depth of 50 cm. Water was maintained at 23°C during the task. The tank was virtually divided into four equal quadrants. Visual cues were placed in the room. The protocol was adapted from Netto et al. [23].

The task consisted of six training sessions and one testing session. Rats had daily sessions of four trials per day in order to locate the platform, submerged 2 cm in the water and placed in the same quadrant in every training session. For each trial, the animals were positioned facing the wall at each cardinal point. Rats were allowed to search the platform for 60 s and, in case of failure, were gently guided. All animals remained for 15 s on the platform after reaching or being guided to it. The latency to reach the platform was counted daily. The interval between trials was of 15–20 min. In the testing day, the platform was removed and the time spent in the quadrant where the platform was placed was counted.

2.3.5. Statistical analysis

Data which did not follow normal distribution were analyzed by the Mann–Whitney test (developmental milestones, food preference training, open field, Morris water maze test and motivation to seek palatable food test). Repeated measures ANOVA was performed for pups' weight gain, Morris water maze training and motivation to seek palatable food training and two-way ANOVA was performed for the food preference test. Significant ANOVA was followed by Bonferroni post hoc test. Softwares GraphPad Prism ® 6.0 and SPSS ® 24 were used.

3. Results

3.1. Effects of gestational CR on litter size and pups' weight gain

Gestational CR showed no effect on litter size [$t(38) = 0$; $p > 0.9999$]. Both groups showed a mean of 8.9 in the
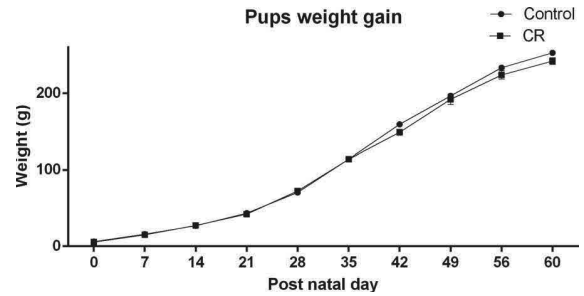

Figure 2. Effect of intrauterine caloric restriction (CR) on pups' weight throughout life. Results are expressed as mean ± S.E.M. for $n = 20$. (repeated measures ANOVA).

number of pups. Control: 8.9 ± 0.44, CR:8.9 ± 0.61 (mean + S.E.M).

Concerning pups' weight gain throughout life, ANOVA showed no difference between groups [$F(1) = 2,21; p = 0.154$] and no interaction between group and PND [$F(2.3) = 1.596; p = 0.211$]. Post hoc tests were not performed since there was no alteration in ANOVA (Figure 2).

3.2. Effects of gestational CR on developmental milestones

A battery of tests was performed in order to determine any change in pups' development caused by maternal CR treatment (Table 1). One pup of each sex was used per litter. We observed an alteration in the sucking reflex implementation day. Female CR pups presented this reflex earlier than the control group. In males, there was no alteration. Negative geotaxis implementation day did not change for the females but the males showed a significant difference, CR pups responded to the uncomfortable position earlier than the control group. We observed no alteration in the following parameters.

Table 1. Effect of intrauterine caloric restriction (CR) on developmental milestones.

Test	Sex	Control ($n = 8$)	CR ($n = 10$)	p value
Teeth appearence (days)	F	10.0 ± 0.3	9.8 ± 0.3	0.80
	M	10.0 ± 0.4	10.3 ± 0.3	0.62
Eye opening (days)	F	14.5 ± 0.2	14.5 ± 0.2	>0.99
	M	14.6 ± 0.2	14.5 ± 0.3	0.96
Fur appearence (days)	F	14.7 ± 0.2	14.5 ± 0.2	0.53
	M	14.6 ± 0.2	14.6 ± 0.2	>0.99
Rooting (days)	F	6.2 ± 0.5	5.3 ± 0.4	0.11
	M	5.8 ± 0.8	4.3 ± 0.6	0.10
Rightening reflex (days)	F	2.3 ± 0.3	2.0 ± 0.0	0.18
	M	2.2 ± 0.2	2.1 ± 0.1	0.56
Cliff avoidance (days)	F	5.7 ± 0.4	5.1 ± 0.2	0.23
	M	6.6 ± 0.4	5.9 ± 0.3	0.21
Suckling reflex (days)	F	3.1 ± 0.4	2.0 ± 0.3	<0.05*
	M	2.5 ± 0.5	2.9 ± 0.5	0.58
Negative geotaxis (days)	F	6.6 ± 0.6	6.5 ± 0.7	0.85
	M	6.9 ± 0.3	5.5 ± 0.4	<0.05*

Results are expressed as mean ± S.E.M. for $n = 8$–10. *$p < 0.05$ (Student's t tests).
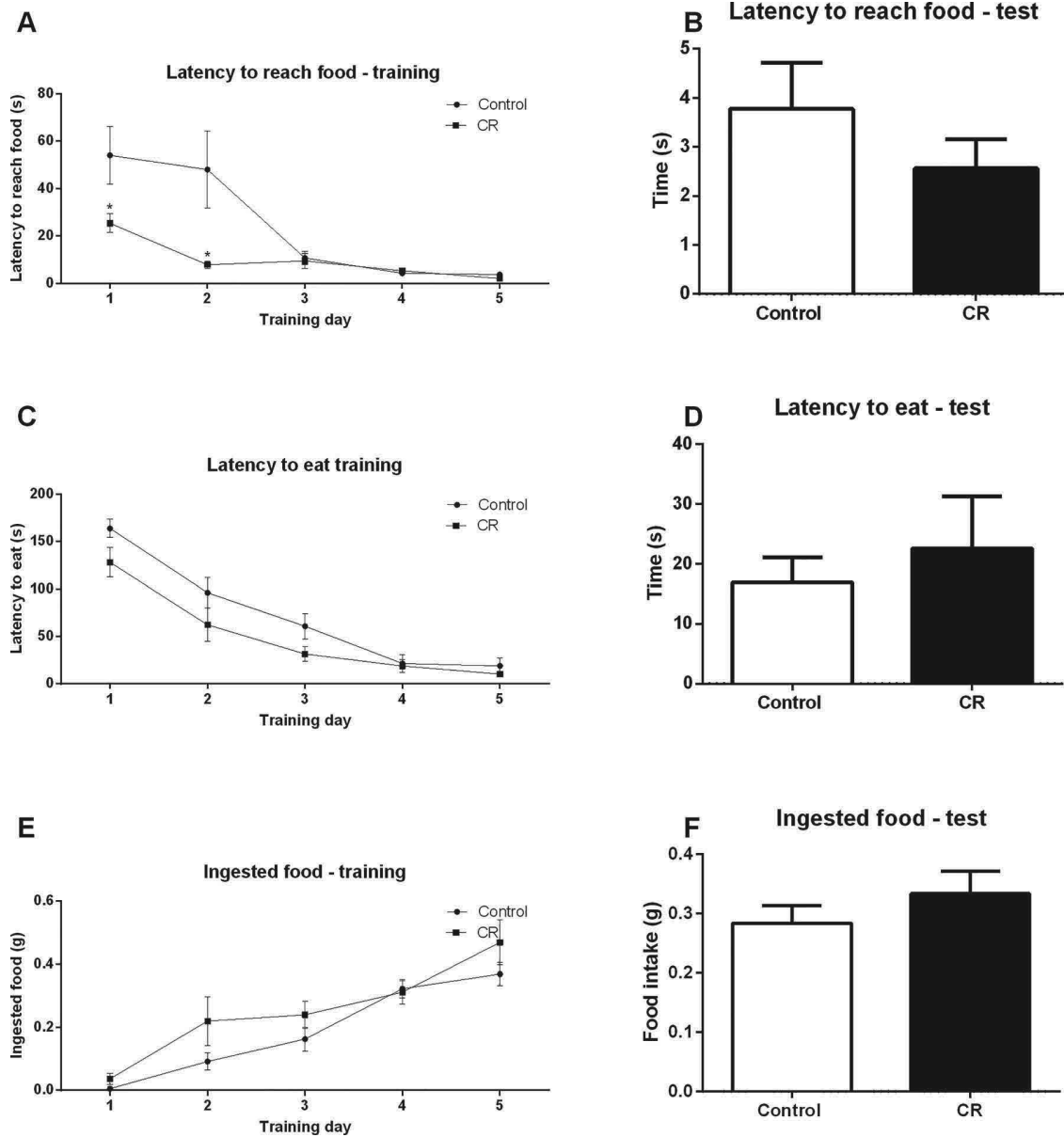
Figure 3. Effect of intrauterine caloric restriction (CR) on pups' latency to reach food in training (A) and testing (B) sessions, latency to eat in training (C) and testing (D) sessions, and ingested food in training (E) and testing (F) sessions of the motivation to seek palatable food test preformed on PND21. Results are expressed as mean ± S.E.M. for $n = 16–18$. *$p < 0.05$, (repeated measures ANOVA for training and Mann-Whitney for testing). When ANOVA was significant, Bonferroni posthoc test was performed.

3.3. Effects of gestational CR on feeding behavior

We observed several alterations in pups' feeding behavior, especially in the adult age.

Concerning motivation to seek palatable food on PND21, ANOVA showed a difference between groups [$F(1) = 7.093$; $p < 0.05$] and interaction between group and training day [$F(1.918) = 4.611$; $p < 0.05$] in the latency to reach food. Bonferroni post hoc test showed that CR pups had smaller latency to reach food on testing days 1 and 2 [$p < 0.05$] (Figure 3(A)). ANOVA showed a difference between groups in the latency to

start eating [$F(1) = 5.76$; $p < 0.05$], although there was no interaction between testing day and group [$F = (2.509) = 1.098$; $p = 0.366$] (Figure 3(B)). No difference was observed in the amount of ingested food between groups [$F(1) = 3.118$; $p = 0.098$]. Interaction between testing day and group was also absent [$F(4) = 1.443$; $p = 0.231$].

In the testing day, there was no difference in the three parameters evaluated.

On PND 60, no difference in the latency to reach food was found between groups [$F(1) = 3.774$; $p = 0.078$].
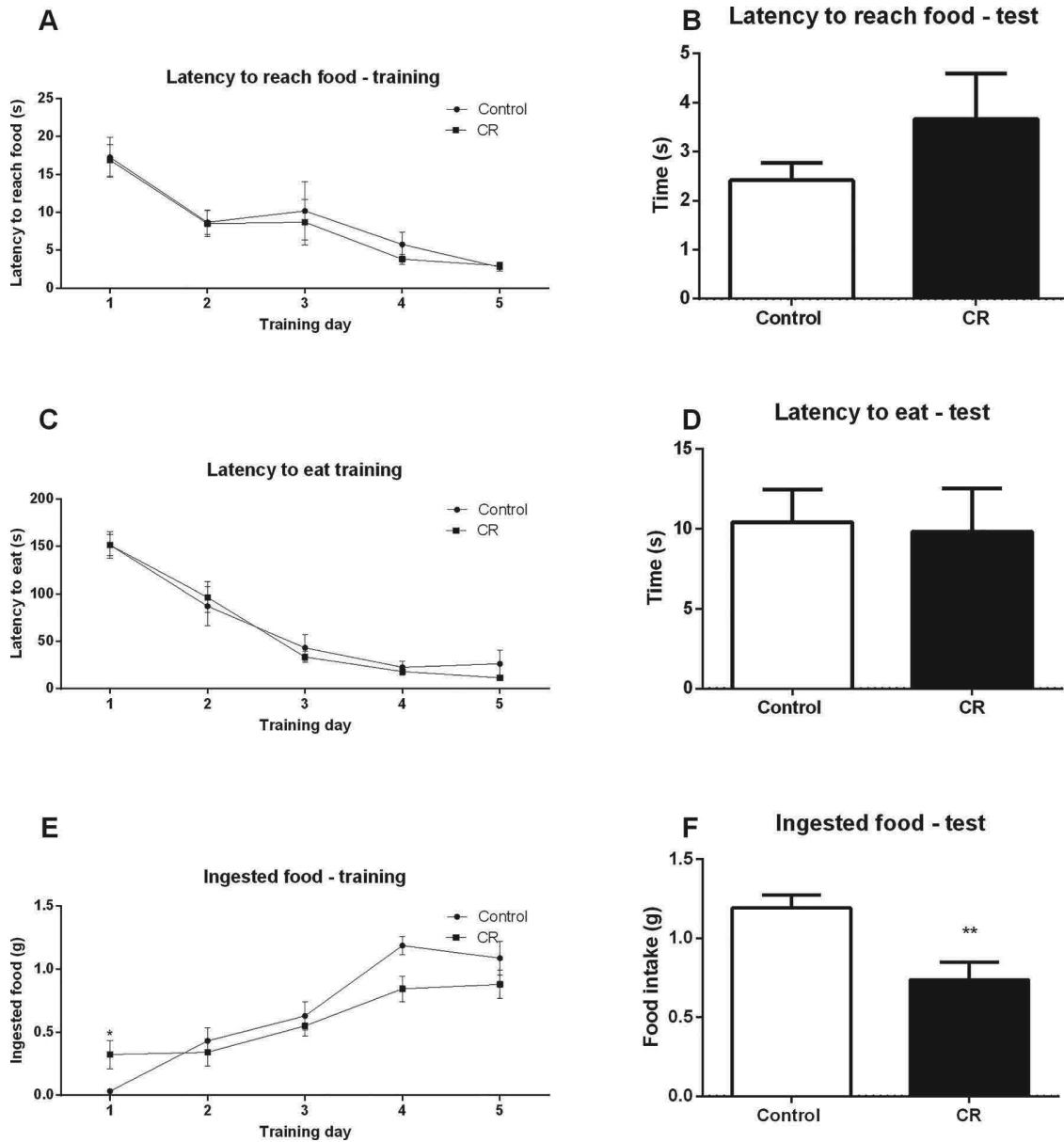
Figure 4. Effect of intrauterine caloric restriction (CR) on pups' latency to reach food in training (A) and testing (B) sessions, latency to eat in training (C) and testing (D) sessions, and ingested food in training (E) and testing (F) sessions of the motivation to seek palatable food test performed on PND60. Results are expressed as mean ± S.E.M. for $n = 12$ *$p < 0.05$, (repeated measures ANOVA for training and Mann-Whitney for testing). When ANOVA was significant, Bonferroni posthoc test was performed.

Interaction between testing day and groups was also absent [$F(4) = 0.642; p = 0.636$] (Figure 4(A)). Latency to eat also did not show difference between groups [$F(1) = 0.208; p = 0.657$] or interaction between testing day and group [$F(2.777) = 0.268; p = 0.834$] (Figure 4(B)). Concerning the amount of food ingested, ANOVA showed no difference between groups [$F(1) = 0.002; p = 0.962$]. However, an interaction between testing day and group was found [$F(2.017) = 4.633; p < 0.05$]. Bonferroni post hoc test showed that CR pups ingested more food on training day 1 [$p < 0.05$].

In the testing day, CR pups exhibited decreased consumption when compared to their control counterparts.

Concerning food preference, we observed that when only chow was available for consumption, there was no difference on PND21 or PND60. When Froot Loops® was available, PND21 pups showed no difference in consumption. However, PND60 CR pups ate significantly less than control. When Cheetos® was offered to PND21 pups in the open field arena, CR pups ate more than control pups. On PND60 there was no significant difference (Table 2).

Table 2. Effect of intrauterine caloric restriction (CR) on food preference test.
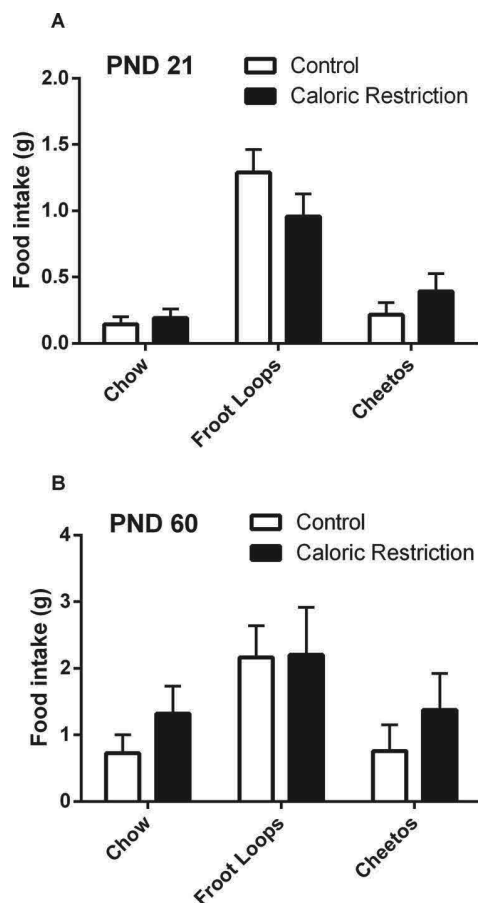
Parameter	PND	Control	CR	p value
Chow consumption (g)	21	0.37 ± 0.09	0.41 ± 0.11	0.71
	60	0.68 ± 0.29	0.83 ± 0.26	0.81
Froot Loops® consumption (g)	21	1.05 ± 0.16	0.85 ± 0.13	0.33
	60	2.20 ± 0.58	0.82 ± 0.32	<0.05*
Cheetos® consumption (g)	21	0.53 ± 0.10	0.89 ± 0.08	<0.05*
	60	1.68 ± 0.27	1.27 ± 0.29	0.26

Results are expressed as mean ± S.E.M (Mann-Whitney test). 21 = post natal day 21; 60 = post natal day 60. n = 15–19 *p < 0.05.

Table 3. Effect of intrauterine caloric restriction (CR) on open field test.

Parameter	PND	Control	CR	p value
Distance traveled (m)	21	15.6 ± 4.5	16.7 ± 3.8	0.24
	60	16.1 ± 4.4	20.0 ± 3.4	<0.05*
Time in central zone (%)	21	4.4 ± 2.6	4.9 ± 3.7	0.89
	60	9.5 ± 6.7	9.2 ± 4.1	0.84

Results are expressed as mean ± S.E.M (Mann-Whitney test). 21 = post natal day 21; 60 = post natal day 60. n = 14–18 *p < 0.05.

In the testing day, when pups were offered chow, Froot Loops® and Cheetos® at the same time, there was no difference between groups in the consumption of any kind of food on PND21 or PND60 (Figure 5).

3.4. Effects of gestational CR on exploratory behavior and memory

Gestational CR did not affect pups' exploratory behavior. In the open field task, CR pups exhibited higher traveled

distance on PND60, which indicates higher locomotor activity. However, the time spent in the central zone (%) was not different between groups neither on PND21 nor in PND60 (Table 3), showing no difference in anxiety-like behavior.

Gestational CR effect on pups' spatial memory was null. ANOVA showed no difference between groups [F(1) = 0.101;p = 0.756] or interaction between testing day and group [F(5) = 1.204;p = 0.317] in weaned pups (Figure 6(A)). In young adult pups, ANOVA also did not show difference between groups [F(1) = 0.501;p = 0.494] or interaction between groups [F(5) = 0.847;p = 0.522] (Figure 6(B)). In the testing day, we did not observe any alteration on weaned neither on young adult pups (Figure 7).
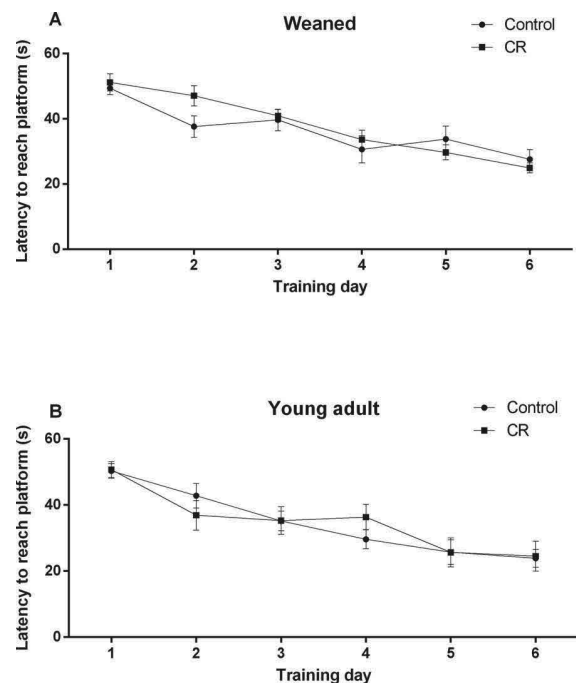

Figure 5. Effect of intrauterine caloric restriction (CR) on pups' preference for chow, Froot Loops® or Cheetos® on PND 21 (A) and PND 60 (B) on food preference test. Results are expressed as mean ± S.E.M. for n = 12–19. (two-way ANOVA).

Figure 6. Effect of intrauterine caloric restriction (CR) on pups' latency to reach platform in the Morris water maze training sessions on weaned (A) and young adult (B) pups. Results are expressed as mean ± S.E.M. for n = 12–17. (repeated measures ANOVA).
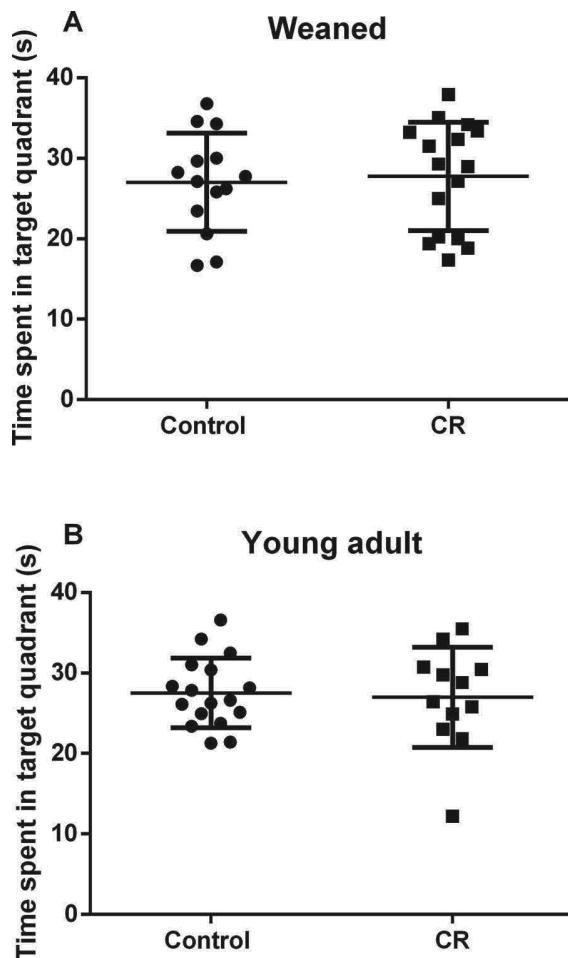
Figure 7. Effect of intrauterine caloric restriction (CR) on pups' time spent in target quadrant in the Morris water maze testing day on weaned (A) and young adult (B) pups. Results are expressed as mean ± S.E.M. for $n = 12$–17 (Mann-Whitney test).

4. Discussion

In this work, we obtained relevant results concerning the development and behavior of pups delivered to gestational CR dams. First key evidence, which contributes to confirm that malnutrition prevention successfully worked in the animal model used here and elsewhere [18] is that no difference in birth weight was observed between groups. This evidence indicates that the pups do not present thrifty phenotype characteristics, such as higher weight gain throughout life and, consequently, overweight in adulthood [7]. CR dams gained less weight than their control peers, as described elsewhere [18].

Data from the literature show that even without malnutrition prevention, 20% CR should not affect pups' birth weight. Contrastingly, the authors observed decreased litter size (number of pups) and increased weight gain after PN week 12 in their model, which includes food restriction (FR) starting before pregnancy

[9]. When severe restriction (50%) without micronutrient supplementation is applied during pregnancy, pups show a rapid catch-up growth [24], which was not observed in our study.

Concerning developmental milestones and motor behavior tests, the hypothesis that gestational CR did not delay pups' development is reinforced. We tested eight parameters well validated in the literature [19–21] and none of them indicated that pups presented any delay. Actually, female pups showed the suckling reflex earlier than control groups, as well as male pups responded earlier to the negative geotaxis test. To our knowledge, our work is a novelty in evaluating these parameters under gestational CR conditions. Besides, literature shows that developmental milestones may be affected by specific nutrients restriction, such as iron [25, 26] and stressor factors *in utero*, such as nicotine exposure [27].

Feeding behavior was also affected by gestational CR, especially in the adult age. Interestingly, CR pups ate less Froot Loops ® in the motivation to seek palatable food testing day on PND60, although latency to reach food and to start eating was similar to control. In the food preference test, there was no difference in the testing day; however, CR pups ate less Froot Loops ® in the second training session, when this was the only food available.

We also observed that juvenile CR pups ate more Cheetos ® in the third training session of the food preference test when this was the only food available. This is a contrasting result since feeding behavior was improved in adulthood. Pups' weight gain did not seem to be affected by this behavior.

Concerning restriction during pregnancy, data from the literature shows that protein or nutrient restriction during pregnancy promotes hyperphagia in the offspring [28, 29]. Evidence on human subjects shows that survivors of World War II submitted to nutrient deprivation on extermination camps showed an increased preference for calorie-dense food consumption later in life [30]. Moreover, we can observe that exposure to famine during intrauterine life makes individuals eat more high-energy food and be less physically active, as shown on those who were born from women that were pregnant during the Dutch famine [31]. Taken together, the effects on animals and humans clearly demonstrate that intrauterine undernourishment increases the preference for palatable food. In our work we observed the opposite effect, once again demonstrating that when malnutrition is responsibly prevented, CR positive effects are extended to feeding behavior.

Feeding behavior is controlled especially by the hypothalamus, which's appetite regulatory systems develop during gestation in humans and until 2 weeks-of-age in

rodents [32, 33]. Undernourishment during hypothalamic development modifies the density of NPY and POMC neurons, by impairing cell proliferation and axonal elongation, directly influencing food consumption and body weight [29, 34]. The endocannabinoid system also plays a key role in appetite regulation. Data from the literature show that food restriction before and during pregnancy decreased predominant endocannabinoid levels both in the hippocampus and hypothalamus. In the same work, which evaluated pups on PN weeks 8 and 12, the authors showed diminished chocolate consumption in female pups on PN week 12 [9].

On the other hand, overweight and obesity during gestation are also linked to increased weight gain after birth, cardiovascular diseases [35] and psychiatric disorders [36]. Disturbingly, the number of children classified as overweight or obese has increased by 1.5 fold worldwide in this decade [37]. Maternal overnutrition clearly affects hypothalamic circuitry, and although obesity development and cardiovascular diseases are pointed as a consequence of this factor [38], the effects on hypothalamic feeding control were diverse [39–41].

Actually, the relation between birth weight and complications later in life has been defined as a 'U-shaped' curve [42]. Then, it is important to define where our intervention is placed in this curve. According to the results presented here, we believe that our intervention is at the bottom of the curve.

When carrying dietary studies on animal models, it is always important to take into account that animals used as control are overfed and sedentary [43]. In this sense, we hypothesize that 20% CR with micronutrients supplementation may represent a healthy diet when compared to *ad libitum* feeding in rodents, and its effects, as we demonstrate, are extended to feeding behavior, which certainly influences weight gain throughout life.

Gestational CR showed no effect on anxiety-like behavior in our work, measured by time spent in the central zone in the open field task. Ramirez-Lopez et al. [9] showed in their work that FR also did not impact anxiety-like behavior in the open field test. In turn, elevated plus maze test showed anxiety-like responses on PN week 12 in their work. Concerning locomotor activity in the open field, they found no difference [9]. Contrastingly, we observed increased locomotor activity in PND 60 CR pups, showing increased exploratory behavior in these animals. Concerning CR effects on spatial memory, no evidence was found, since there were no significant differences in the Morris water maze test elicited by gestational CR.

In conclusion, our data showed that gestational CR elicited positive effects in the offspring, such as decreased preference for palatable food in adulthood and normal neuromotor development. We believe that when the restriction is moderate and the only alteration between groups is the number of calories eaten and not the micronutrient content of the diet, it can bring benefits to the next generation, avoiding chronic metabolic diseases. Investigating what molecular mechanisms are involved in the adaptations elicited by gestational CR, especially on feeding behavior, should be helpful for clinical research.

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6. Discussão

A restrição calórica de 20% aplicada durante a gestação promoveu alterações nas diferentes regiões encefálicas dos filhotes, desde o nascimento até a idade adulta. Estas alterações incluem o conteúdo de oxidantes, a atividade de enzimas antioxidantes, a concentração de antioxidantes não-enzimáticos e os parâmetros de dano oxidativo a lipídeos e a proteínas. O aumento do conteúdo de oxidantes em DPN0 observado na maior parte das estruturas avaliadas, através do aumento da oxidação da diclorofluoresceína (DCFH), parece levar à adaptação hormética das defesas antioxidantes, que estão ativadas nas idades subsequentes.

Enquanto a oxidação da DCFH retorna ao nível do controle na idade adulta na maioria das estruturas avaliadas, verificamos uma ativação da SOD, que estava diminuída na maior parte das estruturas ao nascer, e apresentou níveis iguais ou superiores ao do controle na idade adulta. A CAT estava aumentada em diferentes idades, e a GPx, cuja atividade estava diminuída no cerebelo, retornou aos níveis do controle. Nas demais estruturas, a atividade desta enzima não sofreu alterações, exceto pelo hipocampo, onde sua atividade estava aumentada na idade adulta. A Grx demonstrou atividade igual à do controle ao nascer, exceto pelo córtex pré-frontal, onde estava diminuída, e sua atividade aumentou durante o desenvolvimento. A tiorredoxina-redutase estava aumentada no cerebelo em DPN0 e no córtex pré-frontal em DPN0 e DPN60.

No que se refere aos antioxidantes não-enzimáticos, a GSH demonstrou modulação semelhante à das enzimas antioxidantes, exibindo níveis

diminuídos no nascimento e aumento do conteúdo nas idades posteriores, exceto pelo hipocampo, onde este antioxidante estava igual ao controle em todas as idades avaliadas. A vitamina C, outro antioxidante não-enzimático, estava diminuída ou igual ao controle nas idades iniciais e aumentada na idade adulta, exceto no hipocampo, onde o efeito negativo das idades iniciais foi abolido, mas não revertido. No cerebelo, a concentração dessa vitamina não apresentou alteração.

Em DPN60 a RC durante a gestação parece proteger os filhotes contra o dano oxidativo, com diminuição do conteúdo de oxidantes em todas as estruturas avaliadas, ativação de várias defesas antioxidantes enzimáticas e não-enzimáticas, além da diminuição do dano oxidativo a proteínas no cerebelo e hipocampo. Em relação ao dano oxidativo a lipídeos, verificamos que este estava aumentado em todas as estruturas cerebrais avaliadas, com exceção do córtex pré-frontal, no dia do nascimento. O efeito pró-oxidativo foi abolido nas demais idades no cerebelo, hipocampo e hipotálamo. No córtex pré-frontal, além de não haver alteração em DPN0, a peroxidação lipídica estava diminuída em todas as idades subsequentes.

Comparando nossos resultados com os resultados publicados na literatura, verificamos que a RC em camundongos adultos foi capaz de aumentar a expectativa de vida, diminuindo a produção de oxidantes e aumentando a atividade antioxidante (Ruetenik e Barrientos, 2015). Em revisão realizada por Walsh e colaboradores, foi verificado que em modelos animais adultos de RD, apenas 1% dos estudos demonstraram diminuição na atividade antioxidante enzimática em roedores, evidenciando que a RD exerce

importante papel na ativação das defesas antioxidantes, pelo menos quando aplicada na vida adulta (Walsh *et al.*, 2014).

Por outro lado, dados de nosso grupo de pesquisa demonstraram que a RA gestacional severa (40%) foi capaz de diminuir a atividade de enzimas antioxidantes no cerebelo e córtex total de mães e filhotes recém nascidos, entretanto, não foi capaz de promover dano oxidativo a lipídeos ou proteínas (Stone *et al.*, 2016). Resultados publicados anteriormente na literatura demonstraram que a RA gestacional também teve influência negativa na homeostase redox: tantos os filhotes como as mães exibiram um menor potencial antioxidante total (Agale *et al.*, 2010).

O aumento da atividade antioxidante não-enzimática, por sua vez, já foi demonstrado em modelos animais adultos de RC, com aumento da GSH promovido por essa intervenção (Santin *et al.*, 2011). Quando aplicada durante a gestação, a RC também teve efeito positivo sobre a atividade antioxidante não-enzimática. Partadiredja e colaboradores, embora tenham realizado RA de 25% com objetivo de promover desnutrição durante a gestação em camundongos, observaram um aumento do conteúdo de GSH (Partadiredja *et al.*, 2009). Apesar da modulação positiva no que diz respeito ao principal antioxidante não-enzimático, os autores verificaram diminuição na atividade antioxidante enzimática na prole. Também foi avaliada a expressão das enzimas antioxidantes, na qual não houve nenhuma alteração significativa.

Em modelos animais adultos, a RC aumenta a expressão do fator nuclear eritróide 2 relacionado ao fator 2 (NRF2) que atua em vias de sinalização responsáveis pelo aumento da atividade antioxidante enzimática

(Martin-Montalvo e de Cabo, 2013) e não-enzimática, especialmente a GSH (Cho *et al.*, 2006). O aumento da atividade antioxidante nas idades adultas em resposta à produção de antioxidantes nas idades iniciais pode estar relacionado com a ativação destas vias de sinalização, cuja avaliação vai além dos objetivos desta tese.

Os resultados apresentados no capítulo I concordam com os resultados a respeito do metabolismo mitocondrial exibidos no Capitulo II, onde observamos que a massa e o potencial de membrana mitocondrial estão diminuídos na maior parte das estruturas em DPN0 e, durante o desenvolvimento, esses parâmetros de função mitocondrial sofrem adaptação, estando aumentados na maior parte das estruturas em DPN60. Dados da literatura demonstram que em modelos animais adultos, o aumento da função mitocondrial está associado, por exemplo, à preservação da atividade neuronal durante o envelhecimento (Lin *et al.*, 2014).

Observa-se que, em geral, o aumento da função mitocondrial parece acompanhar o aumento das concentrações de NO. O papel do NO na indução da biogênese mitocondrial em animais adultos submetidos à RC já foi demonstrado por outro grupo, esse oxidante promove a ativação do PGC-1α, que por sua vez, age no controle da biogênese mitocondrial (Cerqueira *et al.*, 2012). Além disso, a modulação da biogênese mitocondrial induzida pela dieta está ausente em animais *knock out* para óxido-nítrico-sintase endotelial (eNOS) (Nisoli *et al.*, 2005).

Curiosamente, verificamos que o conteúdo de superóxido mitocondrial está diminuído ou inalterado mesmo em idades onde a função mitocondrial está elevada em relação ao controle, demonstrando maior atividade mitocondrial acompanhada de menor concentração de superóxido, ou seja, maior eficiência mitocondrial (van der Bliek *et al.*, 2017). A relação entre a RC e o aumento da biogênese e eficiência mitocondrial está demonstrada em cultura de células incubadas com o soro de animais que sofreram RC na vida adulta (Lopez-Lluch *et al.*, 2006).

Sabe-se que a biogênese mitocondrial também é regulada por NRFs, os quais são ativados pelo receptor ativado por proliferador de peroxissomo (PPAR) e pelo PGC-1α (Lopez-Lluch *et al.*, 2006). A SIRT1 também está relacionada com a promoção da biogênese mitocondrial através da ativação do PGC-1α por desacetilação (Rodgers *et al.*, 2005), ou fosforilação mediada pela AMPK (Jager *et al.*, 2007). A AMPK também age diminuindo a atividade da TOR, que por sua vez, ativa fatores de transcrição FoxO.

Além dos efeitos sobre parâmetros de biogênese mitocondrial, o PGC-1α também aumenta a expressão de enzimas antioxidantes (St-Pierre *et al.*, 2006). Foi demonstrado que a RC manteve a expressão do PGC1-α após o envelhecimento em modelos animais adultos em relação aos animais controle de mesma idade (Baker *et al.*, 2006). Dada a contribuição das vias de sinalização apresentadas para alterações da homeostase redox e do metabolismo mitocondrial desencadeadas pela RC, é de grande importância avaliar o imunoconteúdo dessas proteínas em animais submetidos à RC

gestacional, objetivo o qual vai além do escopo da presente tese, sendo uma perspectiva de futuros trabalhos.

Além da SIRT1, outra desacetilase da mesma família também deve ser alvo de futuros estudos, a SIRT3, uma isoforma mitocondrial. A superexpressão desta enzima também promove a biogênese mitocondrial, especialmente quando estimulada pela RC (Civitarese *et al.*, 2007, Gesing *et al.*, 2011). A SIRT3 também atua na ativação dos complexos do STEM (Ahn *et al.*, 2008, Finley *et al.*, 2011), cuja modulação sob nosso protocolo ocorreu principalmente no cerebelo.

Embora diversos trabalhos demonstrem os efeitos positivos RC sobre a biogênese mitocondrial, a RA de 30% durante a gestação parece ter efeito negativo na prole. Apesar de promover o aumento da biogênese mitocondrial, esta intervenção demonstrou anormalidades nas mitocôndrias placentárias das mães, reduzindo a eficiência bioenergética (Mayeur *et al.*, 2013). A falta da prevenção da desnutrição no modelo aplicado pode ter efeito importante, além da intensidade da restrição.

No capítulo III, verificamos que nosso modelo não promoveu atraso no desenvolvimento físico e neuromotor dos filhotes, validando a RC gestacional como alternativa viável de intervenção, pelo menos em ratos. Vistos os efeitos positivos demonstrados nos capítulos I e II, foi realizada uma bateria de testes de desenvolvimento motor, além de parâmetros observacionais de desenvolvimento físico dos filhotes (Fox, 1965). Em nenhum dos parâmetros avaliados o grupo RC demonstrou atraso no desenvolvimento em relação ao grupo controle. Na verdade, os filhotes fêmeas do grupo RC foram capazes de

executar o reflexo de sucção mais cedo que os filhotes do grupo controle; enquanto que os machos responderam mais cedo ao teste da geotaxia negativa, onde o filhote é colocado em uma posição desconfortável e tem o desafio de retornar à posição mais confortável.

Além desses parâmetros, outro dado que confirma essa hipótese é o ganho de peso dos filhotes. A maioria dos trabalhos que relaciona a RD no período gestacional com adaptações negativas ao longo da vida cita a diminuição do ganho de peso dos filhotes durante o período intrauterino como um dos fatores determinantes para os efeitos negativos da RD (Akitake *et al.*, 2015). Em nosso trabalho, embora o ganho de peso materno tenha sido significativamente menor no grupo RC, os filhotes deste grupo não apresentaram diferença no peso em relação ao controle em nenhuma das idades avaliadas, demonstrando que não apresentam características do fenótipo poupador, que prevê o ganho de peso aumentado ao longo da vida (Barker, 1990).

Na verdade, mesmo sem a prevenção da desnutrição, a restrição moderada (20%) não foi capaz de alterar o peso dos filhotes no nascimento (Ramirez-Lopez *et al.*, 2016), embora essa intervenção tenha demonstrado uma diminuição no tamanho da ninhada (número de filhotes), efeito que não observamos com a RC de 20% e nem com a RA de 40% em trabalho anteriormente publicado (Stone *et al.*, 2016). Já a RA severa (50%) promove diminuição do peso ao nascer seguida de rápido aumento do ganho de peso pós-natal   (Lee *et al.*, 2013), ilustrando o fenótipo poupador descrito no

parágrafo anterior. A RA de 40% também promove diminuição do peso ao nascer nos filhotes (Stone *et al.*, 2016).

Sabendo que o aumento no ganho de peso desencadeado pela RD durante a gestação também está associado à preferência por alimentos hiperpalatáveis e a hiperalimentação no geral quando o indivíduo atinge a idade adulta (Vickers *et al.*, 2000, Orozco-Solis *et al.*, 2009), verificamos as alterações promovidas por nosso modelo no comportamento alimentar dos filhotes através de dois testes distintos, o de motivação na busca por alimento hiperpalatável e o de preferência alimentar. Além de não exibir preferência por alimentos hiperpalatáveis, os filhotes do grupo RC demonstraram menor consumo de Froot Loops ® no teste de motivação na busca por alimento hiperpalatável no DPN60. Apesar de não haver diferença entre os grupos no teste de preferência alimentar, os filhotes do grupo RC também comeram menos Froot Loops ® no segundo dia de treino, quando havia apenas este alimento disponível, demonstrando menor preferência pelo alimento doce na idade adulta.

Evidências em humanos também demonstram alterações no comportamento alimentar relacionadas à restrição alimentar. Sobreviventes dos campos de concentração nazistas na segunda guerra mundial que passaram por privação nutricional demonstraram maior preferência por alimentos com alta densidade calórica após saírem da condição desumana que viveram (Polivy *et al.*, 1994). Os filhos de mulheres que estavam grávidas durante o cerco a Leningrado (Stanner e Yudkin, 2001) e a invasão alemã contra a Holanda (Schulz, 2010), dois crimes contra a humanidade cometidos pelo

regime nazista, demonstraram maior consumo de alimentos ricos em calorias e menor atividade física, culminando no aumento de várias desordens metabólicas.

O comportamento alimentar é controlado principalmente pelo hipotálamo, cujo sistema regulador do apetite se desenvolve durante a gestação em humanos e até a segunda semana de vida pós-natal em roedores (Bouret, 2010, Gali Ramamoorthy *et al.*, 2015). A desnutrição durante o desenvolvimento hipotalâmico modifica a densidade de neurônios relacionados à produção do neuropeptídeo Y e da pró-opiomelanocortina (POMC), prejudicando a proliferação celular e o alongamento axonal, e dessa maneira, influenciando diretamente o consumo alimentar e o peso corporal (Vickers *et al.*, 2000, Yura *et al.*, 2005). Dados experimentais demonstram que em ratos, a restrição alimentar de 50% aumenta a preferência por alimentos doces (Alves *et al.*, 2019).

Por outro lado, o excesso de peso e a obesidade durante a gestação também estão relacionados ao aumento de peso após o nascimento, doenças cardiovasculares (Drake e Reynolds, 2010) e desordens psiquiátricas (Mehta *et al.*, 2014), influenciando no controle alimentar exercido pelo hipotálamo (Penfold e Ozanne, 2015).

Em relação aos outros parâmetros comportamentais, verificamos que a RC no período gestacional não teve efeito no comportamento do tipo ansioso, medido aqui pelo tempo gasto pelo animal na zona central no teste de campo aberto. Outros trabalhos na literatura demonstram que a restrição alimentar durante a gestação não impactou neste tipo de comportamento no mesmo

teste, entretanto, no teste do labirinto em cruz elevado, demonstrou um aumento no comportamento do tipo ansioso em filhotes de 12 semanas de idade (Ramirez-Lopez *et al.*, 2016).

Além disso, Ramirez-Lopez- e colaboradores não verificaram nenhuma diferença em relação à atividade locomotora nesse teste (Ramirez-Lopez *et al.*, 2016), enquanto que no presente trabalho os animais demonstraram maior distância percorrida, exibindo um aumento no comportamento explorador.

Em relação à memória espacial, a RC não parece ter nenhum efeito, tanto nos animais adolescentes como nos animais adultos, já que não foram encontrados resultados significativos no teste do labirinto aquático de Morris.

A comparação dos resultados obtidos até então com os dados da literatura, incluso dados de nosso grupo de pesquisa, que motivaram a realização do presente trabalho, no que tange à homeostase redox, a função mitocondrial, o desenvolvimento físico e motor, e o comportamento alimentar, explicita a importância de prevenir a desnutrição nos protocolos de RC gestacional, além disso, demonstra que a RC aplicada no período gestacional parece ser uma alternativa mais saudável em relação ao consumo *ad libitum* utilizado como padrão para animais de biotérios. Já foi demonstrado que esses animais têm o metabolismo alterado, o que leva ao aumento do estresse oxidativo e diminui a expectativa de vida (Martin *et al.*, 2010). A dieta aqui utilizada como intervenção pode ser comparada a uma redução saudável no consumo de calorias em humanos, visto que as condições de sedentarismo e superalimentação dos animais de biotério são, infelizmente, comparáveis com a realidade de grande parte da população ocidental, que é sedentária e

superalimentada (Owen *et al.*, 2010), sendo que uma a cada cinco mulheres grávidas estão acima do peso (Fisher *et al.*, 2013).

A relação entre o peso ao nascer e o desfecho na idade adulta é definido como uma curva em forma de "U" (Ong, 2006), tanto a desnutrição como a hiperalimentação materna influenciam negativamente o desfecho da prole (Cunha Fda *et al.*, 2015). Todas as evidências explicitadas nessa tese indicam que o papel dos profissionais da saúde na prática clínica e na elaboração das políticas públicas em relação à nutrição durante a gestação não pode se limitar a prescrever uma "dieta balanceada", mas sim levar em consideração o estado nutricional individual e das populações para intervir na alimentação da gestante da forma mais oportuna possível.

7. Conclusão

A RC gestacional parece ter papel neuroprotetor, já que aumenta a atividade mitocondrial e a capacidade antioxidante do cérebro dos filhotes, reduzindo o dano oxidativo e promovendo uma melhora no desenvolvimento físico e motor e no comportamento alimentar.

Considerando os resultados apresentados nesta tese e os dados obtidos através de ampla revisão bibliográfica, fica claro que intervenções nutricionais no período gestacional tem consequências que perduram por toda a vida do indivíduo, evidenciando a necessidade de investimento na pesquisa nesta área e em políticas públicas de promoção de saúde na gestação.

Em ratos, a RC durante a gestação, com prevenção responsável da desnutrição promoveu diversos efeitos benéficos, além de não atrasar o desenvolvimento dos filhotes. Portanto, parece ser uma estratégia promissora para promoção de saúde, considerando o atual perfil nutricional das gestantes em nosso país e no mundo. Mais pesquisas relacionadas ao tema são necessárias para melhor orientar a prática clínica em relação à nutrição materna.

8. Financiamento

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2. ANEXO I

Carta de aprovação da Comissão de Ética no Uso de Animais (CEUA-UFRGS)

U F R G S — **PRÓ-REITORIA DE PESQUISA**
UNIVERSIDADE FEDERAL — Comissão De Ética No Uso De Animais
DO RIO GRANDE DO SUL

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 30044
Título: Efeito da restrição calórica durante o período gestacional sobre parâmetros comportamentais e bioquímicos mitocondriais em ratos

Vigência: 05/11/2015 à 30/09/2019

Pesquisadores:

Equipe UFRGS:

CRISTIANE MATTE – coordenador desde 05/11/2015
VINICIUS STONE SILVA – Aluno de Doutorado desde 05/11/2015

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 25/01/2016 - Sala 330 do Anexo I do Prédio da Reitoria - Campus Centro- Universidade Federal do Rio Grande do Sul - Porto Alegre, em seus aspectos éticos e metodológicos, para a utilização de 187 ratos Wistar machos (60-90 dias de vida), 374 fêmeas adultas (60 dias de vida), e 704 filhotes provenientes do Biotério do departamento de Bioquímica da UFRGS, de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Segunda-Feira, 7 de Março de 2016

BRUNO CASSEL NETO
Vice Pró-Reitor de Pesquisa