Universidade Federal do Rio Grande do Sul
Instituto de Ciências Básicas da Saúde
Departamento de Bioquímica Prof. Tuiskon Dick
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

Avaliação do efeito da suplementação com naringenina associada ao exercício físico materno sobre a homeostase redox e o metabolismo energético em encéfalo de ratos Wistar

Pauline Maciel August

Orientadora: Profª Drª Cristiane Matté

Porto Alegre, 2016

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"*Pies para qué los quiero, si tengo alas pa' volar*"

Frida Kahlo

Resumo

A exposição da gestante a diversos fatores ambientais pode interferir drasticamente na programação metabólica do feto, podendo aumentar o risco de desenvolvimento de doenças na vida adulta, em casos como da exposição ao estresse ou restrição calórica. Por outro lado, pode resultar em uma modulação positiva, gerando uma criança mais preparada contra os possíveis insultos sofridos durante a vida, como ocorre através da prática de exercício físico durante a gestação. Considerando que a suplementação com antioxidantes tem demonstrado impedir a sinalização celular e o estabelecimento de adaptações metabólicas quando aliado ao exercício físico, nesse estudo nós avaliamos parâmetros de metabolismo energético e homeostase redox no encéfalo da prole submetida ao exercício físico materno, aliado ou não à suplementação com naringenina. Ratas Wistar adultas foram divididas em quatro grupos: (1) sedentário, (2) sedentário suplementado com naringenina, (3) exercício de natação, e (4) exercício de natação suplementado com naringenina. Os grupos 3 e 4 praticaram natação 30 minutos ao dia, 5 dias por semana, durante 4 semanas (incluindo uma semana de adaptação antes do acasalamento), enquanto os grupos 1 e 2 foram apenas submersos na água. A suplementação com naringenina foi realizada uma vez ao dia, na dose de 50 mg/kg, durante toda a prenhez, imediatamente antes do exercício físico. A prole foi eutanasiada aos 7 dias de vida, quando cerebelo, córtex parietal e hipocampo foram dissecados para as análises bioquímicas. Nossos resultados demonstraram que tanto a suplementação com naringenina quanto a prática de exercício materno causaram um aumento nas defesas antioxidantes da prole, e também um aumento na atividade da cadeia transportadora de elétrons, uma indicação de biogênese mitocondrial. A suplementação com naringenina inibiu a atividade das desidrogenases do ciclo do ácido cítrico, provavelmente interferindo no sítio de ligação do NAD^+. Quando os tratamentos foram aliados, demonstrou-se a abolição dos efeitos isolados em vários parâmetros, avaliados no encéfalo dos filhotes. Também verificamos que a estrutura cerebral mais suscetível aos efeitos da naringenina e do exercício materno é o cerebelo. Concluímos que as intervenções utilizadas, suplementação com naringenina e exercício gestacional, causaram relevantes modulações metabólicas no encéfalo da prole, sugerindo cautela nas intervenções durante a gestação.

Abstract

Pregnant woman's exposure to various environmental factors dramatically interferes in the fetus metabolic programming, increasing the risk for diseases in adulthood, in cases such as exposure to stress or calorie restriction. On the other hand, a positive modulation also is possible, able to prevent future insults, as occurs through physical exercise during pregnancy. Whereas antioxidant supplementation has been shown to prevent the adaptive signaling pathways when combined with exercise, in this study we evaluated some parameters of energy metabolism and redox homeostasis in the brain of the offspring submitted to maternal exercise, ally or not to naringenin supplementation. Female adult Wistar rats were divided into four groups: (1) sedentary, (2) sedentary supplemented with naringenin, (3) swimming exercise, and (4) swimming exercise supplemented with naringenin. Groups 3 and 4 practiced swimming for 30 minutes a day, 5 days a week for 4 weeks (including one week of adaptation prior to mating); while groups 1 and 2 were just submerged in the water. Supplementation with naringenin was performed daily immediately before exercise, at a dose of 50 mg/kg, throughout pregnancy. The offspring was euthanized at 7 days of life when cerebellum, hippocampus, and parietal cortex were dissected for biochemical analysis. Our results demonstrated that both strategies, naringenin supplementation and maternal exercise training, increased the antioxidant defenses in offspring's brain, as well as the electron transport chain activity, an indication of mitochondrial biogenesis. Naringenin supplementation inhibited the activity of citric acid cycle's dehydrogenases, probably by interfering with the NAD^+ binding site. When the treatments were allies, it proved to abolish the separate effects on various parameters evaluated in puppies. It was also found that the brain structure more susceptible to the effects of naringenin and maternal exercise is the cerebellum. We concluded that the interventions used, naringenin supplementation and gestational exercise, bring substantial metabolic modulations in the offspring's brain, suggesting caution in interventions during pregnancy.

Lista de abreviaturas

α-KGDH - α-Cetoglutarato-desidrogenase

Acetil-CoA - Acetil-Coenzima A

ATP - Adenosina trifosfato

BDNF - Fator neurotrófico derivado do encéfalo

CAT – Catalase

CS - Citrato-sintase

CTE - Cadeia transportadora de elétrons

DCFH - Diclorofluoresceína

DNA - Ácido desoxirribonucleico

EROs – Espécies reativas de oxigênio

ERNs – Espécies reativas de nitrogênio

$FADH_2$ - Flavina adenina dinucleotídeo reduzido

GPx - Glutationa-peroxidase

GSH - Glutationa reduzida

GTP - Guanosina trifosfato

HPA - Hipotálamo-hipófise-adrenal

IDH - Isocitrato-desidrogenase

IL - Interleucina

LDL - Lipoproteína de baixa densidade

MDA - Malondialdeído

MDH - Malato-desidrogenase

mRNA - Ácido ribonucleico mensageiro

NAD^+ - Nicotinamida adenina dinucleotídeo

$NADH.H^+$ - Nicotinamida adenina dinucleotídeo reduzido

NF-κB - Fator nuclear κB

Nrf1 - Fator nuclear respiratório 1

Nrf2 - Fator de transcrição nuclear eritroide 2 p45-relacionado

PGC-1α - Coativador de transcrição 1α do receptor ativado por proliferação peroxissomal

PND - Dia pós-natal

PPARα - Receptor ativado por proliferado de peroxissoma α

SDH - Succinato-desidrogenase

SOD – Superóxido-dismutase

CAC – Ciclo do ácido cítrico

TFAM - Fator de transcrição mitocondrial A

TNFα - Fator de necrose tumoral α

VEGF - Fator de crescimento endotelial vascular

Sumário

1. Introdução

1.1. Metabolismo energético cerebral

Apesar de o cérebro apresentar uma alta demanda energética (Tomasi, Wang and Volkow 2013), dispõe de um baixo conteúdo de adenosina trifosfato (ATP, do inglês *adenosine triphosphate*), sendo demonstrada uma pausa na atividade em torno de 7 a 10 segundos após uma interrupção na circulação sanguínea (Ames 2000). A glicólise e a fosforilação oxidativa são vias metabólicas que contribuem, de forma relevante, para a manutenção da homeostase energética cerebral (Pellerin and Magistretti 2004).

Na glicólise aeróbica uma molécula de glicose é oxidada a duas de piruvato, produzindo duas de nicotinamida adenina dinucleotídeo reduzidas ($NADH.H^+$) e duas de ATP, numa sucessão de reações que ocorrem no citoplasma. Na glicólise anaeróbica o piruvato é reduzido a lactato, regenerando a nicotinamida adenina dinucleotídeo (NAD^+) e permitindo a continuação da glicólise (Nelson and Cox 2014).

Tanto o piruvato proveniente da glicose quanto os aminoácidos e ácidos graxos, todos provenientes da alimentação, são oxidados à acetil-Coenzima A (acetil-CoA) para posterior oxidação no ciclo do ácido cítrico (CAC), que ocorre na matriz mitocondrial e irá finalizar a oxidação destas moléculas combustíveis orgânicas a dióxido de carbono (CO_2), sintetizando uma molécula de guanosina trifosfato (GTP, do inglês *guanosine triphosphate*) e conservando energia na forma de três $NADH.H^+$ e uma flavina adenina dinucleotídeo reduzido ($FADH_2$) (Nelson and Cox 2014).

As coenzimas reduzidas são então reoxidadas pelos complexos I

($NADH.H^+$) e II ($FADH_2$) da cadeia de transporte de elétrons (CTE), situada namembrana mitocondrial interna, a qual conserva a energia de transferência dos elétrons em um gradiente de prótons. Ao final, os elétrons são transferidos para o oxigênio molecular, produzindo água, e a energia conservada é utilizada na síntese de 36 moléculas de ATP pela fosforilação oxidativa, sendo esse processo mais eficiente em liberação de energia do que a glicólise anaeróbica (Nelson and Cox 2014).

Oitenta por cento da energia fetal é proveniente da glicose, vinda da mãe (Garg and Devaskar 2006). Mesmo a glicólise anaeróbica sendo considerada importante durante o desenvolvimento fetal, devido, entre outros fatores, à capacidade do cérebro imaturo de utilizar lactato, além de corpos cetônicos, como fonte energética; a maior parte desta glicose é utilizada aerobicamente, sendo o fornecimento de oxigênio materno suficiente para o metabolismo oxidativo (du Plessis 2009, Singer 1999, Vannucci and Vannucci 2000). A taxa de utilização de glicose cerebral no neonato representa em torno de 70% da utilizada no cérebro adulto, e aumenta de acordo com o desenvolvimento de cada região cerebral (Chugani 1998, Vannucci and Vannucci 2000). Já foi verificado que, em condições normais, cerca de 60% da energia utilizada pelo cérebro de ratos de sete dias é proveniente da glicose (Vannucci, Yager and Vannucci 1994).

Além de ser importante no fornecimento de energia, a glicólise também propicia substratos essenciais no desenvolvimento cerebral, tais como lipídios, fundamentais na proliferação neuronal e na mielinização, enquanto a CTE é mais importante para a manutenção da atividade sináptica em todos os períodos da vida (Raichle 2010, Tekkok et al. 2005, Bauernfeind et al. 2014).

A mitocôndria tem papel importante não só no fornecimento de energia, mas também no desenvolvimento embrionário e na homeostase celular (Benkhalifa et al. 2014, Harvey et al. 2011). O processo de divisão ou aumento da mitocôndria é chamado de biogênese mitocondrial, e envolve os genomas nuclear e mitocondrial (Onyango et al. 2010, Larsson and Clayton 1995). O coativador de transcrição 1α do receptor ativado por proliferação peroxissomal (PGC-1α, do inglês *peroxisome proliferator-activated receptor-gamma coactivator 1*α) é considerado um regulador chave na biogênese mitocondrial, estimulando, através do fator nuclear respiratório 1 (Nrf1, do inglês *nuclear respiratory fator 1*), a síntese do fator de transcrição mitocondrial A (TFAM, do inglês *transcriptional factor A, mitochondrial*), que levará a duplicação do ácido desoxirribonucleico (DNA, do inglês *deoxyribonucleic acid)* mitocondrial (Vina et al. 2009).

Embriões de ratos Wistar no início da gestação demonstram um aumento na diferenciação mitocondrial, enquanto no final da gestação a proliferação é mais pronunciada (Alcolea et al. 2006, Piko and Taylor 1987), sendo o período pós-natal mais importante nas variações de densidade e volume mitocondrial, enquanto peso e tamanho se mantém mais estáveis do desenvolvimento até a vida adulta (Hagberg et al. 2014).

1.2. Homeostase redox

Reações de oxidação e redução, onde ocorre a transferência e o recebimento de elétrons, respectivamente, são chamadas de redox e ocorrem constantemente nos organismos vivos, sendo essenciais para a homeostase celular (McCord 2000, Nordberg and Arner 2001, Gutowski and Kowalczyk

2013, Forman, Ursini and Maiorino 2014, Buettner 1993).

No metabolismo energético, as reações redox são parte fundamental da retirada de elétrons de compostos celulares mais reduzidos, transferindo-os às coenzimas NAD^+ e FAD, às quais são reoxidadas na CTE. Nesse processo é comum o vazamento de elétrons e a produção de espécies reativas. Essas englobam os radicais livres que são definidos como espécies capazes de manter-se independentemente e que possuem um ou mais elétrons desemparelhados na última camada eletrônica (Gutteridge and Halliwell 2000). Dentre os compartimentos celulares, retículo endoplasmático, peroxissomos e a mitocôndria são as organelas mais importantes na sua formação (Halliwell and Gutteridge 2007, Jones 2006, Mailloux 2015).

Biologicamente, as espécies reativas mais relevantes são as de oxigênio (ERO), tais como o radical ânion superóxido ($O_2^{\bullet-}$), o peróxido de hidrogênio (H_2O_2), e o radical hidroxil ($^{\bullet}OH$); e as de nitrogênio (ERN), destacando o óxido nítrico ($NO^{\bullet}$) e o peroxinitrito ($ONOO^-$) (De Tullio and Asard 2012, Halliwell 2006b).

O ânion $O_2^{\bullet-}$ não é muito reativo, porém ao reagir com $NO^{\bullet}$ ocorre a formação de $ONOO^-$, causador da maior parte do dano oxidativo a aminoácidos (Beckman and Koppenol 1996). O H_2O_2 também se torna mais danoso quando reage com metais de transição, tais como Cu^{2+} e Fe^{2+}. Por meio da reação de Fenton, catalisada por Fe^{2+}, ocorre a formação de $^{\bullet}OH$ (Halliwell and Gutteridge 2007, McCord 2000). O radical $^{\bullet}OH$, por sua vez, é a ERO com maior reatividade, não existindo defesa enzimática endógena capaz de diminuir sua ação, o que juntamente com a sua capacidade de causar dano a quase todas

as biomoléculas, o torna o radical livre mais danoso (Cadenas and Davies 2000, Halliwell 2006b).

Apesar de serem geradas em processos habituais do organismo e também estarem envolvidas em processos fisiológicos, o excesso de espécies reativas pode levar ao dano a biomoléculas, devido a sua alta reatividade, precisando haver um equilíbrio entre produção e eliminação; o que é mediado por defesas antioxidantes enzimáticas e não enzimáticas. Quando ocorre um desequilíbrio, com aumento exagerado do conteúdo de espécies reativas e/ou redução das defesas antioxidantes, é gerado o estado de estresse oxidativo, que está relacionado com diversas doenças (Halliwell 2006a, Nordberg and Arner 2001, Thanan et al. 2015, Mei et al. 2015).

Para ser definida como antioxidante, uma substância deve conseguir proteger biomoléculas do dano oxidativo, quando em concentrações menores em relação ao substrato oxidável (Halliwell 2011). Antioxidantes enzimáticos são moléculas endógenas que agem na detoxificação de ERO com maior eficiência, enquanto os antioxidantes não enzimáticos são na maior parte obtidos através da alimentação e agem doando elétrons diretamente às espécies reativas, diminuindo sua reatividade (Tokarz, Kaarniranta and Blasiak 2013, Halliwell and Gutteridge 2007).

Entre os antioxidantes enzimáticos destacam-se a superóxido-dismutase (SOD), a catalase (CAT) e a glutationa-peroxidase (GPx). A SOD age especificamente na dismutação do $O_2^{\bullet-}$ formando H_2O_2 e O_2, e pode ser encontrada nas isoformas MnSOD, dependente de manganês e presente em mitocôndrias; CuZnSOD, dependente de cobre e zinco, e encontrada no citoplasma, meio extracelular e organelas tais como peroxissomos e núcleo;

além da EcSOD, também dependente de cobre e zinco (McCord and Fridovich 1969, Halliwell and Gutteridge 2007, Matté 2015). A CAT é uma enzima dependente de Fe^{2+}, contendo um grupamento heme em seu centro ativo, que age exclusivamente na dismutação do H_2O_2, formando H_2O e O_2, e é encontrada especialmente em peroxissomos (Sies 2014, Halliwell and Gutteridge 2007, Matté 2015). A GPx catalisa a redução do H_2O_2 utilizando glutationa reduzida (GSH), e também age eliminando hidroperóxidos orgânicos (ROOH) e $ONOO^-$. Em mamíferos é amplamente distribuída, sendo encontrada em oito diferentes isoformas, no meio extracelular, membranas, citosol, mitocôndrias, entre outros (Brigelius-Flohe and Maiorino 2013, Halliwell and Gutteridge 2007, Matté 2015).

Entre os antioxidantes não enzimáticos está a GSH, um tripeptídeo endógeno que é utilizado por antioxidantes enzimáticos e também age diretamente reduzindo $^{\bullet}OH$ e $ONOO^-$ (Halliwell and Gutteridge 2007). Entre os exógenos, estão a vitamina C, que age diretamente como sequestradora de radicais livres e espécies reativas não radicais (Halliwell 1999, May 2000); e a vitamina E, importante pela sua lipossolubilidade, evitando a oxidação de lipídios ao reagir diretamente com radicais livres (Machlin and Bendich 1987, Halliwell and Gutteridge 2007), sendo regenerada pela GSH e vitamina C após ser oxidada (Halliwell and Gutteridge 2007).

O encéfalo é mais suscetível ao dano oxidativo em relação a outros tecidos, pois além do seu alto consumo de oxigênio que leva a uma maior produção de espécies reativas (Sokoloff 1999), alto conteúdo de ferro, favorecendo a formação de radical hidroxil (Gerlach et al. 1994), e seu alto conteúdo de ácidos graxos suscetíveis a lipoperoxidação (Aureli et al. 2015), o

encéfalo também possui um menor conteúdo de defesas antioxidantes quando comparado aos demais tecidos (Ho et al. 1997, Halliwell and Gutteridge 2007). As enzimas SOD e GPx tem sua atividade diminuída no cérebro de ratos durante a gestação em relação a vida adulta, e ocorre um aumento a partir do nascimento, enquanto a enzima CAT tem uma alta atividade na gestação e logo após o nascimento, depois sua atividade é reduzida até chegar aos níveis de vida adulta (Aspberg and Tottmar 1992). Em análise do sangue do cordão umbilical de bebês saudáveis e prematuros, foram encontrados maiores níveis de potencial antioxidante total e vitamina C, em relação à vida adulta (Lindeman et al. 1989), sendo encontrada uma relação da atividade de antioxidantes com o peso ao nascer (Sullivan and Newton 1988).

1.2. Programação metabólica na gestação

Na década de 60 surge a hipótese do genótipo poupador, sugerindo uma influência do ambiente intrauterino e da genética sobre a doença na vida adulta, a partir da observação de macrossomia em crianças nascidas de mães diabéticas (Neel 1962). Já na década de 70, verificou-se uma maior taxa de obesidade em crianças nascidas de uma gestação com baixo fornecimento de energia durante o primeiro semestre, enquanto a mesma exposição no terceiro semestre levou a uma menor taxa de obesidade (Ravelli, Stein and Susser 1976).

A partir da década de 80 uma sequência de estudos levou à hipótese de Barker, que relaciona condições inadequadas no ambiente intrauterino a uma adaptação negativa no metabolismo do feto, que leva a um ambiente pós-natal com maior risco para doenças cardiovasculares. A má nutrição durante a gestação poderia levar a um desenvolvimento anormal de alguns tecidos, no

caso as células β do pâncreas, buscando priorizar órgãos essenciais, levando ao aumento na incidência de doenças metabólicas em longo prazo (Hales and Barker 1992).

Essa hipótese foi descrita após detecção da correlação entre baixo peso ao nascer e maior taxa de mortalidade por doenças cardíacas (Barker et al. 1989), entretanto, mais tarde o baixo peso ao nascer também foi relacionado ao maior risco para diabetes mellitus tipo 2, hiperlipidemia e hipertensão (Barker et al. 1993), maiores níveis de glicose no plasma (Robinson et al. 1992), e resistência à insulina (Eriksson et al. 2002).

Outros fatores gestacionais também influenciam negativamente a saúde do feto. Exposição ao etanol durante a gestação leva a atrasos e anormalidades neurológicas, devido a alterações na metilação do DNA e na expressão gênica no hipocampo, alteração na resposta regulatória renal e aumento da ativação do eixo hipotálamo-pituitária-adrenal (HPA) (Marjonen et al. 2015, Schambra et al. 2015, Godino et al. 2015, Schneider et al. 2002, Mattson, Crocker and Nguyen 2011).

O estresse materno durante a gestação é associado ao maior risco para distúrbios neurológicos, tais como esquizofrenia, transtorno do aspecto autista, depressão e distúrbio do déficit de atenção (Bale et al. 2010, Bale 2014), aumentando os níveis de citocinas e hormônios do estresse na placenta e aumentando a ativação do eixo HPA na prole (Bale 2015). Já foi demonstrada a persistência dos efeitos negativos do estresse pré-natal até a adolescência em humanos. Meninos de onze anos demonstram um menor volume no hipocampo direito quando expostos ao estresse pré-natal relacionado a um desastre natural (Dufoix et al. 2015). Além disso, existe uma relação positiva

entre gestantes depressivas com o aumento do risco para depressão em suas filhas aos dezoito anos de idade (Quarini et al. 2016).

Estas modulações causadas pelo ambiente materno à prole parecem ter participação de mecanismos epigenéticos, os quais promovem modificações covalentes em histonas e em bases do DNA, mas sem modificar a sua sequência (Bale et al. 2010, Bale 2015, Franklin and Mansuy 2010).

1.4. Dieta materna e consumo de polifenois

Como citado anteriormente, a alimentação materna também tem grande influência na programação fetal, e os primeiros achados sobre programação metabólica envolveram a desnutrição materna (Hales and Barker 1992).

A dieta hiperlipídica também causa efeitos deletérios, sendo demonstrado, em modelo animal, um aumento nos níveis dos marcadores inflamatórios, tais como o fator de necrose tumoral α (TNFα, do inglês *tumor necrosis factor* α) e a interleucina (IL) 1β no sangue das mães durante a gestação (Ashino et al. 2012). Na prole, a dieta hiperlipídica parece aumentar os níveis de corticosterona e glicose plasmática aos 21 dias, além do percentual de gordura corporal e a pressão sistólica aos 21 dias e na vida adulta (Desai et al. 2014, Guberman et al. 2013), podendo causar alterações no endofenótipo, provavelmente epigenéticas, no córtex cerebral da prole independente da dieta pós-natal (Manousopoulou et al. 2015).

Uma dieta materna rica em sal também demonstra causar malefícios, como disfunção cardíaca e vascular da prole, independente da dieta pós-natal (Maruyama et al. 2015). A deficiência de proteína no período pré-natal pode causar efeitos negativos, sendo demonstrado em modelo animal um menor peso e maior pressão arterial aos três meses de idade (de Belchior et al. 2015),

e aumento no colesterol plasmático e resistência à insulina na vida adulta (Erhuma et al. 2007, Fernandez-Twinn et al. 2005), aumentando o risco para disfunção cardíaca.

Polifenois são compostos presentes em vegetais, frutas, grãos e outros alimentos, e são divididos em ácidos fenólicos (ácidos hidroxibenzoicos e hidroxicinâmicos), estilbenos, flavonoides (flavanois, flavonas, flavonois, flavanonas, isoflavonas e proantocianidinas) e lignanas (Pandareesh, Mythri and Srinivas Bharath 2015, Manach et al. 2004). Entre os efeitos já demonstrados dos polifenois na saúde estão a sua ação antioxidante, neuroprotetora e anti-inflamatória. Atuam na eliminação de espécies reativas, sendo mais efetivos dependendo da sua estrutura fenólica, inibindo a oxidação da lipoproteína de baixa densidade (LDL, do inglês *low density lipoprotein*) em humanos (Gharras 2009), aumentando o conteúdo de GSH em cultura celular e modelo animal e inibindo a secreção de citocinas inflamatórias (Rzepecka-Stojko et al. 2015, Pandareesh et al. 2015, Mileo and Miccadei 2016, Dickinson et al. 2003, Cui, Li and Zhu 2015, Cardinal et al. 2015, Impellizzeri et al. 2015).

A naringenina é um polifenol da classe dos flavonoides, subclasse flavanona, encontrada principalmente em frutas cítricas (Dou et al. 2013). Também apresenta efeitos benéficos à saúde comuns aos polifenois, tais como antioxidante (Wang et al. 2012, Khan et al. 2012) e neuroprotetor, demonstrando atravessar a barreira hematoencefálica, reduzindo o estresse oxidativo e a inflamação através da inibição da ativação do fator nuclear κ B (NF-κB, do inglês *nuclear fator* κ *B*) (Raza et al. 2013, Youdim et al. 2004, Muthaiah et al. 2013).

Quanto ao uso de polifenois durante a gestação, seus efeitos ainda não

estão bem elucidados. Em modelo animal já foram demonstrados diversos benefícios para a prole, com reversão quase completa dos danos causados por um período de hipóxia durante a gestação através da suplementação com resveratrol (Bourque et al. 2012).

A suplementação com quercetina foi capaz de prevenir o dano causado por estresse materno pré-natal (Toumi et al. 2013), e aumento da expressão gênica de antioxidantes enzimáticos tais como SOD, CAT e GPx em fígado e pulmão da prole já adulta, foi demonstrada com a ingestão materna de quercetina e genisteína (Vanhees et al. 2013).

Entretanto, em humanos já foi encontrada relação negativa com o consumo de alimentos ricos em polifenois no terceiro trimestre da gestação, levando a uma constrição do canal arterial coronário fetal, possivelmente devido ao seu efeito anti-inflamatório (Zielinsky and Busato 2013, Zielinsky et al. 2010, Zielinsky et al. 2012).

1.5. Exercício físico materno

Por muitos anos a prática do exercício físico durante a gestação não foi indicada, devido à falta de maior conhecimento dos efeitos sobre a gestante e também sobre a prole, como o possível efeito teratogênico que o aumento da temperatura corporal poderia causar (Shiota 1988), e também o efeito do impacto do exercício físico de maior intensidade (Gorgati 2008).

A prática foi recomendada apenas em 2002 pelo Colégio Americano de Obstetras e Ginecologistas (ACOG 2002), que em 2015 atualizou a diretriz, mantendo a mesma prescrição de 20 a 30 minutos de exercício moderado por dia, para gestantes sem complicações médicas prévias (ACOG 2015).

O exercício materno traz benefícios à criança já ao nascer, pois

aumenta a taxa de nascimentos com peso adequado, reduzindo o risco para macrossomia em torno de 30%, o que parece manter-se ao menos até a infância, onde crianças nascidas de uma gestação ativa demonstram menor peso e menor percentual de gordura corporal desde o nascimento até os cinco anos de idade (Clapp 1996, Siebel, Carey and Kingwell 2012, Wiebe et al. 2015, Currie et al. 2014). Em modelo animal, o exercício materno demonstra efeito a longo prazo na prole em relação ao peso corporal, com aumento na massa magra e diminuição do percentual de gordura nos machos (Sheldon et al. 2015, Carter et al. 2012), melhora da homeostase da glicose em machos e fêmeas (Carter et al. 2012), e melhora na saúde vascular (Bahls et al. 2014).

O exercício materno também tem efeito protetor contra um ambiente estressor. Em modelo animal demonstrou reduzir parte do dano neuronal e alterações comportamentais em ratos jovens causadas pelo estresse diário ao final da gestação (Bustamante et al. 2013). Ainda, preveniu a diminuição dos níveis de fator de crescimento endotelial vascular (VEGF, do inglês *vascular endothelial growth factor*) e fator neurotrófico derivado do encéfalo (BDNF, do inglês *brain derived neurotrophic factor*) em caso de privação materna, também reduzindo a corticosterona sérica aos níveis do controle em ratos de 24 dias (Uysal et al. 2011). Neste sentido, o exercício materno parece proteger também contra insultos no período pós-natal, prevenindo a perda neuronal causada por períodos de hipóxia do 4º ao 8º dia de vida (Akhavan et al. 2012), e evitando a esteatose hepática e outros danos induzidos por dieta rica em lipídios em ratos machos na vida adulta, que demonstram ainda aumento de biogênese mitocondrial (Sheldon et al. 2015), melhora na sensibilidade à insulina e redução na expressão de IL 6 (Wasinski et al. 2015).

O encéfalo da prole também é alvo da prática de exercício materno, com aumento das defesas antioxidantes e da biogênese mitocondrial em ratos de sete dias de vida (Marcelino et al. 2013), melhora na aprendizagem e memória aos 21, 36 e 120 dias (Akhavan et al. 2008, Dayi et al. 2012), aumento na expressão do ácido ribonucleico (RNA, do inglês *ribonucleic acid*) mensageiro de BNDF (Parnpiansil et al. 2003, Kim et al. 2007), neurogênese no hipocampo no período pré-púbere e na vida adulta (Lee et al. 2006, Dayi et al. 2012), além de aumentar a expressão de leptina em ratos adultos (Dayi et al. 2012). Todas estas adaptações benéficas causam um melhor desenvolvimento e função cerebral na prole, que em longo prazo demonstram ter efeito inclusive na redução do risco de desenvolvimento da doença de Alzheimer, com redução em parâmetros que são comuns a diversas doenças neurodegenerativas como estresse oxidativo e inflamação, e melhora na plasticidade cerebral (Herring et al. 2012).

1.6. Suplementação com antioxidantes na prática do exercício físico

A prática de exercício físico leva à produção de ERO, que é dependente da intensidade da atividade física. As espécies reativas irão participar tanto da sinalização para adaptação, mediadas principalmente por NF-κB, fator de transcrição nuclear eritroide 2 p45-relacionado (Nrf2, do inglês *nuclear factor erythroid 2-related factor 2*), e PGC-1α (Sachdev and Davies 2008, Ji et al. 2004, Gomez-Cabrera, Domenech and Vina 2008b, Muthusamy et al. 2012, Piantadosi and Suliman 2012, Olsen, Cornelius and Gregersen 2015); quanto da indução de dano oxidativo, quando em excesso (Halliwell 2006a, Nordberg and Arner 2001, Thanan et al. 2015, Mei et al. 2015).

O uso de antioxidantes na prevenção de doenças tem se mostrado contraditório, não sendo demonstrada relação com a diminuição do risco para câncer e doenças cardiovasculares (Myung et al. 2013, Myung and Yang 2013, Valko et al. 2004). Alguns estudos mostram que a suplementação aumenta a incidência dessas doenças (Omenn et al. 1996), inclusive estimulando a proliferação de células cancerígenas, visto que as ERO são importantes no estímulo da apoptose em células malignas (Verhaegen et al. 1995, Saintot et al. 1996, Maxwell 1999, Mates and Sanchez-Jimenez 2000).

Sua suplementação aliada à prática de exercício físico parece inicialmente trazer benefícios, impedindo o dano oxidativo causado pela contração muscular, porém este impedimento tem demonstrado evitar a própria adaptação do exercício, que em longo prazo é benéfica e considerada antioxidante *per se* (Xiao 2015, Gomez-Cabrera et al. 2015, Gomez-Cabrera et al. 2008b, Powers et al. 2010). Em modelo animal, a suplementação com quercetina aliada ao exercício físico demonstrou causar aumento do dano oxidativo a proteínas no cerebelo de ratos de 60 dias, e aboliu o aumento da expressão de PGC-1α causada pelo exercício isolado (Casuso et al. 2015). A suplementação com vitamina C em ratos também preveniu o aumento da expressão de SOD, GPx, Nrf1, PGC-1α e TFAM induzida pelo exercício no músculo de ratos de 90 dias (Gomez-Cabrera et al. 2008a).

Em humanos, já foi demonstrada a prevenção da biogênese mitocondrial no músculo induzida pelo exercício com a suplementação de vitamina C e E, em um protocolo experimental de 11 semanas (Paulsen et al. 2014a), enquanto um protocolo de 4 semanas com as mesmas vitaminas demonstrou reduzir o aumento da SOD e de TFAM induzido pelo treino no músculo (Morrison et al.

2015).

2. Objetivos

2.1. Objetivo Geral

O objetivo geral deste trabalho foi avaliar se e como a suplementação com naringenina altera as adaptações metabólicas encefálicas verificadas na prole de ratas Wistar submetidas ao exercício físico durante a gestação.

2.2. Objetivos Específicos

- Avaliar parâmetros de homeostase redox, denominados conteúdo de espécies reativas através da oxidação da diclorofloresceina (DCFH), conteúdo de malondialdeído (MDA), níveis de vitamina C e GSH; além da atividade das enzimas antioxidantes SOD, CAT e GPx em cerebelo, córtex parietal e hipocampo da prole no sétimo dia pós-natal (PND), a qual foi submetida ao protocolo de exercício materno e suplementação pré-natal com naringenina;

- Avaliar parâmetros bioquímicos mitocondriais através da quantificação da atividade das enzimas citrato-sintase (CS), isocitrato-desidrogenase (IDH), α-cetoglutarato-desidrogenase (α-KGDH, do inglês *α-ketoglutarate dehydrogenase*), succinato-desidrogenase (SDH) e malato-desidrogenase (MDH), e dos complexos I-IV da cadeia respiratória nas mesmas estruturas encefálicas da prole submetida a exercício e suplementação com naringenina maternos.

3. Resultados

3.1. Capítulo I:

Combined approaches of aerobic physical exercise and naringenin supplementation during pregnancy alters offspring's brain redox status in Wistar rats

Autores: Pauline Maciel August; Daniela Pereira Stocher; Vinícius Stone; Yasmini D. Silva; Régis Hözer; Tiago B. Salomon, Mara S. Benfato and Cristiane Matté

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Combined approaches of aerobic physical exercise and naringenin supplementation during pregnancy alters offspring's brain redox status in Wistar rats

Pauline Maciel August[2]; Daniela Pereira Stocher[1]; Vinícius Stone[2]; Yasmini D. Silva[1]; Régis Hözer[1]; Tiago B. Salomon[3], Mara S. Benfato[3,4] and Cristiane Matté[1,2]

[1] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

[2] Programa de Pós graduação em Ciências Biológicas: Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

[3] Programa de Pós-graduação em Biologia Molecular e Celular, Instituto de Biociências, Universidade Federal do Rio Grande do Sul.

[4] Departamento de Biofísica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul.

Corresponding author: Cristiane Matté, PhD, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo (laboratório 23), CEP 90035-003, Porto Alegre, RS, Brazil, Phone: +55 51 3308 5548, Fax: +55 51 3308 5535, e-mail: matte@ufrgs.br

Abstract

Maternal environment during pregnancy is decisive to fetus metabolic programming. Diverse disturbing factors, such as stress, hormones, diet and physical exercise during pregnancy could leave long-term effects on offspring. We previously showed that swimming exercise improves brain's antioxidant network and induces mitochondrial biogenesis. Considering that recent evidence show that antioxidant supplementation might cancel the metabolic adaptations induced by exercise, we evaluated the offspring brain redox status in response to maternal swimming allied or not to naringenin intake by pregnant rats. Female Wistar rats were divided into four groups: (1) sedentary (2) sedentary with naringenin intake (3) swimming exercised (4) swimming exercised with naringenin intake. The exercised groups practice thirty-minute swimming exercise for 5 days/week, during 4 weeks, while sedentary groups were only immersed in 32 ºC water. Naringenin was administered daily during pregnancy, at 50 mg/kg, before exercise. Offspring was euthanized at 7^{th} day of life, when cerebellum, parietal cortex, and hippocampus were dissected, to analyze redox status parameters. Our results showed that naringenin intake during pregnancy enhanced malondialdehyde (MDA) and reduced glutathione (GSH) content in cerebellum and glutathione peroxidase (GPx) activity in parietal cortex; while maternal exercise enhanced superoxide dismutase (SOD) and GPx activities and GSH content in cerebellum, as well as GPx activity and GSH content in parietal cortex. The effects of both treatments were maintained on some parameters and canceled in others. In conclusion, the antioxidant supplementation and exercise practice during pregnancy significantly modify antioxidant responses in offspring brain, suggesting a positive metabolic neuromodulation.

Keywords: metabolic programming, flavonoids, oxidative stress, adaptation, free radical

Introduction

The benefits of bioactive enriched diet and regular exercise practice on health are incontestable. Modern life habits, with easier access and higher food consumption, leads to dietary deficiencies that are related to higher mortality rates, allied to enhanced risk to cancer development, obesity, hypertension, and other cardiovascular diseases [1-3].

Insofar, healthier diets including fruits, vegetables, and oilseeds are associated to lower blood pressure levels and reduced risk of cardiovascular disease development [4].

While the initial response to high ATP demand during physical exercise leads to increased reactive oxygen species production by the respiratory chain, the long term practice brings relevant adaptations on the antioxidant defenses, characterizing exercise itself an antioxidant therapy [5].

The regular practice of physical exercise is considered a non-pharmacological treatment, even preventing cardiovascular diseases, diabetes, and neurological diseases [6-8]. Nevertheless, physical exercise practice during pregnancy was not indicated up to 2002 by American College of Obstetricians and Gynecologists [9].

Nowadays, physicians are prudent, and a recently published update maintained the indication of at least 20-30 minutes of moderate exercise a day, comprising 150 minutes/week [10]. In addition, aquatic activities are preferred, due to positive effects of immersion as less visceral blood flow, increased cardiac output, and reduced impact in joints (Katz 2003).

It has been established that regular practice of moderate physical exercise during pregnancy leads to a significant metabolic change for both,

mother and fetus [11-14].

Pregnant women exhibited decreased risk of preeclampsia and gestational diabetes [15-18], while their children presented further guidance and regulation capacity on the fifth day after birth [19], as well as a better oral language score at five years old [13]. In addition, animal models subjected to prenatal exercise showed a better offspring brain growth and development, due to increased hippocampal neurogenesis and leptin expression [20, 21].

Another crucial interference during pregnancy is the mother diet. Over or poor nutrition during pregnancy cause fetus impairments, resulting in macrosomia or low birth weight, respectively. Prenatal nutrient availability impacts the adulthood, altering the risk factors for insulin resistance, diabetes, and cardiovascular diseases [22-26].

In this sense, naringenin, an flavonoid found in citrus fruits [27], has demonstrated protective effects on health as antioxidant [28, 29], antihyperglycemic [30], anticarcinogenic [31], antidepressant [32], and anti-inflammatory activities [33]. Although there is a lack of studies concerning the intake of flavonoids during pregnancy, Vanhees and colleagues [34] showed increased enzymatic and non-enzymatic antioxidant capacity in liver and lung, and a reduced DNA damage induced by oxidative stress in adult offspring, triggered by flavonoids administration during pregnancy.

With the increasing demand for healthier living habits, the combination of physical activity and antioxidant supplementation is natural. The final objective with this healthy protocol is to achieve cellular protection against stress, including oxidative stress.

Nonetheless, recent evidence support the idea that exogenous

antioxidants might block the metabolic adaptations induced by exercise practice; including mitochondrial biogenesis [35, 36], Nrf2 and PCG-1α nuclear translocation [37], even MAPKs, as p38 and ERK1/2 phosphorylation [38].

Therefore, the present study aims to evaluate the effect of naringenin intake allied to swimming exercise practice by dams on offspring's brain redox homeostasis. We select the brain as a target due to its high energy demand and consequently the high rate of oxygen consumption, that makes the brain susceptible to oxidative attack, coupled with reduced antioxidant defenses and high content of iron and copper, which can easily react with reactive species, producing hydroxyl radical [39-41].

Experimental Procedures

Animals and reagents

Forty-eight adult female (90 days of age) and 24 adult male Wistar rats (60 days of age) with an average weight of 200 and 250 g respectively were obtained from the Central Animal House of Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air conditioned constant temperature (22 $\pm$ 1°C) colony room. The animals had free access to water and a 20% (w/w) protein commercial chow.

The experiments were approved by the local Ethics Commission (Comissão de Ética no Uso de Animais - Universidade Federal do Rio Grande do Sul, CEUA/UFRGS) under the number 26542, and followed according 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.

Experimental design

Female rats were randomly divided into four groups (n=12): 1) sedentary rats receiving vehicle (1 mL/Kg p.o.); 2) sedentary rats receiving naringenin (50 mg/Kg p.o.); 3) swimming exercise receiving vehicle (1 mL/Kg p.o.); or 4) swimming exercise receiving naringenin (50 mg/Kg p.o.).

The experimental design is presented in Figure 1. The administration of naringenin and/or vehicle was started only after mating, while the maternal exercise began one week previous to mating, in order to aquatic environment habituation. During the exercise protocol four animals were kept in each cage, except for mating (one male per two females). Pregnancy was diagnosed by the presence of a vaginal plug. From the 20th day after the onset of pregnancy, we isolated the pregnant dams (one per cage), and the rats were observed twice a day (at 9 and 18 h), to verify the litter's birth. The day corresponding to the offspring's birth was defined as postnatal day 0 (PND0).

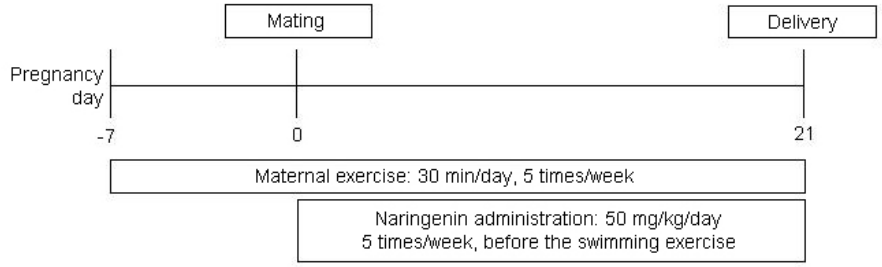

Figure 1. Experimental design

The offspring was left with the mother until PND7, when the offspring was euthanized by decapitation without anesthesia. Cerebellum, parietal cortex, and hippocampus were dissected, and stored at -80°C until the completion of the biochemical assays. It was used one pup from each offspring for each technique, in order to eliminate the litter effect.

Naringenin supplementation

Naringenin was suspended in sunflower oil (1 mL/kg), which was also used as vehicle. The oral treatments, given by gavage, were given just before the swimming exercise, according the weight of each animal (measured daily), and initiated with the onset of the second week of swimming protocol, as depicted in Figure 1. The scheme of naringenin administration is due its increasing availability in plasma immediately after it is ingested [42, 43], and its dose was defined according its neuroprotective action demonstrated in literature [44, 45].

Swimming exercise protocol

The maternal exercise protocol was adapted from [21], as described in [46]. The rats were divided into control and exercised groups. In the exercised group, rats were submitted to swimming in a pool filled with 32±1ºC water on 5 day/week for 4 weeks. Each swimming session lasted for 30 minutes, and always took place between 9 and 12 a.m. Each rat was isolated for the swim, which was conducted using an apparatus designed specifically for rat swimming. Within this apparatus, each room measures 30x30x90 cm (width x length x depth), preventing the animals from touching the bottom of the tank. The animals were left free to swim, without any extra weight, and were gently stimulated to swimming. This protocol has moderate intensity. Control rats were

immersed in water, carefully dried, and returned to the housing boxes.

Biochemical Assays

Cerebellum, parietal cortex, and hippocampus were prepared for oxidative stress assays as previously described [46].

Malondialdehyde contents

Concentration of malondialdehyde (MDA), a product of lipid peroxidation, was 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 MDA was 5.6 min. MDA levels was expressed as mg/g of protein [47].

Dichlorofluorescein oxidation

The reactive oxygen/nitrogen species content was assessed through the 2',7'-dichlorofluorescein (DCFH) oxidation method [48]. In a 96-well plate, 50 µL of diluted sample was incubated at 37 °C/ 30 min, in the dark, with the addition of 200 µL of DCFH diacetate (H_2DCF-DA). H_2DCF-DA is cleaved by cellular esterases and form DCFH, a non-fluorescent compound, that is oxidized by reactive species present in the sample, producing a fluorescent compound, DCF. DCFH oxidation was measured fluorimetrically, using a 488 nm excitation and 525 nm emission wavelength. A standard curve of DCF (0.25-10 mM) was performed in parallel with the samples. The results were expressed as nmol/mg protein.

Antioxidant enzymes activity

Superoxide dismutase (SOD, EC 1.15.1.1) activity was evaluated by quantifying the inhibition superoxide-dependent autoxidation of epinephrine, verifying the absorbance of the samples at 480 nm [49]. SOD activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit. The data were expressed as units/mg protein.

Catalase (CAT, EC 1.11.1.6) activity was assayed according to Aebi [50] by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100 and 10 mM potassium phosphate buffer, pH 7.0. One CAT unit is defined as 1 µmol of hydrogen peroxide consumed per minute and the specific activity is reported as units/mg protein.

Glutathione peroxidase (GPx, EC 1.11.1.9) activity was measured according to the method described by Wendel [51] using *tert*-butyl hydroperoxide as substrate. NADPH disappearance was monitored spectrophotometrically at 340 nm in a medium containing 2 mM glutathione, 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.

Non-enzymatic antioxidants measure

The content of reduced glutathione (GSH) was measured according the method described by Browne and Armstrong [52]. Initially, the proteins were precipitated with meta-phosphoric acid (1:1, v:v), after centrifugation (5.000 x g at 25ºC/ 10 min), 40 µL of supernatant was incubated with 15 µL of 7.5 mM o-phthaldialdehyde and 185 µL of 120 mM sodium phosphate buffer pH 8.0,

containing 5 mM ethylene diamine tetraacetic acid (EDTA), at room temperature during 15 min. GSH reacts with the fluorophore o-phthaldialdehyde. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. A blank sample and a standard curve of GSH (0.001-1 mM) were performed in parallel. The data are expressed as nmol/mg protein.

Vitamin C levels were measured by High-performance liquid chromatography (HPLC), in the same assay used to quantify MDA. Under these conditions, the retention time of vitamin C was 3.0 min. Vitamin C was expressed as mg/g of protein [47].

Protein concentration assay

Protein concentration was measured by the method of Lowry et al. [53], using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by two-way ANOVA, using GraphPad Prism 6.0 software. All analyzes were followed by a Tukey post-test for multiple comparisons for parametric data. Data were presented as mean ± SEM, and were considered statistically significant when $p<0.05$.

Results

Maternal swimming exercise and naringenin supplementation did not alter litter's weight and size

Table 1 shows maternal exercise practice and naringenin supplementation on litter's weight and size. We observed that the treatments are not capable to cause significant differences on both litter's weight [$F(1,63)=0.02684;p=0.8704$] and size [$F(1,63)=3.020;p=0.0872$].

Table 1. Effect of maternal exercise and naringenin supplementation during pregnancy on litter's weight and size.

	Control	*Naringenin*	*Exercised*	*Exercised + naringenin*	*p value*
Litter weight (weight/pup)	6,8 (5,2 – 7,8)	6,7 (6,2 - 7,5)	6,8 (5,6 – 8)	6,8 (5,3 – 7,9)	0.8704
Litter size (number of pups)	*8,3 (1 – 13)*	*9,6 (6 – 11)*	*8,6 (4 – 14)*	*7,4 (1 – 13)*	*0.0872*

Data are expressed as mean (minimum-maximum). Results were evaluated by ANOVA followed by Tukey's test, for n=14-19.

Swimming exercise and naringenin supplementation alters the reactive species levels and antioxidant parameters in offspring's cerebellum

Figure 2 shows MDA content and DCFH oxidation in the offspring's cerebellum. Interestingly, MDA levels were enhanced in naringenin supplementation group ($p<0.01$, Tukey's test), while the reactive species levels, evaluated by DCFH oxidation, was not significantly increased [$F(1,24)=1.24;p=0.0802$]. Maternal exercise did not alter MDA levels, however, two way ANOVA showed a significant difference on DCFH oxidation levels [$F(1,24)=5.946;p=0.0225$], increasing it. In addition, two way ANOVA showed an interaction between both treatments (naringenin and exercise) on MDA concentration [$F(1,22)=14.74;p=0.0009$], but not for DCFH oxidation [$F(1,24)=0.1766;p=0.6781$].

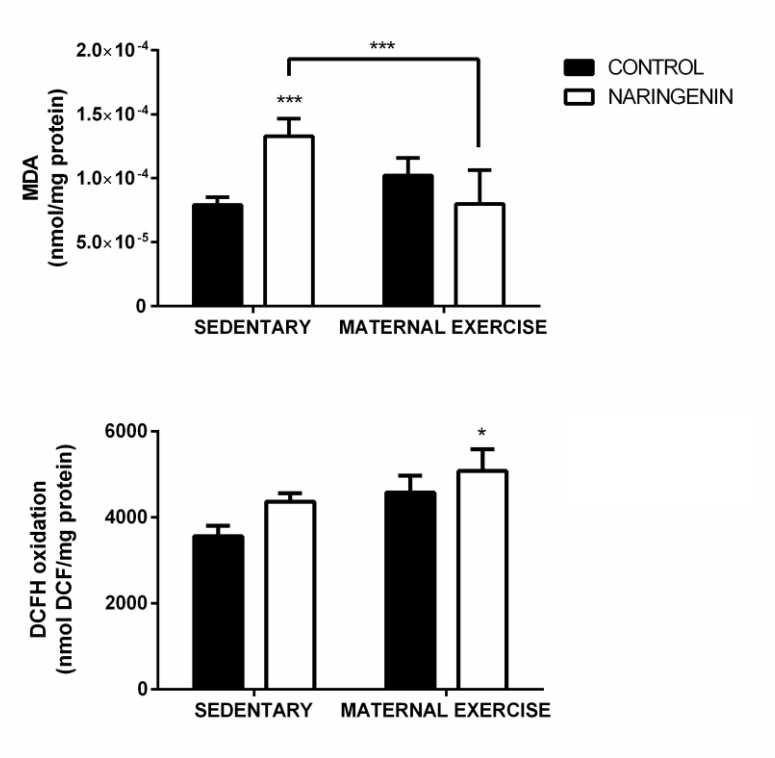

Figure 2. Effect of maternal naringenin supplementation and/or swimming exercise on (A) malondialdehyde (MDA) and (B) 2'7'-dichlorofluorescein (DCFH) oxidation levels in offspring's cerebellum. Results are expressed as mean + SEM for *n*=7 performed in triplicate. Results were analyzed by two-way ANOVA followed by Tukey's test. *p<0.05; ***p<0.001

Figure 3 shows the maternal exercise practice and naringenin supplementation effect on antioxidant enzymes activities in offspring's cerebellum. SOD activity was enhanced in exercise group [F(1,16)=7.255;p=0.0160], and when allied to naringenin intake, the effect disappears, as showed by the statistical analysis [F(1,16)=1.204;p=0.2888].

GPx activity was also enhanced in exercise group [F(1,23)=14.58;p=0.0009], and the effect was maintained even allied to naringenin supplementation. CAT activity was not affected by neither of the treatments [F(1,18)=0.03174;p=0.8606].

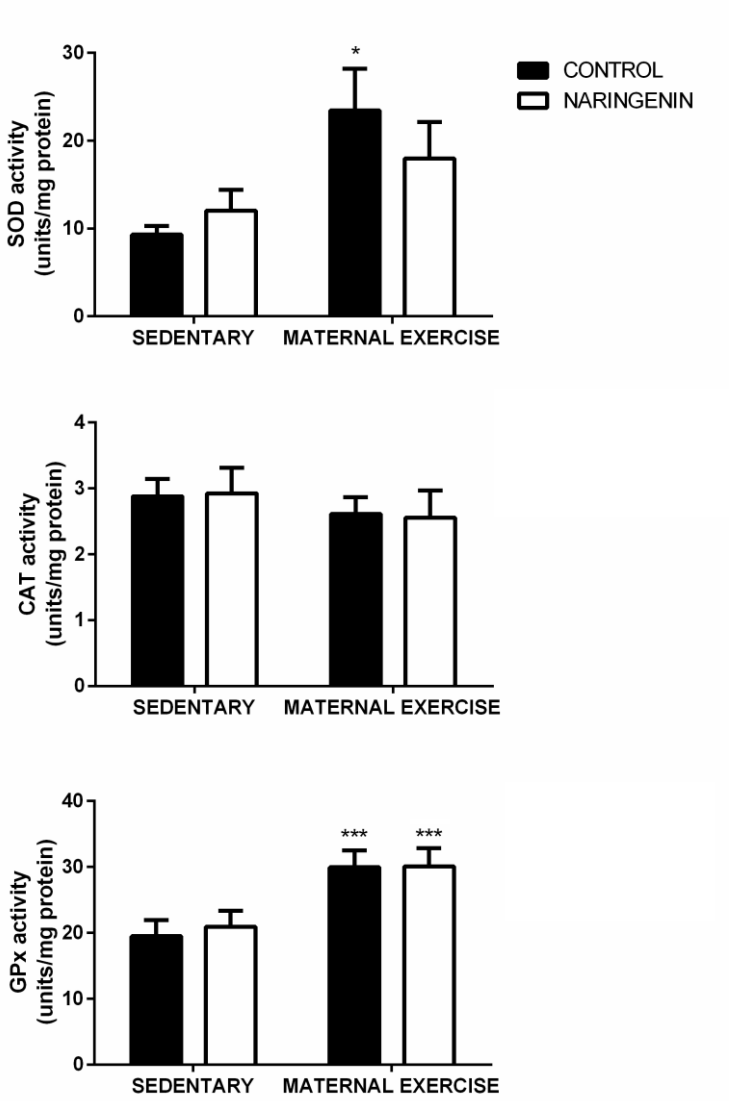

Figure 3. Effect of maternal naringenin supplementation and/or swimming exercise on (A) superoxide dismutase (SOD), (B) catalase (CAT), and (C) glutathione peroxidase (GPx) activities in offspring's cerebellum. Results are expressed as mean + SEM for *n*=6-7 performed in triplicate. Results were analyzed by two-way ANOVA followed by Tukey's test. *p<0.05; ***p<0.001

In agreement with the increment in enzymatic antioxidants, Figure 4 shows that GSH content in offspring's cerebellum was enhanced by isolated

maternal naringenin intake and swimming exercise [F(1.18)=21.15;p=0.0002] but not when allied, as demonstrated by two way ANOVA [F(1.18)=0.04404;p=0.8361]. We also measured vitamin C concentration, that was not affected [F(1,24)=1.985;p=0.1717].

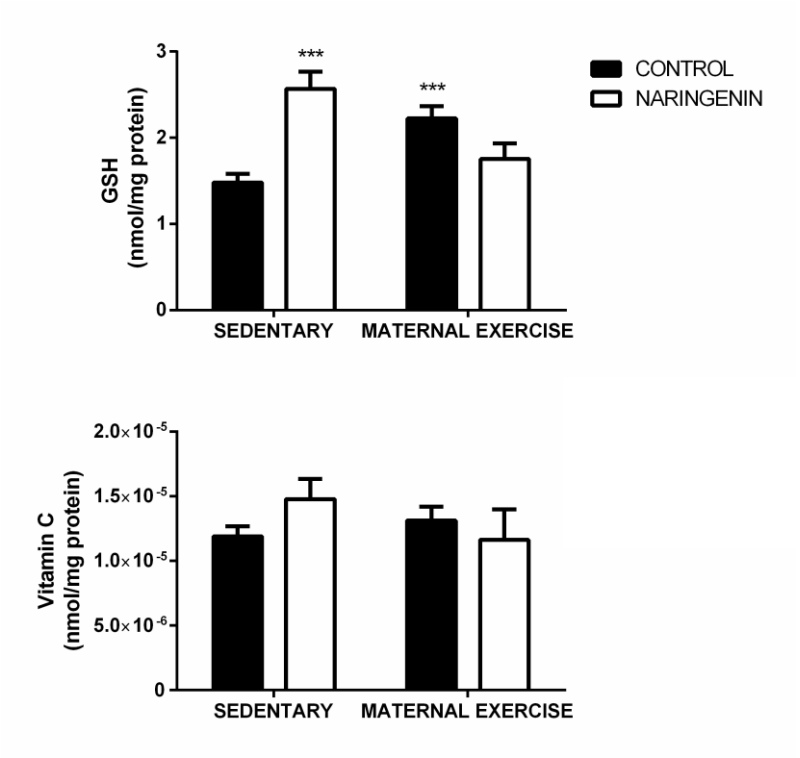

Figure 4. Effect of maternal naringenin supplementation and/or swimming exercise on (A) reduced glutathione (GSH) and (B) vitamin C levels in offspring's cerebellum. Results are expressed as mean+SEM for *n*=7 performed in triplicate. Results were analyzed by two-way ANOVA followed by Tukey's test. ***p<0.001

Swimming exercise and naringenin supplementation enhances antioxidant status in offspring's parietal cortex

Parietal cortex did not present any alteration in MDA content [F(1,21)=0.06199;p=0.8058] or DCFH oxidation [F(1,21)=0.1870;p=0.6698], as showed in Figure 5.

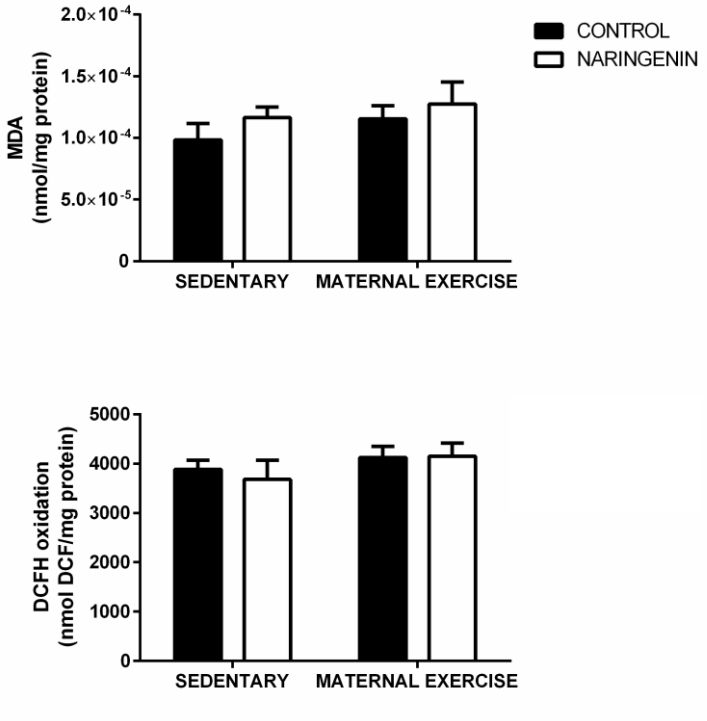

Figure 5. Effect of maternal naringenin supplementation and/or swimming exercise on (A) malondialdehyde (MDA) and (B) 2'7'-dichlorofluorescein (DCFH) oxidation levels in offspring's parietal cortex. Results are expressed as mean+SEM for *n*=7 performed in triplicate. Results for two-way ANOVA followed by Tukey's test.

On the other hand, antioxidant network was affected. Figure 6 shows the maternal exercise practice and naringenin supplementation effect on SOD, CAT, and GPx activities in offspring's parietal cortex. Despite the tendency of enhanced activity, SOD was not altered [F(1,17)=1.128;p=0.3031], as well as CAT activity [F(1,18)=1.360;p=0.9991]. In accord with the data found in cerebellum, GPx activity was enhanced in isolated naringenin [F(1,19)=8.978;p=0.0074] and exercise groups and also when both factors were allied [F(1,19)=12.38;p=0.0023].

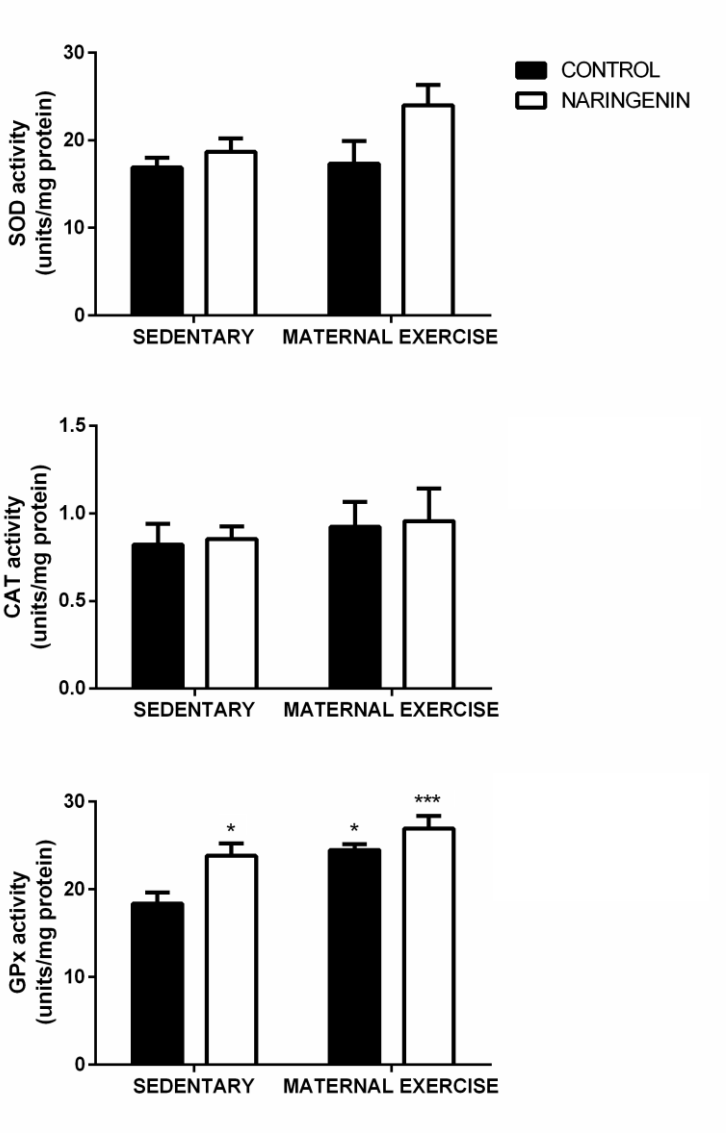

Figure 6. Effect of maternal naringenin supplementation and/or swimming exercise on (A) superoxide dismutase (SOD), (B) catalase (CAT), and (C) glutathione peroxidase (GPx) activities in offspring's parietal cortex. Results are expressed as mean+SEM for *n*=7 performed in triplicate. Results were analyzed by two-way ANOVA followed by Tukey's test. *p<0.05; ***p<0.00

In agreement, Figure 7 shows GSH content was enhanced in isolated exercise group, and also with naringenin supplementation addition [F(1,20)=9.038;p=0.0070]. Similarly to found in cerebellum, vitamin C levels were not affected [F(1,23)=1.341;p=0.2588].

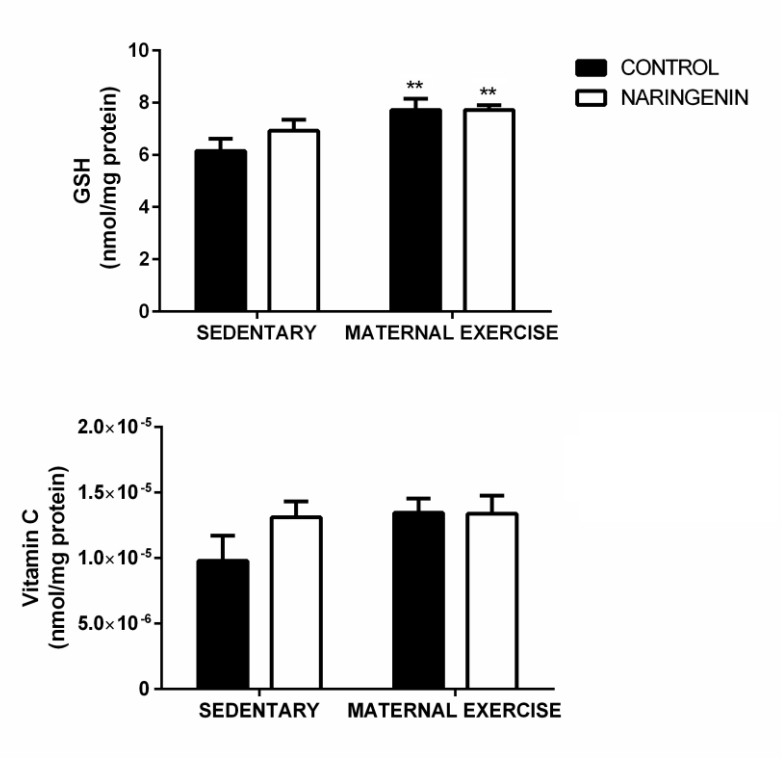

Figure 7. Effect of maternal naringenin supplementation and/or swimming exercise on (A) reduced glutathione (GSH) and (B) vitamin C levels in offspring's parietal cortex. Results are expressed as mean+SEM for *n*=6-7 performed in triplicate. Results were analyzed by two-way ANOVA followed by followed by Tukey's test. **p<0.01

Swimming exercise and naringenin supplementation did not affect redox status in offspring's hippocampus

The maternal exercise practice and naringenin supplementation did not cause statistical significant alterations in MDA content [F(1,20)=0.7543;p=0.3954], DCFH oxidation [F(1,21)=0.0003307;p=0.9857], SOD [F(1,17)=2.734;p=0.1166], CAT [F(1,22)=3.018;p=0.0963] and GPx [F(1,21)=2.746;p=0.1124] activities, as well as in GSH [F(1,21)=0.01287;p=0.9107], and vitamin C levels [F(1,20)=0.006530;p=0.9364] (Table 2).

Table 2. Effect of maternal exercise and naringenin supplementation during pregnancy on redox status in offspring's hippocampus

	Control	Naringenin	Exercised	Exercised + naringenin	p value
MDA content	$8x10^5$ ($6x10^5$ - $1x10^4$)	$9x10^5$ ($7x10^5$ - $1x10^4$)	$9x10^5$ ($6x10^5$ – $1x10^4$)	$9x10^5$ ($7x10^5$ – $1x10^4$)	0.3954
DCFH oxidation	6396 (5192 – 7929)	5716 (3347 – 7998)	7043 (5300 – 8096)	6335 (3185 – 8780)	0.9857
SOD activity	32,5 (26,5 – 45,2)	33,8 (24,4 – 45,8)	40,4 (25,2 – 55,6)	29,3 (27 – 29,9)	0.1166
CAT activity	1,3 (0,9 – 1,6)	1,2 (0,7 – 1,4)	1,6 (1,3 – 1,9)	1,2 (0,6 – 2)	0.0963
GPx activity	18,7 (17,4 – 20,4)	20 (14,5 – 26,8)	22 (19,2 – 24,2)	18,6 (13,5 – 23,8)	0.1124
GSH content	4,8 (3,3 – 6,8)	4 (3,1 – 5,6)	4,4 (4 – 5,4)	3,6 (2,4 – 6,5)	0.9107
Vitamin C levels	$7x10^6$ ($6x10^6$ – $1x10^5$)	$7,9x10^6$ ($6x10^6$ – $1x10^5$)	$8x10^6$ ($7 x10^6$ – $1x10^5$)	$9x10^6$ ($8x10^6$ – $2x10^5$)	0.9364

Data are expressed as mean (minimum-maximum). Results were evaluated by ANOVA followed by Tukey's test, for n=6-7.

Discussion

Understanding the effect of physical exercise practice combined with antioxidants consumption has gained attention in recent years. Initially, it was believed that antioxidant supplementation owns protective effect on exercise-induced muscle damage. At the moment, literature evidence have contested this theory, showing that reactive species produced as a result from exercise possesses adaptive effects, and when eliminated by antioxidants, the beneficial metabolic adaptations were not verified [35, 54, 55].

In this sense, exercise *per se* is able to increase the muscular antioxidant capacity, and the antioxidant supplementation might not be the most appropriate conduct to achieve health and physical performance improvement [5].

Herein, it was aimed to assess whether the combined approach of maternal swimming exercise practice and naringenin supplementation could change the offspring's brain adaptations exerted by each individually. Some of the redox homeostasis parameters were evaluated, and appears that naringenin intake by dams, as swimming exercise, induces positive antioxidant adaptations when isolated. Nevertheless, when both strategies were allied some of the benefits disappear.

Referring to the weight of offspring and litter size, the treatments did not cause significant change. Exercise during pregnancy in humans does not cause changes in birth weight, only reduces the risk for macrosomia or underweight [13, 56, 57]. Regarding antioxidant supplementation during pregnancy, results differ. Melatonin supplementation during pregnancy has been shown to reduce offspring weight and litter size in Wistar-Kyoto and Sprague-Dawley rats [58], while vitamin E could not alter litter size in rabbits [59].

MDA is a lipid peroxidation product, used as a marker for lipid damage assessment [60]. Maternal exercise did not cause changes in the offspring's brain MDA content, agreeing with other studies, in which, the treadmill exercise practice on adult male rats for 30 days/ 30 minutes a day did not alter MDA levels in rat's hippocampus [61]. Also, another protocol of maternal exercise using treadmill during 30 minutes a day, 5 days a week, during pregnancy also did not affect MDA levels in offspring's hippocampus [62]. In agreement, we previously measured carbonyl levels, an index of protein oxidation, and it was not affected by maternal swimming [46].

At the same time, we found that naringenin supplementation during pregnancy enhanced MDA levels in offspring cerebellum, causing no effect in

other brain structures. Naringenin supplementation at 50 mg/kg during 8 days did not cause MDA levels alterations in rat kidney [63], and during 4 weeks did not affect MDA levels in adult male rat cortex [64]. Our data suggest that naringenin supplementation during pregnancy caused increased lipid oxidation in the offspring's cerebellum. Although GSH levels were increased, it seems not to be enough to restrain the oxidative effect.

Although DCFH oxidation involves many reactive oxygen/nitrogen species detection (ROS/RNS), it is more sensible to hydrogen peroxide, peroxynitrite, and hydroxyl radicals [65, 66]. Offspring's cerebellum showed increased DCFH oxidation levels when the maternal exercise practice was allied to naringenin supplementation, despite the slight increment verified in the treatments isolated. In agreement, we already demonstrated that swimming exercise performed during pregnancy increases DCFH oxidation in offspring's cerebellum [46]. In addition, naringenin showed to increase DCFH oxidation levels in human lung embryonic fibroblasts cell culture, when incubated with 75 or 150 µM [67].

Considering the increment in reactive species verified in litter's cerebellum, and its potential to induce the expression of antioxidant enzymes by diverse signaling pathways, we evaluated the central antioxidant network.

SOD catalyzes the $O_2^{\bullet-}$ dismutation, forming O_2 and H_2O_2 [68, 69]. Offspring showed an increasing in SOD activity in exercised group in the cerebellum, while in the parietal cortex maternal exercise practice allied to naringenin supplementation showed a tendency to increase.

Regarding the effect on SOD activity, the results are diverting in the literature. Marques-Aleixo and colleagues [70] did not found alterations after 12

weeks of treadmill training in male rats' cerebellum, while Casuso and colleagues [71], also evaluating male rats' cerebellum, found decreased SOD activity after 6 weeks of treadmill exercise, isolated and with quercetin supplementation addition. Both studies were done 48 hours after the last exercise training and evaluated the exercised rat directly.

CAT exclusively utilizes H_2O_2 as substrate, forming H_2O and O_2 inside the peroxissome [69, 72]. We did not find any alteration on this parameter, in agreement to Chtourou and colleagues [64], which reported that naringenin supplementation (50 mg/kg) during four weeks did not affect CAT activity in cerebral cortex. Another study showed that aquatic exercise practice also did not change CAT activity in the cerebral cortex, performed 1 hour a day, 5 days a week [73].

In contrast, we previously showed that CAT was increased by maternal swimming exercise in cerebellum, parietal cortex, and hippocampus from pups delivered from exercised dams [46]. At this moment, we could not explain the diverse results found in different moments, however, we speculate that the administration of oil as vehicle to exercised dams might alter this parameter, considering that CAT eliminates hydrogen peroxide produced by lipid β-oxidation in peroxisomes [69, 74].

GPx requires GSH to promote the reduction reaction of H_2O_2 in H_2O, and can also act removing organic hydroperoxides and peroxynitrite [69]. We found increased GPx activity in exercised group and also in exercise allied to naringenin supplementation group in offspring's cerebellum and parietal cortex, as well as an enhanced activity in parietal cortex from pups subjected to maternal naringenin supplementation. The results on exercise effect are in

accord with our previous data, showing increased GPx activity in cerebellum and parietal cortex in pups delivered from exercised dams [46].

Other studies demonstrate that naringenin enhances GSH oxidation in a dose-dependent effect, through peroxidase/H2O2 system [75], and its glycosylated form naringin increases GPx activity in P388 cells, when treated during 60 minutes with 1 mM of the flavanol [76]. At the same time, Jaguetia and colleagues [77] found that HepG2 cells treated with naringin at 1 mmol during 1 hour did not alter GPx activity, and Wistar rats treated with naringin at 25, 50 100 mg/kg during 5 weeks also did not show GPx activity modulation in striatum [78].

The enhanced antioxidant enzymes activities can be related to exercise and flavonoid induction of transcription factors activation, as PGC-1α and Nrf2, which, when activated, are translocated to the nucleus, eliciting the transcription of antioxidant defenses, such as GPx and SOD enzymes [79-81]. Considering the experimental conditions in our biochemical assays, using a substrate saturated medium and optimal conditions; we could suggest that the activity of the enzyme positively correlates with their expression or content in the tissue.

In a similar way, GSH content was enhanced in naringenin group, and also in exercise group in offspring's cerebellum. However, when the treatments are allied, the effect disappears. Offspring's parietal cortex also showed enhanced GSH content in exercise group, which maintains the levels when combined to prenatal naringenin supplementation. We believe that GSH increment can be explained by increased synthesis, prompted by Nrf2 activation, which can increase the expression of GSH synthase and maintain GSH levels even in response to increased GPx activity [80, 82].

We did not found any alterations on antioxidant enzymes in offspring's hippocampus, although exercise practice has demonstrated enhanced SOD and GPx densities in hippocampus [83], as well as CAT and GPx activities in offspring's hippocampus, as previously demonstrated by our group [46]. The absence of effect of naringenin supplementation at 50 mg/kg on SOD, CAT, GPx activities and GSH content in hippocampus was also reported by Chtourou and colleagues [84].

Conclusion

In conclusion, we found a clear antioxidant effect on offspring's brain delivered from dams supplemented with naringenin and/or exercised during pregnancy. Some of the benefits are maintained when both treatments are allied, some of them not, depending on the parameter evaluated. We also verified that diverse brain structures are affected differently. Undeniably, the milieu that dams are submitted during pregnancy compromises and induces adaptations in the redox metabolism of descendants. Whether our results might be extrapolated to human condition, we propose that antioxidant strategies, polyphenols intake or physical exercise, could improve brain antioxidant defenses.

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Conflict of interest

The authors declare no conflicts of interest or competing interest.

3.2. Capítulo II:

Evidence that naringenin supplementation and swimming exercise during pregnancy enhances respiratory chain activity in offspring's brain: positive effect when isolated and null effect when allied

Autores: Pauline Maciel August; Mateus Grings; Daniela Pereira Stocher; Guilhian Leipnitz; and Cristiane Matté

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Evidence that naringenin supplementation and swimming exercise during pregnancy enhances respiratory chain activity in offspring's brain: positive effect when isolated and null effect when allied

Pauline Maciel August[2]; Mateus Grings[2]; Daniela Pereira Stocher[1]; Guilhian Leipnitz[1,2]; and Cristiane Matté[1,2]

[1] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

[2] Programa de Pós-graduação em Ciências Biológicas: Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

Corresponding author: Cristiane Matté, PhD, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo (laboratório 23), CEP 90035-003, Porto Alegre, RS, Brazil, Phone: +55 51 3308 5548, Fax: +55 51 3308 5535, e-mail: matte@ufrgs.br.

Abstract

Maternal intake of flavonoids and exercise practice during pregnancy directly affect the fetus development, bringing adaptive effects. Herein, we assessed whether maternal swimming allied to naringenin intake by pregnant rats alters the energetic metabolism in offspring's brain. Female Wistar rats were divided into four groups: (1) sedentary (2) sedentary with naringenin intake (3) swimming exercise (4) swimming exercise with naringenin intake. The exercised groups were submitted to thirty-minute swimming protocol on 5 days a week, for 4 weeks; while sedentary groups were immersed in 32 ºC water, without practice swimming exercise. Offspring was euthanized at 7^{th} day of life, when brain structures were dissected and the activities of the respiratory chain complexes were assessed. Our results showed that maternal exercise increases complex IV activity in cerebellum, and complex II activity in parietal cortex of offspring. Nevertheless, naringenin intake by dams profoundly altered the progeny's respiratory chain. The flavonoid intake increases SDH activity in cerebellum and hippocampus, complex II activity in all structures evaluated, and complex IV activity in cerebellum of offspring. When combined, naringenin intake and exercise practice during pregnancy, the effect on offspring's brain disappear. Our data suggest that antioxidant supplementation during exercise practice at gestation can avoid positive neuroadaptations on offspring, which are consistent with their similar mechanisms of action. Considering that both antioxidant strategies, polyphenol rich diet and physical exercise, might interact metabolically and eliminate the benefits of each other; we believe that the excess of health habits during pregnancy might be evaluated with attention by physicians.

Keywords: metabolic programming, flavonoids, oxidative stress, adaptation

Introduction

Prenatal exposure to a negative/positive environment brings health effects that could least for a long-term period, including adulthood (Bale 2015). It has been shown that stressors applied during pregnancy strongly affect the metabolic programming of the fetus, causing an adaptation to the stress that will lead to an exaggerated response after birth, and may predispose to various diseases in adulthood (Xiang et al. 2015, Maftei et al. 2015, Felisbino-Mendes, Villamor and Velasquez-Melendez 2014, Gross et al. 2013, Barker 1995).

In this context, cardiovascular diseases, diabetes mellitus and other chronic diseases are among the leading causes of death worldwide, according to the World Health Organization (WHO 2014). Those diseases are strongly associated with inadequate diets and also the lack of practice of physical activities, which lead to increased weight gain and hence increased mortality risk (McGavock, Anderson and Lewanczuk 2006, Ye et al. 2012, Das 2015, Myles 2014, Wilmot et al. 2012, Wellburn et al. 2015).

We have been searching for positive prenatal influences, such as diet and physical exercise, which could lead to postnatal benefits. The practice of physical exercise during pregnancy is indicated by The American College of Obstetricians and Gynecologists (ACOG 2015) for at least 20-30 minutes a day for women without medical complications, and has demonstrated benefits to pregnant women, during and after pregnancy, and also to the offspring, from birth to adulthood (Schlussel et al. 2008, Prather, Spitznagle and Hunt 2012, Clapp 1996, Hochner et al. 2012).

Women who keep active during pregnancy had lower risk to giving birth

to a baby with low or high birth weight (Siebel, Carey and Kingwell 2012), and it is also found lower weight and lower fat percentage in five-year-old children born to active pregnancies (Clapp 1996). Other benefits are demonstrated in animal models, including improvement on brain development, cognition, neurogenesis (Dayi et al. 2012, Lee et al. 2006), as well as enhanced insulin sensitivity, glucose homeostasis, and endothelial function in adulthood (Carter et al. 2013, Bahls et al. 2014). Maternal exercise also demonstrates improve mitochondrial biogenesis and enhance function in brain's offspring, probably related to activation of peroxisome proliferator-activated receptor gamma-coactivator-1 alpha (PGC-1α) and nuclear factor erythroid 2-related factor 2 (Nrf2) by reactive species (Park et al. 2013, Marcelino et al. 2013).

As a part of a healthier life, antioxidant intake has increased in the society. Diet has been supplemented with polyphenol family members, as flavonoids found in plants (Myburgh 2014, Iwashina 2015). Its frequent consumption is associated with decreased risk for chronic diseases, as diabetes and cardiovascular diseases (Yamada et al. 2011, Yang et al. 2015, Ponzo et al. 2015), also affecting energetic metabolism and enhancing mitochondrial biogenesis (Kim et al. 2015, Bernatoniene et al. 2014).

Naringenin is a flavanol commonly found in citrus fruits such as tangerine, grapefruits, and lemon. It has been shown antioxidant activity, protection against oxidative stress and cognitive damage and also prevention of hyperglycemia in diabetes model (Khan et al. 2012, Wang et al. 2012, Myburgh 2014, Annadurai et al. 2012). In addition, flavonoid supplementation during pregnancy has been associated with a reduced risk of preterm birth, reduced oxidative damage, and enhanced antioxidant activity in the offspring (Vanhees

et al. 2013, Toumi et al. 2013, Wall et al. 2013).

Recently, the association of antioxidants and exercise has been challenged. As has been reported by several studies, the use of antioxidants prevents the establishment of metabolic adaptations promoted by exercise (Gomez-Cabrera, Domenech and Vina 2008b, Casuso et al. 2013), and the flavonoids supplementation is capable to preventing the mitochondrial biogenesis caused in the brain in response to exercise (Casuso et al. 2014).

In view of the lack of studies showing the combination of exercise and antioxidant intake during pregnancy, the objective of this study was to evaluate whether these two antioxidant strategies, allied or not, might interfere in offspring's electron transport chain (ETC) activity, evaluated in cerebellum, parietal cortex, and hippocampus of rats.

Experimental Procedures

Animals and reagents

Forty-eight adult female (90 days of age), and 24 adult male Wistar rats (60 days of age), with an average weight of 200 and 250 g respectively, were obtained from the Central Animal House of Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained in a 12/12-h light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room. The animals had free access to water and a 20% (w/w) protein commercial chow.

The experiments were approved by the local Ethics Commission (Comissão de Ética no Uso de Animais - Universidade Federal do Rio Grande do Sul, CEUA/UFRGS) under the number 26542, and followed national animal

rights regulations (Law 11.794/2008), the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised 1996) and Directive 2010/63/EU. 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.

Experimental design

Female rats were randomly divided into four groups (n= 12 each): 1) sedentary rats receiving vehicle (1 mL/Kg p.o.); 2) sedentary rats receiving naringenin (50 mg/Kg p.o.); 3) swimming exercised rats receiving vehicle (1 mL/Kg p.o.); 4) swimming exercised rats receiving naringenin (50 mg/Kg p.o.).

The experimental design is presented in Figure 1. The administration of naringenin and/or vehicle was started only after mating, while the maternal exercise began one week previous to mating, in order to adapt the animals to the aquatic environment. During the exercise protocol four animals were kept in each cage, except for mating (one male per two females). Pregnancy was diagnosed by the presence of a vaginal plug. From the 20th day after the onset of pregnancy, we isolated the pregnant dams (one per cage), and the rats were observed twice a day (at 9 a.m. and 6 p.m.), to verify the litter's birth. The day corresponding to the offspring's birth is defined as postnatal day 0 (PND0).

The offspring was left with the mother until PND7, when both, mother and offspring, were euthanized by decapitation without anesthesia. Cerebella, parietal cortices, and hippocampi were dissected, and stored at -80°C until the completion of the biochemical assays. One pup from each offspring was used for each assay, in order to eliminate the litter effect.

Naringenin supplementation

Naringenin (50 mg/Kg) was suspended in sunflower oil (1 mL/kg), which was used as vehicle. The oral treatments, administered by gavage, were given just before the swimming exercise, according the weight of each animal (measured daily), and initiated with the onset of the second week of swimming protocol. The scheme of naringenin administration is due its increasing availability in plasma immediately after it is ingested (Wang et al. 2014, Mata-Bilbao Mde et al. 2007), and the dose was defined according to its neuroprotective action reported in literature (Raza et al. 2013, Sabarinathan, Mahalakshmi and Vanisree 2011).

Swimming exercise protocol

The maternal exercise protocol was adapted from (Lee et al. 2006), as described in (Marcelino et al. 2013). The rats were divided into control and exercised groups. In the exercised group, rats were submitted to swimming in a pool filled with 32±1ºC water on 5 day/week for 4 weeks. Each swimming session lasted for 30 minutes, and always took place between 9 and 12 a.m. Each rat was isolated for the swim, which was conducted using an apparatus designed specifically for rat swimming. Within this apparatus, each room measures 30x30x90 cm (width x length x depth), preventing the animals from touching the bottom of the tank. The animals were left free to swim, without any extra weight, and were gently stimulated to swimming. This protocol has moderate intensity. Control rats were immersed in water, carefully dried, and returned to the housing boxes.

Biochemical assays

Sample preparation:

Cerebellum, parietal cortex, and hippocampus were dissected, weighed, and homogenized (1:20, w/v) in SET buffer, pH 7.4. The homogenates were centrifuged at 800xg for 10 min and the supernatants were

kept at -80 ºC until being used for respiratory chain complexes activities determination.

Respiratory Chain Complexes Activities:

Mitochondrial respiratory chain enzyme activities (complexes II, II–III, and IV) were measured in homogenates with a protein concentration varying from 1.5 to 5.0 mg protein.mL^{-1}.

The activities of NADH-cytochrome c oxidoreductase (complex I–III) was assayed according to the method described by Schapira and colleagues (1990), succinate:DCIP-oxidoreductase (complex II), succinate: cytochrome c oxidoreductase (complex II–III), and SDH (succinate: phenazine oxireductase) activity were determined according to the method of Fischer et al. (1985), and the activity of cytochrome c oxidase (complex IV) according to Rustin et al. (1994). The methods used to measure these activities were slightly modified, as described previously (da Silva et al. 2002). The activities of the respiratory chain complexes were calculated as nmol.min^{-1}.mg $protein^{-1}$.

2.5.3 Protein determination: Protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by two-way ANOVA, using GraphPad Prism 6.0 software. All analyzes were followed by a Tukey post-test for multiple comparisons for parametric data. Data were presented as mean + SEM, and were considered statistically significant when $p<0.05$.

Results

Swimming exercise and naringenin administration during pregnancy increases respiratory chain complexes activity in offspring's cerebellum

Figure 2 shows the activities of (A) SDH, (B) complex II, (C) complex II-III, and (D) complex IV measured in pups' cerebellum of PND7, after the maternal exercise and/or naringenin administration during pregnancy. We showed that SDH activity was increased with naringenin administration during pregnancy [F(1,19)=5.591;p=0.0289] when compared to sedentary group, not being affected by the other treatments [F(1,19)=3.194;p=0.0899].

In agreement, complex II activity was enhanced with naringenin administration during pregnancy [F(1,18)=4.682;p=0.0442], not being affected by the other treatments [F(1,18)=3.363;p=0.0833]. In addition, activity of complex IV was also increased by maternal naringenin administration [F(1,21)=5.864;p=0.0246], as well as by maternal exercise [F(1,21)=4.705;p=0.0417], when compared to sedentary group.

Interestingly, when maternal exercise was allied to naringenin supplementation no significant difference was observed in cytochrome *c* oxidase activity, evidenced by two way ANOVA interaction [F(1,21)=41.62;p<0.0001], suggesting that one treatment abolished the effect of other. Finally, the electron pathways between complexes I-III [F(1,20)=1.510;p=0.2334] (data no show) and II-III were not altered [F(1,24)=0.3476; p=0.5610].

Naringenin supplementation and maternal aerobic exercise during pregnancy increases complex II activity in litter's parietal cortex

Figure 3 shows the activities of ETC measured in pup's parietal cortex of

PND7, after the maternal exercise and/or naringenin administration during pregnancy. Complex II activity was increased with maternal naringenin administration ($p<0.05$, Tukey's post hoc test) and maternal exercise during pregnancy ($p<0.05$, Tukey's post hoc test), when compared with sedentary group. Two way ANOVA showed a significant interaction between factor (naringenin x maternal exercise) [$F(1,18)=7.560;p=0.0132$], suggesting that one antioxidant strategy nullify the effect of the other.

The electron pathway between complexes II-III [$F(1,24)=0.01128;p=0.9163$] and SDH activity [$F(1,22)=0.4224;p=0.5225$] were not affected in pup's parietal cortex after the maternal practice of swimming exercise and/or naringenin administration during pregnancy.

Complex IV activity was not significantly affected by the treatments, as showed by two way ANOVA [$F(1,20)=4.084;p=0.0569$], except for the statistical significance found through Tukey's posttest when naringenin group was compared to maternal exercise allied to naringenin supplementation, evidencing a reduction of cytochrome *c* oxidase activity in the last [$F(1,20)=5.217;p=0.0334$].

Naringenin supplementation during pregnancy improves the activities of SDH and complex II in the hippocampus of offspring

Figure 4 shows the activities of ETC measured in pup's hippocampus of PND7, after the maternal exercise and/or naringenin administration during pregnancy. The activities of SDH [$F(1,22)=4.673;p=0.0418$] and complex II were increased with maternal naringenin administration [$F(1,19)=5.194;p=0.0344$], when compared to sedentary group. Two way ANOVA also showed an interaction between factors (naringenin x maternal exercise) for SDH

[F(1,22)=10.20;p=0.0042] and complex II activity [F(1,19)=5.228;p=0.0339]. On the other hand, the electron pathway between complexes II-III [F(1,24)=0.1448;p=0.7069] and complex IV activity [F(1,20)=1.332;p=0.2620] was not altered in pup's hippocampus.

Discussion

Since most women tend to gain excessive weight during pregnancy, that is maintained after birth (Pole and Dodds 1999, Herring et al. 2012), this is an important period to encourage the change of old habits and beginning a healthier life.

Usually, diet is replenished with antioxidants and physical exercise practice is encouraged; which will bring benefits to the mother and the fetus that can continue in the postpartum period (Schlussel et al. 2008, Prather et al. 2012, Clapp 1996, Hochner et al. 2012). Physicians recommendations concerning improvements on lifestyle during pregnancy are recent and have been accompanied by several changes lately (ACOG 2015); demanding studies to clarify the effect of pregnancy environment on metabolic programming of the fetus.

Since the coupling of exercise practice and antioxidant supplementation proven not to be as beneficial as thought (Gomez-Cabrera et al. 2008b, Casuso et al. 2013), we have been studying the effect of these antioxidant strategies alone and in conjunction. Even though this two factors cause positive effects when isolated, we sought to evaluate the effects of maternal swimming exercise and naringenin supplementation, isolated or combined, in offspring's brain ETC activity, considering its central role on both processes, ATP and reactive species synthesis (Halliwell and Gutteridge 2007, Nelson and Cox 2014, Jones

2006, Mailloux 2015).

Antioxidant supplementation has prevented the force production during physical exercise, thereby reducing the performance, by interfering in signaling pathways responsible for metabolic adaptation (Coombes et al. 2001, Khawli and Reid 1994, Reid and Moody 1994, Gomez-Cabrera et al. 2015).

In this sense, our hypothesis was that supplementation with naringenin could block the neurometabolic adaptive effects induced by maternal physical exercise in the offspring. We found that ETC was enhanced by maternal naringenin supplementation, as by maternal swimming during pregnancy. Confirming our hypothesis, both healthy strategies allied did not present any significant effect on respiratory complexes.

The ETC activity in response to polyphenols intake is not well studied, especially in the brain. The direct effect of some polyphenols in ETC activity has been evaluated and no significant effects were found. Phloretin, when used in embryonic cardiomyocytes culture at two different concentrations (2.5 and 5.0 mM), did not cause alterations in the activities of complex I, II, III, and IV (Vineetha, Soumya and Raghu 2015).

Epicatechin, administered during 21 days at 20 mg/kg, did not alter SDH and cytochrome *c* oxidase activities, nor ATP levels in rat heart (Stanely Mainzen Prince 2013). The same effect was observed for fisetin, in SDH activity in lung, when administered at 25 mg/kg twice a week during 16 weeks (Ravichandran et al. 2014). Morin, administered at 40 mg/kg for 30 days, also did not alter SDH and cytochrome *c* oxidase activities in mitochondrial heart (Al Numair et al. 2012).

We also studied the effect of maternal exercise on ETC activity in

offspring's brain, and showed increased complex IV in cerebellum and complex II in parietal cortex. Concerning the effect of maternal exercise on offspring, Park et al. (2013) did not find alterations in hippocampus' offspring subjected to 30 minutes/day of maternal exercise, corroborating with our result, but showed increased complex I, cytochrome *c* oxidase, and ATPase activities with 40 minutes of treadmill exercise during pregnancy, and decreased activities of complex I and ATPase with 20 min of maternal exercise showing that the duration of exercise is an important factor in the resulting effect (Park et al. 2013).

Literature presents several studies reporting the direct effect of exercise increasing the activities of complex II and SDH in skeletal muscle of rats, with endurance or strength training practice (Al-Nassan et al. 2012, Kruger et al. 2013, Silva et al. 2013). Moderate exercise on a treadmill during five minutes a day/50 weeks increased cytochrome *c* oxidase activity in rat's brain (Navarro et al. 2004), while ten weeks of voluntary exercise on a running wheel increased cytochrome *c* oxidase activity in rats' spinocerebellum (Garifoli et al. 2003).

In addition, Sampedro-Piquero et al. (2013) showed increased cytochrome *c* oxidase activity at different brain regions after two months of forced exercise program on Rotarod.

After demonstrating the isolated effect of antioxidant supplementation and exercise during pregnancy on mitochondrial respiratory chain, we reach the central question of our study: the reciprocal interference of both factors. The same profile was recently demonstrated in animal and human models. Quercetin inhibits the adaptations caused by exercise in rat skeletal muscle (Casuso et al. 2013) and cerebellum (Casuso et al. 2015), while vitamins C and

E produce the same inhibitory effect on human skeletal muscle (Gomez-Cabrera et al. 2008a, Morrison et al. 2015).

A higher activity of ETC can be stimulated by the same pathways responsible by mitochondrial biogenesis, activated by reactive species. The direct effect of exercise on mitochondrial biogenesis is well demonstrated, through activation of important markers as PGC-1α, Nrf2, and mitochondrial transcription factor A (Tfam) (Muthusamy et al. 2012, Vina et al. 2012, Zhang et al. 2012, Cargnello and Roux 2011, Piantadosi and Suliman 2012). In fact, PGC-1 causes increased expression of ETC components genes (Wu et al. 1999, Scarpulla 2012).

We previous demonstrated that maternal exercise practice using the same swimming exercise protocol can induce mitochondrial biogenesis in offspring's cerebellum and parietal cortex (Marcelino et al. 2013).

Similarly, Park et al. (2013) observed enhanced PGC-1α and Tfam levels in the hippocampus of offspring subjected to maternal exercise for 40 minutes per day during gestation. In agreement, flavonoids also induces mitochondrial biogenesis, *in vitro* and *in vivo*, on several tissues as skeletal muscle, liver, and brain (Rayamajhi et al. 2013, Hiramitsu et al. 2014, Su et al. 2014, Tsutsumi et al. 2014), through Nrf2 activation (Wang et al. 2015, Ji et al. 2015), and PGC-1α/Tfam pathway (Hawley and Morton 2014, Scarpulla 2008), increasing ETC gene expression.

Nonetheless, we could not discard other mechanisms responsible by ETC enhancing. ETC and oxidative phosphorylation uncoupling gives an alternative pathway to protons access mitochondrial matrix, crossing the inner

mitochondrial membrane by uncoupling proteins (UCPs) (Speakman et al. 2004); reducing ATP production (Berentzen et al. 2005, Andrews et al. 2006). UCPs, which facilitates the proton gradient dispersion (Krauss, Zhang and Lowell 2002, Dalgaard and Pedersen 2001), including UCP2 that is found either in the brain, in elevated concentration specially at cerebellum (Richard et al. 1998); are upregulated by physical exercise (Bo et al. 2008, Dietrich, Andrews and Horvath 2008).

In agreement, flavonoids have also shown uncoupling effects. Dorta et al. (2005) exposed isolated mitochondria to various flavonoids, and shown increased Ca^{2+} release and decreased mitochondrial ATP levels. Ortega and Garcia (2009) also found an uncoupler effect for quercetin, while similar effect of naringenin on mitochondria was demonstrated by van Dijk, Driessen and Recourt (2000).

Conclusion

Briefly, we found that maternal exercise practice cause increased ETC activity in offspring's cerebellum and parietal cortex, while maternal naringenin supplementation increased ETC activity in cerebellum, parietal cortex, and hippocampus of pups. Moreover, when combined naringenin supplementation annuls the effect of exercise practice during pregnancy, probably acting by similar biochemical mechanisms. If our results might be extrapolated to clinical condition, considering that polyphenol rich diet and physical exercise might interact metabolically and eliminate the isolated benefits to child; we suggest attentiveness by physicians and pregnant woman avoiding null efforts in healthy.

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Figures

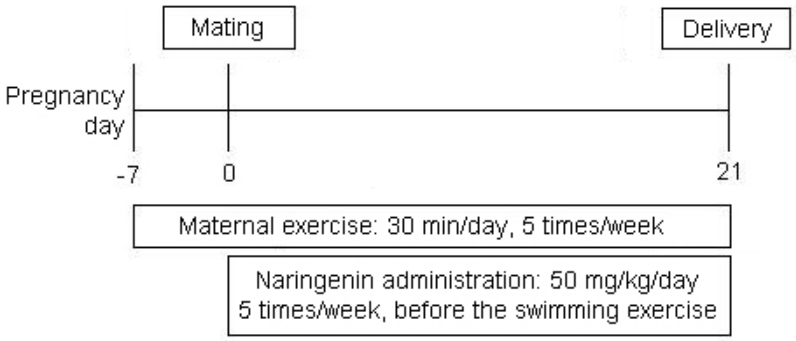

Fig.1 Experimental design.

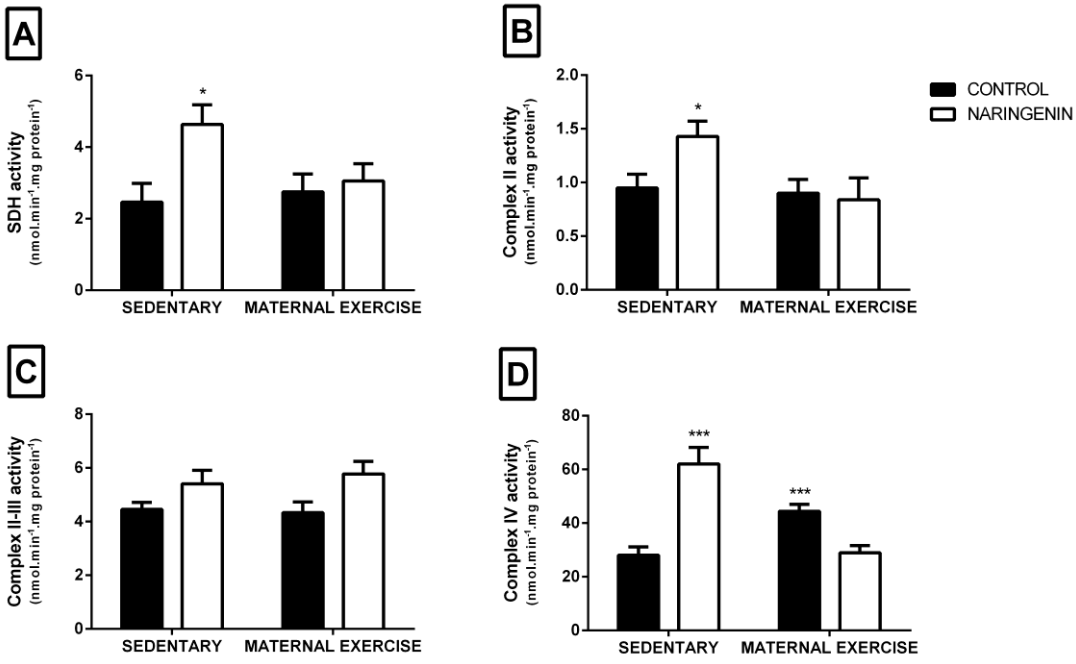

Fig. 2 Effect of naringenin administration and/or swimming exercise during pregnancy on SDH (A), complex II (B), complex II-III (C), and complex IV (D) activities in offspring's cerebellum, evaluated 7 days after birth. Results were expressed as mean+SEM for *n*=5–7 performed in triplicate, and were evaluated by two-way ANOVA (factors: naringenin and maternal exercise), followed by Tukey's post hoc test. *p<0.05 and ***p<0.001.

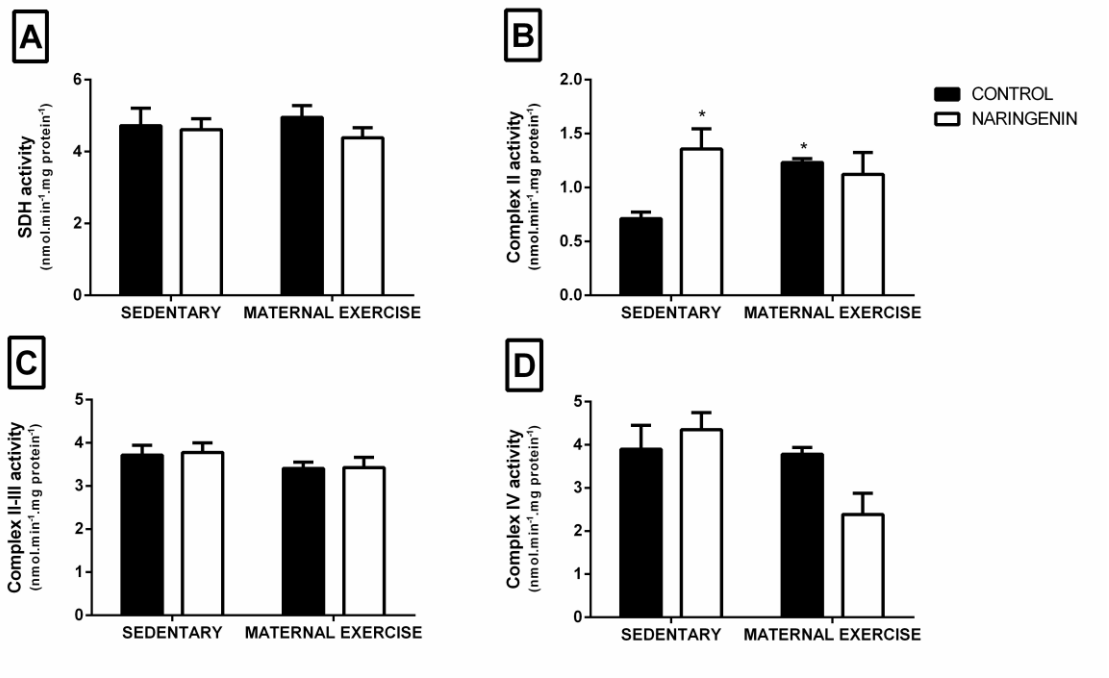

Fig. 3 Effect of naringenin administration and/or swimming exercise during pregnancy on SDH (A), complex II (B), complex II-III (C), and complex IV (D) activities in offspring's parietal cortex, evaluated 7 days after birth. Results are expressed as mean+SEM for *n*=5–7 performed in triplicate, and were evaluated by two-way ANOVA (factors: naringenin and maternal exercise), followed by Tukey's post hoc test. *p<0.05

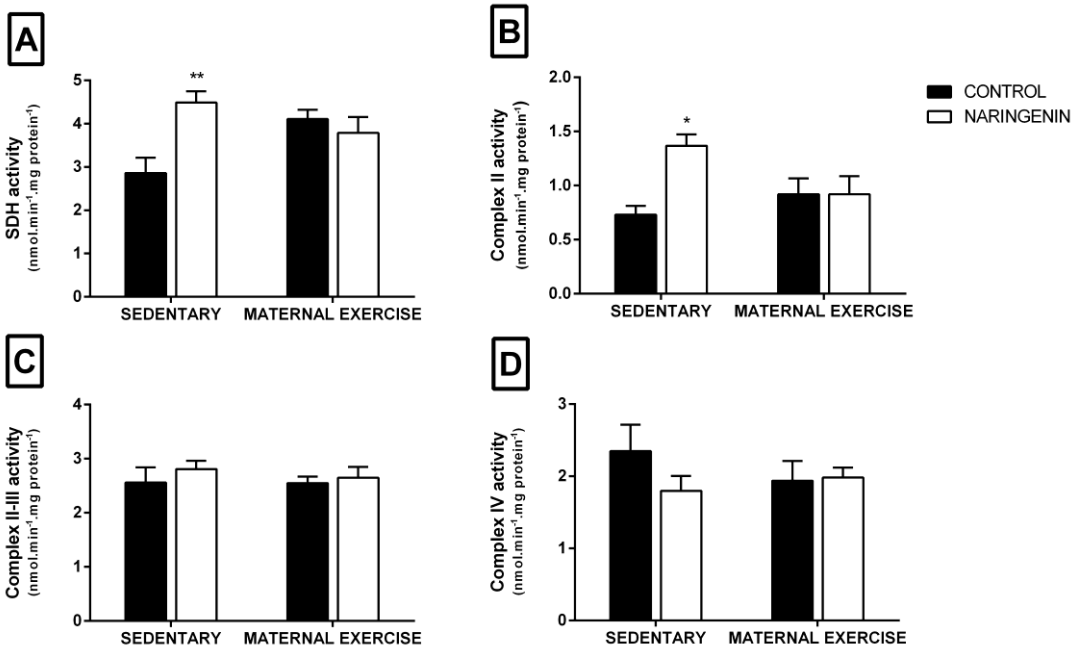

Fig. 4 Effect of naringenin administration and/or swimming exercise during pregnancy on SDH (A), complex II (B), complex II-III (C), and complex IV (D) activities in offspring's hippocampus, evaluated 7 days after birth. Results are expressed as mean+SEM for *n*=5–7 performed in triplicate, and were evaluated by two-way ANOVA (factors: naringenin and maternal exercise), followed by Tukey's post hoc test. *p<0.05 and **p<0.01

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Conflict of interest

The authors declare no conflicts of interest or competing interest.

3.3. Capítulo III:

Naringenin inhibits tricarboxylic acid cycle dehydrogenases activities probably by interacting to NAD$^+$ binding site: experimental and molecular modeling evidence

Autores: Pauline Maciel August; Mateus Grings; Marcelo Sartori Grunwald; Vinícius Stone; Patricia Idalina de Lemos Rodrigues; Daniela Pereira Stocher; José Cláudio Fonseca Moreira; Guilhian Leipnitz and Cristiane Matté

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Naringenin inhibits tricarboxylic acid cycle dehydrogenases activities probably by interacting to NAD^+ binding site: experimental and molecular modeling evidence

Pauline Maciel August[†]; Mateus Grings[†]; Marcelo Sartori Grunwald[†]; Vinícius Stone[†]; Patricia Idalina de Lemos Rodrigues[‡]; Daniela Pereira Stocher[‡]; José Cláudio Fonseca Moreira[‡,†]; Guilhian Leipnitz[‡,†] and Cristiane Matté[‡,†*]

[‡] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

[†] Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

Corresponding author: Cristiane Matté, PhD, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo (laboratório 23), CEP 90035-003, Porto Alegre, RS, Brazil, Phone: +55 51 3308 5548, Fax: +55 51 3308 5535, e-mail: matte@ufrgs.br.

ABSTRACT

Physical exercise practice or antioxidant supplementation during pregnancy has shown bring adaptations to offspring, when isolated. The purpose was to investigate the maternal exercise effect associated with naringenin supplementation on the tricarboxylic acid (TCA) cycle activity in offspring's cerebellum. Adult female Wistar rats were divided into four groups: 1) sedentary; 2) sedentary receiving naringenin (50 mg/Kg p.o.); 3) swimming exercise; 4) swimming exercise receiving naringenin (50 mg/Kg p.o.). The offspring was euthanized at 7^{th} day of life, and the cerebellum was dissected to analyze citrate synthase (CS), isocitrate dehydrogenase (IDH), α-ketogluratate dehydrogenase (α-KGDH) and malate dehydrogenase (MDH) activities. Molecular docking used SwissDock web server and FORECASTER Suite, in order to verify naringenin dehydrogenases' interaction. Naringenin supplementation significantly inhibited IDH, α-KGDH, and MDH in offspring's cerebellum, while maternal exercise was innefective. A similar reduction was observed in purified α-KGDH and MDH submitted to incubation with naringenin. Docking simulations demonstrated that naringenin possibly interacts with dehydrogenases in the NAD^+ binding site. Concluding, naringenin appears to inhibit NAD^+ binding enzymes, and its supplementation must be evaluated with caution, at least during pregnancy.

Keywords: flavonoid, nutrition, pregnancy, metabolic programming, offspring

Pregnancy is the main period for fetus metabolic programming, being affected by pregnant women's environment, reflecting in the child's health throughout life [1]. Early exposure to unsettled milieu, such as maternal obesity, infection or stress, leads to higher triglyceride levels and impaired glucose homeostasis in the offspring, resulting in early obesity and cardiac hypertrophy [2]. In addition, stressful maternal environment increases the risk to neurodevelopmental disorders as schizophrenia, depression, and autism, through alterations into immune system and hypothalamic pituitary-adrenal axis development [3].

Conversely, physical exercise during pregnancy appears to possess a positive effect on progeny. Clinical data show that active women's children set forth higher general intelligence score at five years old, when compared to sedentary ones [4]. Animal models have been used to demonstrate the maternal exercise benefits, such as better offspring's neurodevelopment, cognitive improvement allied to neurogenesis, and enhanced neurotrophic factors levels [5]. In agreement, we already demonstrated that swimming exercise practice during pregnancy is able to positively modulate the antioxidant system in the offspring's brain, associated to mitochondrial biogenesis in Wistar rats [6].

Physical exercise training elicits energetic metabolism changes in response to higher oxygen consumption and energy demand, including in the tricarboxylic acid (TCA) cycle [7]. Exercise practice affects the mitochondrial responses, enhancing complex II and succinate dehydrogenase activities in rat muscle [8], cytochrome c oxidase activity in rat spinocerebellum [9], TCA cycle activity in human skeletal muscle [7c, 10], as well as mitochondrial biogenesis in human and rat skeletal muscle [11]. Despite the exercise effect on mitochondrial

metabolism is well established in muscle and brain of rodents, little is known about the maternal exercise consequences on the progeny.

Flavonoids, in general, also affect mitochondrial function, inducing biogenesis [12] and modifying oxidative phosphorylation [13]. When supplemented during pregnancy, flavonoids prevented stress-induced disorders in pups [14] and reduced DNA damage in adult offspring [15]. Naringenin, the aglycone form of naringin, is a flavanone commonly found in citrus fruits such as tangerine, grapefruits, and lemon. It has shown antioxidant capacity and protection against oxidative stress and cognitive impairment [16]. The use of polyphenols during pregnancy is controversy and has been the subject of an extensive debate in the literature, supporting the relevance of animal models studies in this critical period of development [17].

Considering that literature brings evidence that both, exercise and flavonoid supplementation during pregnancy, elicited relevant offspring's metabolic programming, and recent studies have shown that antioxidant administration can prevent the metabolic adaptations induced by aerobic exercise [18]; this study aimed to analyze the combined effect of maternal swimming exercise and naringenin supplementation by pregnant rats in the TCA cycle activity in offspring's cerebellum. Once verified the significant inhibitory effect of naringenin on dehydrogenases activity, we investigated the mechanism involved.

RESULTS AND DISCUSSION

Nowadays, the society claim for healthier habits, including a massive antioxidant supplementation and at least 30 minutes a day of moderate physical exercise. However, the consumption of high levels of antioxidants, in fact, failed

to bring the expected benefits, beyond stimulating carcinogen cells growth and even increasing the risk of cancer [19]. Many studies have showed that exogenous antioxidants can block the metabolic adaptations induced by physical exercise, preventing the initiation of signaling cascades activated by exercise-generated reactive species; such as the transcription factor peroxisome-proliferator-activated receptor gamma coactivator 1α (PGC-1α), responsible for mitochondrial biogenesis and antioxidant defenses expression [20]. Herein, our first aim was to assess whether the isolated or combined approach of maternal swimming exercise and naringenin supplementation during pregnancy could affect the TCA cycle activity.

Daily maternal supplementation with the polyphenol naringenin significantly inhibited the TCA cycle's dehydrogenases, without affecting CS in offspring's cerebellum. On the other hand, maternal exercise did not alter any of the enzymes evaluated. Figure 1 shows the activities of CS, IDH, α-KGDH, and MDH measured in PND7 pups' cerebellum. Data were evaluated by two way ANOVA, showing a significant interaction between factors for IDH [$F(1,21)=6.304;p=0.0203$], α-KGDH [$F(1,24)=5.062;p=0.0339$], and MDH activities [$F(1,24)=4.423;p=0.0461$] evaluated in pups' cerebellum (Fig. 1B, C, and D; respectively), while CS was not affected [$F(1,23)=0.1100;p=0.7431$] (Fig. 1A). The post-test analysis using Tukey's multiple comparison showed that prenatal naringenin administration significantly reduced the activities of IDH, α-KDH, and MDH; while maternal exercise allied to naringenin supplementation significantly reduced the activity of IDH.

It has been demonstrated that exercise *per se* increases TCA cycle activity in human muscle [7c, 10], as in animal models [21]. The increment in TCA

cycle is probably due to the increased energy demand, and seems to augment according to the higher oxygen consumption [10a]. Albeit physical exercise practice alters muscle TCA cycle activity, we do not know any data showing its effect on the brain, either in adult models or during development.

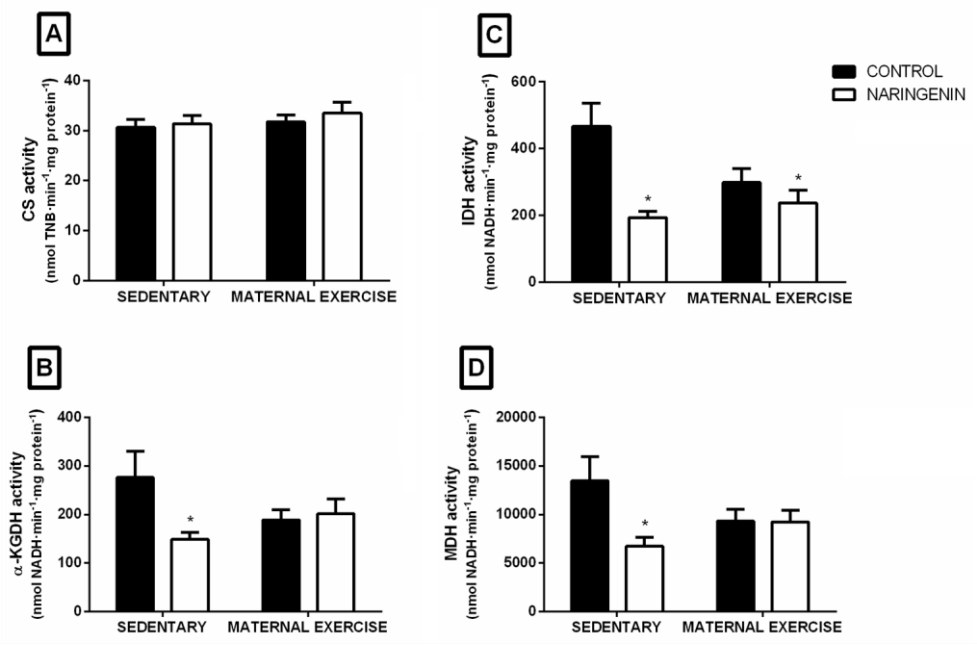

Fig. 1 Effect of naringenin administration and/or swimming exercise during pregnancy on (A) citrate synthase (CS), (B) isocitrate dehydrogenase (IDH), (C) α-ketoglutarate dehydrogenase (KGDH), and (D) malate dehydrogenase (MDH) activities in offspring' cerebellum. Results are expressed as mean+SEM for *n*=8 performed in triplicate. Data were analyzed by two-way ANOVA followed by Tukey's test. *p<0.05

CS is an important TCA cycle marker, synthesizing citrate from acetyl-CoA and oxaloacetate [22], commonly used as a mitochondrial biogenesis marker [23]. In this context, we found that naringenin supplementation during pregnancy did not alter CS activity in the offspring's cerebellum. Resveratrol, another polyphenol, also did not change CS activity in human muscle [24]. In contrast,

quercetin was able to increase CS activity in adult rats' cerebellum after six weeks of 25 mg/kg p.o. supplementation [25]. We also found that naringenin intake during pregnancy causes an important decrease in IDH activity in rat offspring's cerebellum. IDH catalyzes the mitochondrial oxidative decarboxylation of isocitrate to α-ketoglutarate, forming $NADH.H^+$ and CO_2 [22a, 26]. On the other hand, polyphenols as naringin (10, 20, and 40 mg/kg), morin (40 mg/kg), and epicatechin (20 mg/kg), at different periods of administration (21 to 56 days), do not change IDH activity in rat heart [27]. In addition, naringenin supplementation also inhibits α-KGDH, which catalyzes the mitochondrial oxidative decarboxylation of α-ketoglutarate to succinyl-CoA, forming $NADH.H^+$ and CO_2 [7a, 22a]. Conversely, naringin and other flavonoids, at diverse doses, do not change α-KGDH activity in rat heart [27]. MDH, responsible by mitochondrial dehydrogenation of malate to oxaloacetate, forming $NADH.H^+$ [22a, 28], was also inhibited by naringenin. As the previously cited TCA cycle enzymes, diverse flavonoids were not capable of altering the MDH activity in rat heart [27]. However, banana flavonoids decreased MDH activity in male rat liver, administered at 0,1 mg/kg/day during 45 days [29].

Considering that naringenin supplementation during pregnancy reduced specifically the dehydrogenases from TCA cycle, and it was recently found that polyphenols might compete with nicotinamide derivatives by the enzyme site binding [30], we hypothesized that naringenin can surpass the placental barrier [31] and bind specifically the NAD^+ binding site, inhibiting the TCA cycle dehydrogenases. To test this hypothesis, we assessed the effect of naringenin, in a blood compatible concentration [32], on commercially available purified dehydrogenases, which use NAD^+ as coenzyme (α-KGDH and MDH). Figure 2

shows the activities of purified α-KGDH and MDH measured in the presence of naringenin (60 ng/mL) or its vehicle, ethanol. *In vitro* incubation with naringenin inhibited both dehydrogenases, α-KGDH by 60% [F(2,15)=48.69;p<0.0001] and MDH by 70% [F(2,15)=43.53;p<0.0001], which is comparable to the results obtained in the cerebellum of pups submitted to maternal naringenin administration. Although we have showed a clear inhibitory effect of naringenin on purified dehydrogenases, we could not affirm that this mechanism explains the *in vivo* effect verified in the littermates.

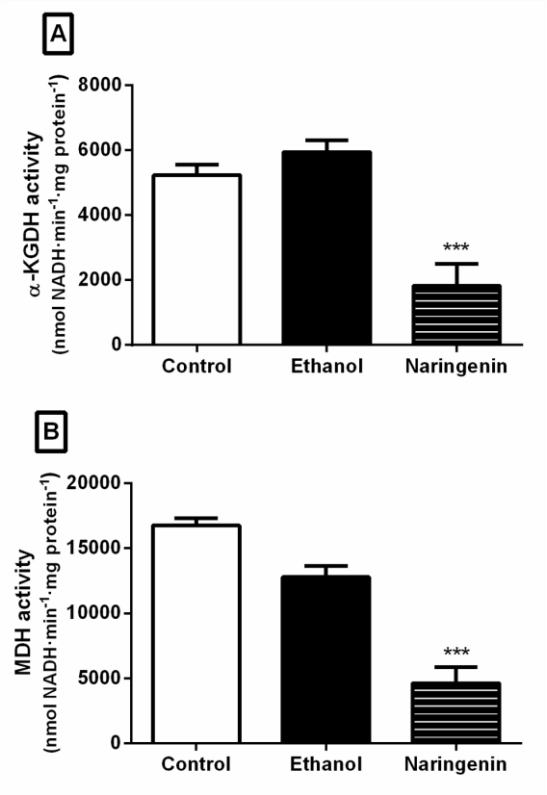

Fig. 2 *In vitro* effect of naringenin on (A) α-ketoglutarate dehydrogenase (KGDH), and (B) malate dehydrogenase (MDH) activities. Results are expressed as mean+SEM for *n*=1 performed in triplicate. Data were analyzed by one-way ANOVA followed by Tukey's test. ***p<0.001

In view of this results, we evaluated if naringenin might interact with dehydrogenases structure. In order to verify this assumption, we performed *in silico* studies, by *FullFitness* analyses. Results were showed on Table 1, displaying the minimum and maximum scoring values (the most negative to most positive values) of the *FullFitness* analyses; where the more negative the value, the more probable to exist a preferable binding site for the ligand on the receptor´s surface. Naringenin is less likely to bind MDH (2CMD), and more likely to bind to α-KGDH (2JGD) and IDH (1V94), all presenting highly negative values.

Table 1. Protein structures verified at Protein Data Base and Scoring values of *FullFitness* analyses

PDB ID	Structure	Minimum scoring value of FullFitness	Maximum scoring value of FullFitness
1ITW	Crystal structure of the monomeric isocitrate dehydrogenase in complex withisocitrate and Mn [33]	-2853	-2837
1V94	Crystal structure of isocitrate dehydrogenase[34]	-3458	-3440
3MBC	Crystal structure of monomeric isocitrate dehydrogenase in complex with $NADP^+$ [35]	-3247	-2687
2JGD	Crystal structure of the E1 component oxoglutarate dehydrogenase[36]	-3935	-3921
1NEK	Crystal structure of succinate dehydrogenase in complex with FAD_2 and oxaloacetate [37]	-2659	-2640
1BMD	Crystal structure of malate dehydrogenase in complex with NAD^+ [38]	-2853	-2841
2CMD	Crystal structure of malate dehydrogenase in complex with citrate [39]	-1262	-1246

Confirmed the possibility of interaction, we performed a second docking calculation utilizing a more refined strategy. The structures under PDB IDs 1BMD and 2CMD were employed on docking calculations on the FORECASTER Suite. 1BMD represents the structure of the enzyme MDH in

complex with NAD^+, while 2CMD represents the structure of MDH in complex with citrate. The software identified the co-crystalized ligands in both enzymes structures and their respective binding site was extracted and later utilized in a targeted docking with the naringenin molecule. This procedure allowed us to compare the score of the dockings for naringenin in two different binding sites in the same enzyme. The score for naringenin docking in the binding site for citrate was 28.309 and the score for the docking in the NAD^+ binding site was -15.593, suggesting a more preferable interaction for naringenin with MDH in the NAD^+ binding site, preventing substrate binding and catalysis, decreasing the dehydrogenases activities. To complement our hypothesis, Fig. 3 shows a putative model for the interaction of naringenin with MDH in the NAD^+ binding site.

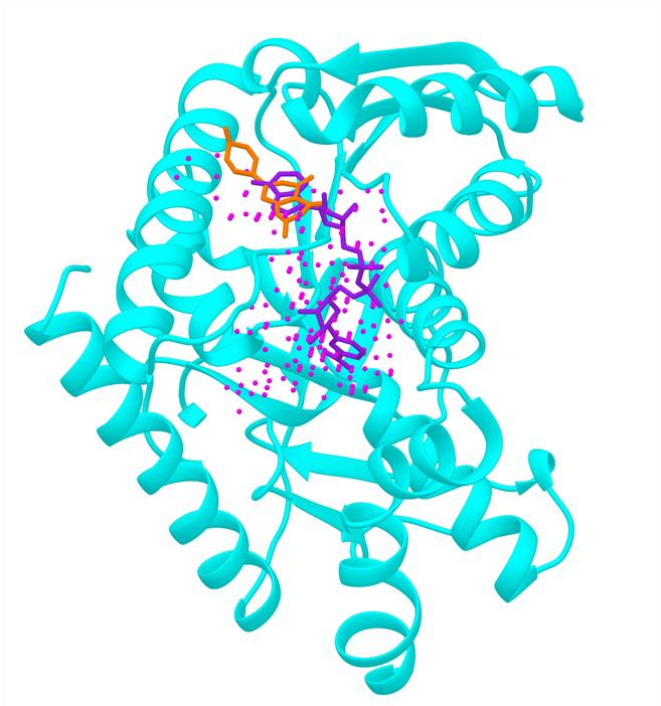

Fig. 3 Docking result for naringenin (orange) interacting with the active site of NAD^+ (purple) in the malate dehydrogenase (MDH) structure (PDB:

2JGD)

In conclusion, maternal diet affects brain metabolic programming of the fetus. We have demonstrated that naringenin supplementation during pregnancy inhibits TCA cycle dehydrogenases in offspring's cerebellum. *In vitro* experiments confirm that naringenin reduces TCA cycle dehydrogenases activities, probably acting directly on NAD^+ site as demonstrated by molecular docking approach. Our findings suggest that polyphenols supplementation during pregnancy should be carefully evaluated, considering the interference in essential enzymes for energy metabolism, jeopardizing brain development.

EXPERIMENTAL SECTION

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

Animals. Forty-eight adult female (90 days of age) and 24 adult male Wistar rats (60 days of age), with an average weight of 200 and 250 g respectively, were obtained from the Central Animal House of Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained in a 12/12-h light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room. The animals had free access to water and a 20% (w/w) protein commercial chow.

The experiments were approved by the local Ethics Commission (Comissão de Ética no Uso de Animais - Universidade Federal do Rio Grande do Sul, CEUA/UFRGS) under the number 26542, and followed national animal rights regulations (Law 11.794/2008), the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised

1996) and Directive 2010/63/EU. We further attest that all efforts were made to minimize the number of animals used and their suffering.

In vivo experimental design. Female rats were randomly divided into four groups (n= 12 each): 1) sedentary rats receiving vehicle (1 mL/Kg p.o.); 2) sedentary rats receiving naringenin (50 mg/Kg p.o.); 3) swimming exercised rats receiving vehicle (1 mL/Kg p.o.); 4) swimming exercised rats receiving naringenin (50 mg/Kg p.o.).

The experimental design is presented in Figure 4. The administration of naringenin and/or vehicle was started only after mating, while the maternal exercise began one week previous to mating, in order to adapt the animals to the aquatic environment. During the exercise protocol four animals were kept in each cage, except for mating (one male per two females). Pregnancy was diagnosed by the presence of a vaginal plug. From the 20th day after the onset of pregnancy, we isolated the pregnant dams (one per cage), and the rats were observed twice a day (at 9 a.m. and 6 p.m.), to verify the litter's birth. The day corresponding to the offspring's birth is defined as postnatal day 0 (PND0).

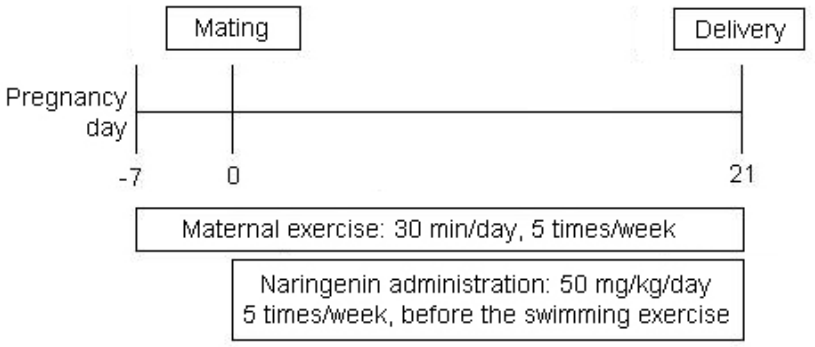

Fig. 4 Experimental design.

The offspring was left with the mother until PND7, when both mother and offspring were euthanized by decapitation without anesthesia. Cerebella were

dissected, and stored at -80°C until the completion of the biochemical assays. One pup from each offspring was used for each assay, in order to eliminate the litter effect.

Naringenin supplementation. Naringenin (50 mg/Kg) was suspended in sunflower oil (1 mL/kg), which was used as vehicle. The oral treatments, administered by gavage, were given just before the swimming exercise, and initiated with the onset of the second week of swimming protocol. The scheme of naringenin administration is due its increasing availability in plasma immediately after it is ingested [32a, 40], and the dose was defined according to its neuroprotective action [41].

Swimming exercise protocol. The maternal exercise protocol was adapted from [5d], as described in [6]. The rats were divided into control and exercised groups. In the exercised group, rats were submitted to swimming in a pool filled with 32±1ºC water on 5 day/week for 4 weeks. Each swimming session lasted 30 minutes, and always took place between 9 and 12 a.m. Each rat was isolated for the swim, which was conducted using an apparatus designed specifically for rat swimming. Within this apparatus, each room measures 30x30x90 cm (width x length x depth), preventing the animals from touching the bottom of the tank. The animals were left free to swim, without any extra weight, and were gently stimulated to swimming. This protocol has moderate intensity. Control rats were immersed in water, carefully dried, and returned to the housing boxes.

Sample preparation. Mitochondrial fraction was isolated from the offspring's cerebellum as described by [42], with slight modifications. Animals were euthanized by decapitation, had their cerebellum rapidly removed and put

into ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1% bovine serum albumin (free of fatty acids) and 10 mM HEPES, pH 7.2. The cerebellum was homogenized 1:10. The homogenate was centrifuged for 3 min at 2000 g. After centrifugation, the supernatant was again centrifuged for 8 min at 12,000 g. The pellet was suspended in isolation buffer containing 4 µL of 10% digitonin and centrifuged for 10 min at 12,000 g. The supernatant was discarded and the final pellet gently washed and suspended in isolation buffer devoid of EGTA, at an approximate protein concentration of 2,5 $mg \cdot mL^{-1}$.

Determination of citrate synthase activity. CS was measured according to [43], by determining DTNB reduction at λ = 412 nm, and calculated as nmol $TNB.min^{-1}.mg\ protein^{-1}$. The reaction mixture contained 5 mM potassium phosphate buffer, pH 7.4, 300 mM sucrose, 1 mM EGTA, 0.1 % BSA, 5 mM MOPS, 0.1 % Triton X-100, 0.1 mM DTNB, 0.1 mM acetyl-CoA and 0.2 mM oxaloacetate.

Determination of isocitrate dehydrogenase activity. IDH was evaluated according to [44], by following NAD^+ reduction fluorimetrically at excitation and emission wave lengths of 340 and 466 nm, respectively. The reaction mixture contained mitochondrial preparations, 33 Mm Tris-HCl buffer, pH 7.4,10 µM rotenone, 1.2 mM $MnCl_2$, 0.67 mM ADP, 0.1 % Triton X-100, 0.6 mM NAD^+ and 5 mM isocitrate. IDH activity was calculated and expressed as nmol $NADH.H^+.min^{-1}.mg\ protein^{-1}$.

Determination of α-ketoglutarate dehydrogenase activity. The activity of α-KGDH complex was evaluated according to [45] and [46] with some modifications. The incubation medium contained mitochondrial preparations, 1 mM $MgCl_2$, 0.2 mM thiamine pyrophosphate, 0.4 mM ADP, 10 µM rotenone, 0.2

mM EGTA, 0.12 mM coenzyme A-SH, 1 mM α-ketoglutarate, 2 mM NAD^+, 0.1% Triton X-100 and 50 mM potassium phosphate, pH 7.4. The reduction of NAD^+ was recorded at wavelengths of excitation and emission of 366 and 450 nm, respectively. α-KGDH activity was calculated and expressed as nmol $NADH.H^+.min^{-1}.mg\ protein^{-1}$.

Determination of malate dehydrogenase activity. MDH activity was measured according to [47]. The incubation medium consisted of mitochondrial preparations, 10 μM rotenone, 0.1% Triton X-100, 0.14 mM $NADH.H^+$, 0.3 mM oxaloacetate and 50mM potassium phosphate, pH 7.4. MDH activity was determined following the reduction of $NADH.H^+$ fluorescence at wavelengths of excitation and emission of 366 and 450 nm, respectively. MDH activity was calculated and expressed as μmol $NADH.H^+.min^{-1}.mg\ protein^{-1}$.

In vitro experimental design. Naringenin (60 ng/mL) was dissolved in ethanol and added in the incubation medium for determination of dehydrogenases (α-KGDH and MDH) activities. α-KGDH complex was evaluated according to [45] and [46]; while MDH activity was measured according to [47], as described above. Naringenin concentration was used due to the level demonstrated of naringenin in blood minutes after ingestion [32].

Protein determination. Protein concentration was measured by the method of Lowry et al. [48] using bovine serum albumin as standard.

Molecular docking, structural analyses and interaction energy calculation. The protein structures corresponding to TCA cycle dehydrogenases enzymes under PDB IDs 1ITW [33], 1V94 [34] and 3MBC [35] were employed in molecular docking calculations, corresponding to IDH; 1BMD [38] and 2CMD [39] corresponding to MDH; 2JGD [36] corresponding to α-KGDH; and,

1NEK [37] corresponding to succinate dehydrogenase, respectively. A detailed description of protein structures particularities was included in Table 1. Naringenin structure was retrieved from ZINC database (ZINC00001785) in .sdf format.

The first round of calculations was performed on the SwissDock web server, a protein-small ligand docking software based on EADockDSS [49]. In this step, a blind-docking approach was employed, this is, several binding modes were generated in the vicinity of all target cavities. Naringenin was free to search the entire enzymes surfaces for its preferential binding sites. Docking calculations were scored based on the *FullFitness* parameter of SwissDock, which considers the sum of the internal energies of ligand, receptor, the interaction energy and the RMSD of the generated clusters.

The second round of docking calculations was performed on the FORECASTER Suite, including the FITTED docking software, from Molecular Forecaster Inc. (Laval, Québec, Canada). It uses a more accurate protein models and is based on a pharmacophore-oriented docking method combined with a genetic algorithm based docking approach. Proteins, ligands and potential bridging water molecules are described as genes and a mixed Lamarckian/Darwinian evolution optimizes the whole complex. In this step, the ligands present in the crystal structures were identified by the software, creating a binding site. Naringenin was then tested to bind in this specific locations and the score of the docking poses was calculated and compared. The lower the score, the more preferable and probable the binding pose. The proposed binding pose image was created on UCSF Chimera [50].

Statistical analysis. Data were analysed by one-way or two-way

ANOVA, using GraphPad Prism 6.0 software. All analysis were followed by Tukey post-test for multiple comparisons for parametric data. Data were presented as mean ± SEM, and were considered statistically significant when $p<0.05$.

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Conflict of interest

The authors declare that they have no conflict of interest.

4. Discussão

A exposição ao ambiente durante a gestação tem grande influência sobre as adaptações metabólicas no feto, que se prepara para a vida pós-natal de acordo com as experiências vividas no período intrauterino (Hales and Barker 1992). Tanto a dieta quanto o exercício praticado pela gestante podem modular o metabolismo da prole, podendo interferir nos benefícios mútuos, o que justifica o objetivo central desse trabalho: avaliar o efeito da suplementação com o polifenol naringenina durante a gestação, aliada ou não à prática de exercício de natação, em parâmetros de homeostase redox e metabolismo energético no encéfalo da prole aos sete dias de vida.

Tanto o exercício materno quanto a suplementação com naringenina, isolados ou aliados, não causaram efeito no tamanho de ninhada e peso da prole no PND1, demonstrando que as alterações que serão mostradas no metabolismo energético cerebral não alteraram o ganho de peso na prole. Em humanos, já foi demonstrado que a prática de exercício materno não causa alterações negativas ao peso ao nascer, apenas reduz o risco para macrossomia (Clapp 1996, Mudd et al. 2012, Siebel et al. 2012). Quanto ao efeito de antioxidantes, a administração de extrato rico em polifenois foi capaz de prevenir o baixo peso causado por uma dieta pobre em proteínas durante a gestação (Costa et al. 2015), enquanto a suplementação com vitamina E ou melatonina não demonstraram efeito nesses parâmetros (Salem and Gomaa 2014, Singh et al. 2013).

Em seguida foi avaliada a homeostase redox encefálica da prole, demonstrando-se que a suplementação materna com naringenina foi capaz de

aumentar o conteúdo de $O_2^{\bullet-}$ mitocondrial no cerebelo, sem afetar as demais estruturas encefálicas avaliadas (*material suplementar*). A naringenina já demonstrou reduzir os níveis de $O_2^{\bullet-}$ em modelo de radiação ultravioleta B em camundongos (Martinez et al. 2015) e quando incubada com fígado de ratos Wistar (Cavia-Saiz et al. 2010). Entretanto, apesar dos polifenois serem considerados sequestradores de espécies reativas, por definição são espécies redox ativas, e podem se tornar pró-oxidantes dependendo da concentração (Sandoval-Acuna, Ferreira and Speisky 2014), causando aumento das espécies reativas e reduzindo a atividade de defesas antioxidantes em linfócitos humanos (Yen et al. 2003).

O exercício físico materno não foi capaz de causar alteração nos níveis de MDA, produto de lipoperoxidação (Lopresti et al. 2014), em nenhuma das estruturas encefálicas avaliadas, concordando com outros estudos que demonstram a ausência de efeito tanto em hipocampo de ratos adultos (Hrncic et al. 2014), quanto em hipocampo da prole submetida ao exercício materno durante a gestação (Park et al. 2013). Em concordância, nosso grupo de pesquisa também demonstrou anteriormente a ausência de dano oxidativo a proteínas no encéfalo da prole, utilizando esse protocolo de natação materna (Marcelino et al. 2013). Por outro lado, a suplementação materna com naringenina causou um aumento no conteúdo de MDA no cerebelo da prole, apesar de polifenois demonstrarem reverter o aumento de MDA causado por dieta hiperlipídica (de Oliveira et al. 2015), e de a naringenina não ter demonstrado alterar este parâmetro em fígado e córtex de ratos suplementados por 8 ou 28 dias, respectivamente (Fouad et al. 2014,

Chtourou, Fetoui and Gdoura 2014). Entretanto, esse dado concorda com o aumento nos níveis de superóxido mitocondrial induzido pela suplementação pré-natal com naringenina.

Embora a adição de antioxidantes já tenha demonstrado prevenir o incremento das espécies reativas induzida pelo exercício (Dalla Corte et al. 2013), nós demonstramos um aumento na oxidação do DCFH no cerebelo da prole submetida aos tratamentos aliados na gestação. A prática de exercício materno aliado com a suplementação com naringenina causou um aumento no conteúdo de espécies reativas no cerebelo, detectadas pela oxidação do DCFH, o qual reage mais especificamente com H_2O_2, $^{\bullet}OH$ e $ONOO^-$ (Halliwell and Whiteman 2004, Jackson 2009), enquanto os tratamentos isolados promoveram uma tendência de aumento. Já está bem demonstrado na literatura o incremento de espécies reativas com a prática de exercício físico (Gomez-Cabrera et al. 2008b), sendo demonstrado um aumento da oxidação do DCFH em músculo esquelético 48 h após uma sessão aguda de exercício aeróbico (Rech et al. 2014). Nós já demonstramos anteriormente que a prática de exercício materno também causa um incremento na oxidação do DCFH no cerebelo da prole (Marcelino et al. 2013). Em adição, a naringenina também já demonstrou efeito no conteúdo de espécies reativas, causando um aumento na oxidação do DCFH quanto incubada em cultura de fibroblastos pulmonares humano com 75 ou 150 µM (Matsuo et al. 2005).

Considerando que as espécies reativas são o sinalizador para a ativação de vias de transcrição responsáveis pelo aumento na atividade do sistema antioxidante, nosso próximo objetivo foi avaliar a atividade de enzimas antioxidantes que atuam sobre EROs. O exercício materno foi capaz de

aumentar a atividade da SOD no cerebelo da prole, bem como a atividade da GPx no cerebelo e no córtex parietal. A suplementação com naringenina isolada causou incremento da atividade da GPx no córtex parietal, e quando os tratamentos foram aliados, o aumento da atividade foi mantida em cerebelo e córtex parietal. Em relação à atividade da SOD, já foi demonstrada diminuição no cerebelo de ratos submetidos a seis semanas de exercício em esteira (Casuso et al. 2015), enquanto um protocolo de 12 semanas de treino não causou alterações na atividade da enzima na mesma estrutura (Marques-Aleixo et al. 2015). Anteriormente já havíamos demonstrado uma tendência de aumento na atividade da SOD em cerebelo da prole submetida ao exercício materno (Marcelino et al. 2013). O exercício regular causa um aumento na atividade da enzima GPx em coração de ratos (Ghiasi et al. 2015), o que também já havia sido demonstrado em nosso grupo, em cerebelo e córtex parietal da prole submetida ao exercício materno (Marcelino et al. 2013).

A ingestão de romã, uma fruta rica em polifenois, demonstrou prevenir a inibição da GPx induzida por modelo experimental de doença de Alzheimer no córtex de camundongos, quando ingerida na dieta durante 15 meses (Subash et al. 2014), enquanto polifenois do quiabo causaram aumento na atividade da enzima em fígado de camundongos após indução de fadiga (Xia et al. 2015). Naringina, a forma glicosilada da naringenina, também já demonstrou aumentar a atividade da enzima quando incubada com células P388 na concentração de 1 mM durante 60 minutos (Kanno et al. 2004).

Considerando que a rede antioxidante também é integrada por moléculas de baixo peso molecular com atividade redox ativa, avaliamos um representante endógeno, a GSH, e um representante obtido da dieta em

humanos e sintetizado por roedores, a vitamina C. Os níveis de vitamina C não foram alterados significativamente por nenhum dos tratamentos; enquanto o conteúdo de GSH foi significativamente afetado. As intervenções na gestação causaram aumento de GSH no cerebelo da prole com os dois tratamentos isolados, mas quando aliados o efeito desaparece. Já no córtex parietal o efeito de aumento no conteúdo de GSH foi demonstrado apenas no grupo de exercício materno, sendo mantido o efeito com a adição da naringenina.

O aumento no conteúdo de GSH pode ocorrer como resposta à ativação do Nrf2, um fator de transcrição sensível a alterações no estado redox, através do aumento da expressão da enzima GSH-sintase; o que explica a manutenção dos níveis do tripeptídeo mesmo com o aumento da atividade da GPx (Wang et al. 2015, Ji et al. 2015). Em humanos, a prática de exercício de alta intensidade parece aumentar a oxidação da GSH no plasma nas primeiras horas após a atividade (Mohr et al. 2015), não demonstrando diferença no conteúdo total de glutationa em indivíduos treinados após um exercício agudo, indiferente da intensidade do treino (Carteri et al. 2015).

Em modelo animal, o treino em esteira durante seis semanas causou aumento do conteúdo de GSH no sangue, e no conteúdo de glutationa total em cerebelo e córtex de ratos adolescentes, 48 h após a última sessão de treinamento (Chalimoniuk et al. 2015). Quanto à naringenina, Galati et al. (1999) já demonstraram um aumento dose-dependente na oxidação *in vitro* da GSH, enquanto o tratamento com resveratrol na dose de 20 mg/kg/dia durante 25 dias não causou alteração no conteúdo de GSH em coração de ratos (Chakraborty, Pujani and Haque 2015), demonstrando uma adaptação diferenciada quando o tratamento foi aplicado durante a gestação e avaliado o

efeito na prole.

Considerando que os tratamentos aplicados durante a gestação, e, em especial, a suplementação com naringenina, aumenta a concentração de espécies reativas, tais como o superóxido mitocondrial; nosso próximo objetivo foi investigar se o exercício materno e a suplementação com naringenina durante a gestação afetam a atividade mitocondrial. Iniciamos com a avaliação dos complexos da CTE, principal fonte celular de espécies reativas formadas pelo vazamento de elétrons dos complexos e redução parcial do O_2 em $O_2^{\bullet-}$.

Quanto à análise do efeito do exercício materno sobre a atividade da CTE, nós demonstramos um aumento da atividade do complexo IV no cerebelo e complexo II no córtex parietal, concordando com diversos trabalhos que demonstram um aumento da atividade da CTE em músculo e encéfalo de ratos com a prática de exercício físico (Al-Nassan et al. 2012, Kruger et al. 2013, Silva et al. 2013, Navarro et al. 2004, Garifoli et al. 2003, Sampedro-Piquero et al. 2013). O efeito parece depender tanto da duração quanto da intensidade do treino. Foi demonstrado em músculo esquelético de camundongos que o treino em esteira durante 4 semanas não causou alteração na atividade dos complexos da CTE, enquanto com 8 semanas de treino, todos os complexos (I, II, III e IV) e SDH encontraram sua atividade aumentada (Silva et al. 2009). Já o treino em esteira feito por ratos durante uma semana, em diferentes níveis de intensidade, demonstrou que o aumento da atividade dos complexos no músculo esquelético ocorria de acordo com o aumento da intensidade, sendo em ordem da passagem de elétrons, primeiro o aumento da atividade do complexo I e por último, a atividade do complexo IV (Pinho et al. 2012). Ainda,

um estudo que avaliou o efeito do exercício materno na prole, não encontrou alteração significativa na atividade da CTE no hipocampo da prole em um protocolo de 30 minutos de exercício materno na esteira, corroborando com os nossos resultados. Entretanto, a prática de 20 min/dia de exercício materno causou diminuição da atividade do complexo I e da ATP sintase, enquanto 40 min/dia causaram o aumento nestes mesmos parâmetros (Park et al. 2013).

Ainda quanto à análise da atividade da CTE na prole, a suplementação materna com naringenina parece ter um efeito mais pronunciado sobre a atividade da cadeia respiratória. Isoladamente, a suplementação pré-natal com naringenina causou um aumento na atividade da SDH e dos complexos II e IV no cerebelo, complexo II no córtex parietal, e SDH e complexo II no hipocampo. Em concordância com nossa hipótese inicial, quando os dois tratamentos foram aliados, o efeito na prole foi anulado.

Quanto ao efeito dos polifenois sobre a atividade da CTE, apesar de vários estudos demonstrarem a ausência de efeito, *in vitro* e *in vivo*, em coração e pulmão de ratos (Vineetha, Soumya and Raghu 2015, Stanely Mainzen Prince 2013, Ravichandran et al. 2014, Al Numair et al. 2012), alguns polifenois já demonstraram inibir a atividade da ATP-sintase (Dadi, Ahmad and Ahmad 2009, Zheng and Ramirez 1999), sendo o resveratrol capaz de ligar-se ao mesmo sítio de outro inibidor da enzima, a aurovertina B, em mitocôndrias isoladas de coração bovino (Gledhill and Walker 2005), enquanto vários tipos de teaflavina reduziram a atividade da CTE e também inibiram a atividade da ATP-sintase (Li, Vik and Tu 2012). O efeito inibitório dos polifenois parece ser concentração-dependente, como demonstrado em preparações mitocondriais de encéfalo de ratos por Zheng and Ramirez (2000), podendo ajudar a

esclarecer o efeito de aumento na atividade da CTE demonstrado na prole submetida à suplementação materna com naringenina.

Tanto o aumento da atividade dos complexos da CTE quanto enzimas antioxidantes demonstrados pode ser estimulado pelas mesmas vias responsáveis pela biogênese mitocondrial, que é induzida pela ativação de marcadores como PGC-1α, Nrf2 e TFAM, que quando ativados estimulam a transcrição de defesas antioxidantes (Olsen et al. 2015, Ji et al. 2015, Lou et al. 2014) e expressão gênica de componentes da CTE (Wu et al. 1999, Scarpulla 2012). Nesse contexto, Park et al. (2013) observaram na prole submetida ao exercício materno durante a gestação, maiores níveis de PGC-1α e TFAM no hipocampo, enquanto nosso grupo demonstrou uma indução da biogênese mitocondrial em cerebelo e córtex parietal da prole (Marcelino et al. 2013).

Os flavonoides também podem induzir a biogênese mitocondrial *in vitro* e *in vivo* em diferentes tecidos (Rayamajhi et al. 2013, Hiramitsu et al. 2014, Su et al. 2014, Tsutsumi et al. 2014), através da ativação das vias sinalizadas por Nrf2 (Wang et al. 2015, Ji et al. 2015) e PGC-1α/TFAM (Hawley and Morton 2014, Scarpulla 2008). A naringenina também já demonstrou aumentar a expressão do receptor ativado por proliferador de peroxissoma α (PPARα, do inglês *peroxisome proliferator–activated receptor* α) no fígado de ratos (Cho et al. 2011), também envolvido na biogênese mitocondrial através da ativação do TFAM (Alam et al. 2014).

Outro mecanismo que poderia ser responsável pelo aumento da atividade da CTE é o desacoplamento da fosforilação oxidativa, que ocorre através das proteínas desacopladoras (UCPs, do inglês *uncoupling proteins*),

permitindo o vazamento dos prótons para a matriz mitocondrial sem a passagem desses pela ATP-sintase (Speakman et al. 2004), causando uma redução na produção de ATP e consequente aceleração da atividade da cadeia respiratória a fim de manter o potencial mitocondrial transmembrana (Berentzen et al. 2005, Andrews et al. 2006). Tanto o exercício físico quanto os flavonoides já demonstraram efeito desacoplador. O exercício pode causar o aumento da regulação da UCP2 (Bo et al. 2008, Dietrich, Andrews and Horvath 2008), que é encontrada em grandes concentrações no encéfalo, em especial no cerebelo (Richard et al. 1998). Quanto aos flavonoides, Dorta et al. (2005) demonstraram que mitocôndrias isoladas expostas a diferentes flavonoides foram capazes de aumentar a liberação de Ca^{2+} e reduzir os níveis de ATP, ambos marcadores de desacoplamento. Ortega e Garcia (2009) também demonstraram um efeito desacoplador da quercetina, e efeito similar também foi demonstrado com a naringenina por van Dijk, Driessen and Recourt (2000).

Na tentativa de explicar o aumento da atividade da cadeia respiratória, avaliamos a atividade do CAC, responsável por fornecer equivalentes redutores à CTE. O CAC é considerado um ciclo anfibólico, pois além de centralizar o metabolismo oxidativo, participando do catabolismo de carboidratos, lipídeos e aminoácidos; também fornece precursores para diversas vias anabólicas, como síntese de aminoácidos (Nelson and Cox 2014). Apesar da prática de exercício físico já ter demonstrado causar aumento da atividade do CAC em humanos e modelo animal (Befroy et al. 2008, Blomstrand et al. 2011, Howarth et al. 2004, Lin et al. 2012, Tikkanen, Naveri and Harkonen 1995, Powers et al. 1992), não encontramos efeito significativo do exercício materno sobre a atividade das enzimas do CAC avaliadas no cerebelo da prole.

Por outro lado, a naringenina possui uma marcada atividade sobre o CAC. Nós encontramos uma diminuição da atividade das enzimas IDH, α-KGDH e MDH no cerebelo da prole submetida à suplementação materna com naringenina, enquanto o mesmo grupo não mostrou alteração na atividade da enzima CS. As estruturas córtex parietal e hipocampo da prole não foram afetadas *(material suplementar)*. Diversos polifenois já demonstraram alterar a atividade do CAC em modelo animal, sendo os resultados variados, com a ausência de efeito (Rajadurai and Prince 2007, Jayachandran, Vasanthi and Rajamanickama 2010, Al Numair et al. 2012, Stanely Mainzen Prince 2013, Olesen et al. 2014), diminuição (Vijayakumar, Presannakumar and Vijayalakshmi 2009) e até mesmo aumento da atividade (Casuso et al. 2015).

Considerando o efeito específico da naringenina sobre as desidrogenases do CAC, realizamos experimentos *in vitro* com as enzimas dependentes de NAD^+ purificadas disponíveis comercialmente (α-KGDH e MDH). Novamente, a naringenina inibiu expressivamente a atividade das desidrogenases, confirmando a redução da atividade causada pela naringenina em uma concentração já demonstrada no sangue logo após a sua ingesta (Mata-Bilbao Mde et al. 2007, Sun et al. 2014). Através da análise bioinformática foi possível demonstrar a interação da naringenina com as enzimas desidrogenases, com energia de ligação alta e negativa, além de uma provável inibição da atividade das enzimas desidrogenases dependentes de NAD^+ por meio da ligação da naringenina especificamente no sítio de ligação da coenzima oxidada. Islam et al. (2015) também demonstraram que diferentes polifenois, incluindo a naringenina, podem inibir a atividade da enzima 3-hidroxi-3-metil-glutaril-Coenzima A-redutase impedindo a ligação do $NADP^+$,

ligando seus aneis fenólicos no mesmo local. Recentemente, um estudo *in vitro* mostrou a ligação do resveratrol no sitio do NAD^+ no complexo I, sendo encontrado um aumento da atividade do complexo em baixas concentrações do polifenol (<5μM), enquanto que, com o aumento da concentração (50 μM), a atividade foi reduzida. Quando feito tratamento com resveratrol 40 mg/kg/dia durante 12 semanas, ocorreu um aumento da atividade do complexo I em mitocôndrias isoladas do cérebro de ratos (Gueguen et al. 2015).

Apesar da redução da atividade do CAC encontrada no cerebelo da prole submetida à suplementação materna com naringenina, demonstramos um aumento na atividade da SDH em cerebelo e hipocampo. O efeito contrário demonstrado pelo aumento da atividade da SHD, desidrogenase que participa tanto do CAC quanto da CTE, é justificada pela utilização do FAD^+ como aceptor de elétrons na reação, sendo que até o momento os polifenois demonstram inibir apenas enzimas dependentes de NAD^+/$NADP^+$, como demonstrado pelo presente e outros trabalhos (Gueguen et al. 2015, Islam et al. 2015). A passagem de elétrons do complexo I-III não foi afetada no cerebelo da prole, corroborando com a menor atividade das desidrogenases dependentes de NAD^+ no CAC, visto que o complexo I recebe os elétrons deste composto na forma reduzida, proveniente do CAC, mas também de outras fontes, como a glicólise e a β-oxidação (Nelson and Cox 2014).

Nós acreditamos que a naringenina suplementada durante a gestação pode atravessar a placenta (Arola-Arnal et al. 2013, Nigam et al. 2015, Cao et al. 2013, Schroder-van der Elst et al. 1998) e estimular a sinalização de diversas vias envolvidas na defesa antioxidante e biogênese mitocondrial nos filhotes. Como a análise experimental se deu aos sete dias de vida da prole,

com ausência de suplementação desde o nascimento, ainda não podemos afirmar se ocorre um depósito do flavonoide durante a gestação, que causa os efeitos demonstrados, ou o mesmo causa uma adaptação que perdura para a vida pós-natal. Apesar de estimular o aumento das defesas antioxidantes, a suplementação com naringenina também demonstrou reduzir a atividade do CAC, efeito reportado também no câncer, onde a rara mutação de enzimas do CAC tem relação com o surgimento de tumores malignos (Schrauwen and Hesselink 2008, Gaster, Nehlin and Minet 2012, Nelson and Cox 2014), além de aumentar o conteúdo de $O_2^{\bullet-}$ e a lipoperoxidação no cerebelo apesar do aumento no conteúdo de GSH. O perfil completo de atividade da naringenina permanece por ser desvendado, podendo atuar como pró-oxidante nesta concentração utilizada, estimulando as vias de sinalização induzidas por espécies reativas e causando as alterações verificadas no perfil antioxidante e na cadeia respiratória. Ainda, a atividade antioxidante de moléculas de baixo peso molecular sempre resulta na formação de eletrófilos, que são responsáveis pela ativação das mesmas vias de sinalização sensíveis às espécies reativas aqui descritas (Kensler, Wakabayashi and Biswal 2007, Uruno et al. 2013).

Em diversos parâmetros avaliados, a combinação dos dois tratamentos promoveu o desaparecimento dos efeitos evidenciados pelos tratamentos de forma individual. Esta interferência já tem sido demonstrada em diversos trabalhos, onde a suplementação com antioxidantes previne a adaptação causada pelo exercício físico em modelo animal (Casuso et al. 2013, Casuso et al. 2015) e humano (Gomez-Cabrera et al. 2008a, Morrison et al. 2015). Sabe-se que a utilização de antioxidantes afeta a homeostase redox e as cascatas

de sinalização induzidas por espécies reativas tais como PGC-1α e Nrf2 (Casuso et al. 2014, Paulsen et al. 2014a, Venditti et al. 2014, Paulsen et al. 2014b, Gomez-Cabrera et al. 2015).

Nós demonstramos que a abolição mais evidente dos efeitos individuais de cada estratégia antioxidante ocorreu nos parâmetros de metabolismo energético, que também são marcadores de biogênese mitocondrial, como a atividade da CTE. Visto que o aumento da biogênese mitocondrial e defesas antioxidantes envolvem vias de sinalização semelhantes, são necessárias mais análises para verificar especialmente se as vias estimuladas por PPARα, PGC-1α, Nrf2 e TFAM são mantidas com a união dos tratamentos, ou se o bloqueio ocorre em outros níveis de sinalização.

Por fim, acreditamos que seja importante uma reflexão a cerca das estruturas encefálicas utilizadas nesse estudo, desde que o cerebelo parece ser mais suscetível às intervenções pré-natais, enquanto o hipocampo foi minimamente afetado. Deve-se restringir a avaliação às condições experimentais aqui utilizadas, e ao modelo animal proposto.

Em todos os parâmetros avaliados o hipocampo demonstrou ser a estrutura menos afetada. Não encontramos alterações na atividade do CAC ou no estado redox, apesar do exercício físico demonstrar afetar o conteúdo de SOD e GPx nessa estrutura (Marosi et al. 2012), o que também foi demonstrado por nosso grupo anteriormente com o aumento da atividade das enzimas CAT e GPx no hipocampo da prole submetida ao exercício materno (Marcelino et al. 2013). Corroborando com os nossos achados, a suplementação com naringenina na dose de 50 mg/kg/dia durante 28 dias não

exerceu alterações em atividade da SOD, CAT e GPx no hipocampo de ratos Wistar (Chtourou et al. 2015).

Já o cerebelo da prole se mostrou mais suscetível a alterações por ambos os tratamentos, seguido do córtex parietal. Já está bem estabelecida a relação do cerebelo com a coordenação motora e de movimento (Morton and Bastian 2007, Pope 2011), bem como sua adaptação em relação ao exercício aeróbico, demonstrando em modelo animal as alterações no volume e densidade das células Purkinje e em diversas proteínas estruturais (Garcia et al. 2012, Huang et al. 2012). Também já foi demonstrada a projeção de sinais realizada do cerebelo ao córtex cerebral (Dean et al. 2010), sendo mantida no córtex motor a aprendizagem motora feita através do cerebelo (Galea et al. 2011).

Pitozzi et al. (2012) também encontraram o efeito antioxidante dos polifenois primeiramente no cerebelo, em relação as outras estruturas encefálicas avaliadas, sendo também uma das estruturas mais afetada em aumento das defesas antioxidantes em ratos Wistar adultos após consumo de chá rico em polifenois durante seis semanas (Linardaki et al. 2011). Em comparação a outras seis estruturas, o cerebelo também demonstrou manter os maiores níveis de polifenois depositados após três semanas de suplementação em porcos (Chen et al. 2015).

Quanto à concentração de naringenina nas frutas, encontramos valores variados. Gharras (2009) cita que é encontrado em média 175 mg de naringenina a cada litro de suco de limão, enquanto em extrato de cascas de frutas cítricas em média 1 mg/g de liofilizado (Nakajima et al. 2016). Já na goiaba vermelha fresca, os valores variam entre 7,9 e 16,7 mg/100g de fruta,

sendo os valores mantidos mesmo após a secagem (Nunes et al. 2016). Entretanto, a forma glicosilada da naringenina, a naringina, é o flavonoide encontrado em maior concentração nas frutas, sendo demonstrado no *grapefruit* e pomêlo maduros a concentração de 2.195 e 560 mg/100g de fruta fresca, respectivamente (Ortuño and A. Garcia-Lidbn 1995). A forma glicosilada do flavonoide não é encontrada na urina de humanos e animais, e sua clivagem e absorção já na forma de naringenina ocorre principalmente no intestino delgado (Orrego-Lagaron et al. 2015), após hidrólise por glicosilases.

Considerando a concentração de naringina como base para dose ingerida de naringenina, seria necessário para um adulto de 70 kg a ingestão de 160g de grapefruit ao dia para utilização da mesma dosagem administrada neste trabalho (Wilcox 1999, Vallejo et al. 2010).

5. Conclusões

O período gestacional é um momento oportuno para melhora dos hábitos de saúde, visto que a mulher tende a ter maior cuidado e ser mais bem instruída por profissionais de saúde no acompanhamento pré-natal. Dado que a tendência na população em geral é a busca por melhor qualidade de vida, a prática de exercícios e a suplementação com antioxidantes tem se tornado comum e sido mais divulgada.

Nós demonstramos que o exercício físico materno causou uma adaptação “benéfica” na prole, com aumento das defesas antioxidantes e de marcadores de biogênese mitocondrial.

A suplementação com naringenina apesar de parecer estimular as mesmas vias “benéficas” do exercício, também aumentou a lipoperoxidação no cerebelo da prole e reduziu a atividade do CAC.

Quando as duas intervenções antioxidantes foram aliadas, o efeito mútuo foi anulado em grande parte dos parâmetros avaliados, demonstrando uma clara inibição da adaptação.

Concluindo, deve haver mais estudos para elucidar o papel do exercício e da suplementação com flavonoides durante a gestação, e cautela na prescrição de intervenções neste período.

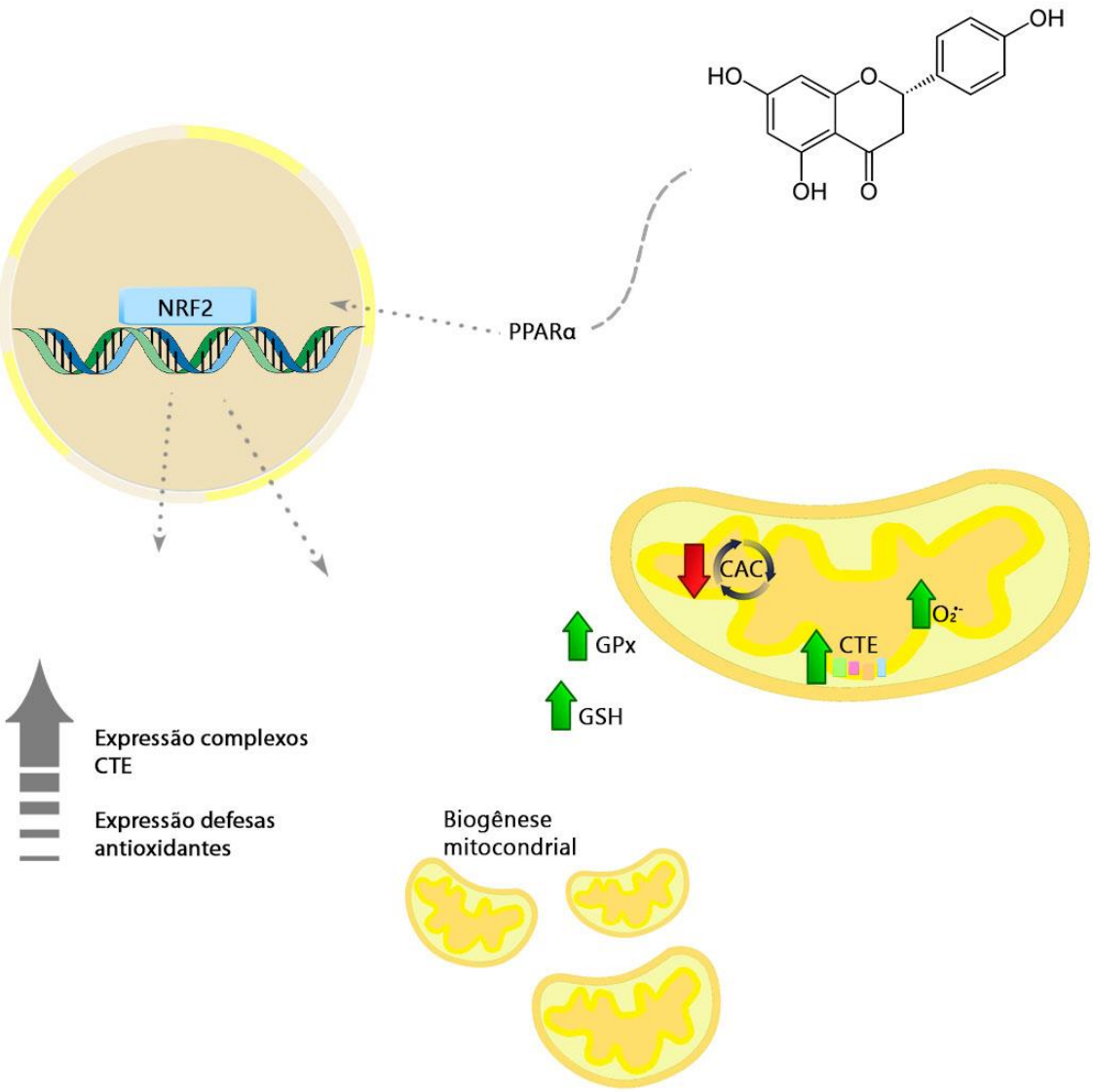

Figura 1. Efeito bioquímico da suplementação materna com naringenina no encéfalo da prole aos sete dias de vida.

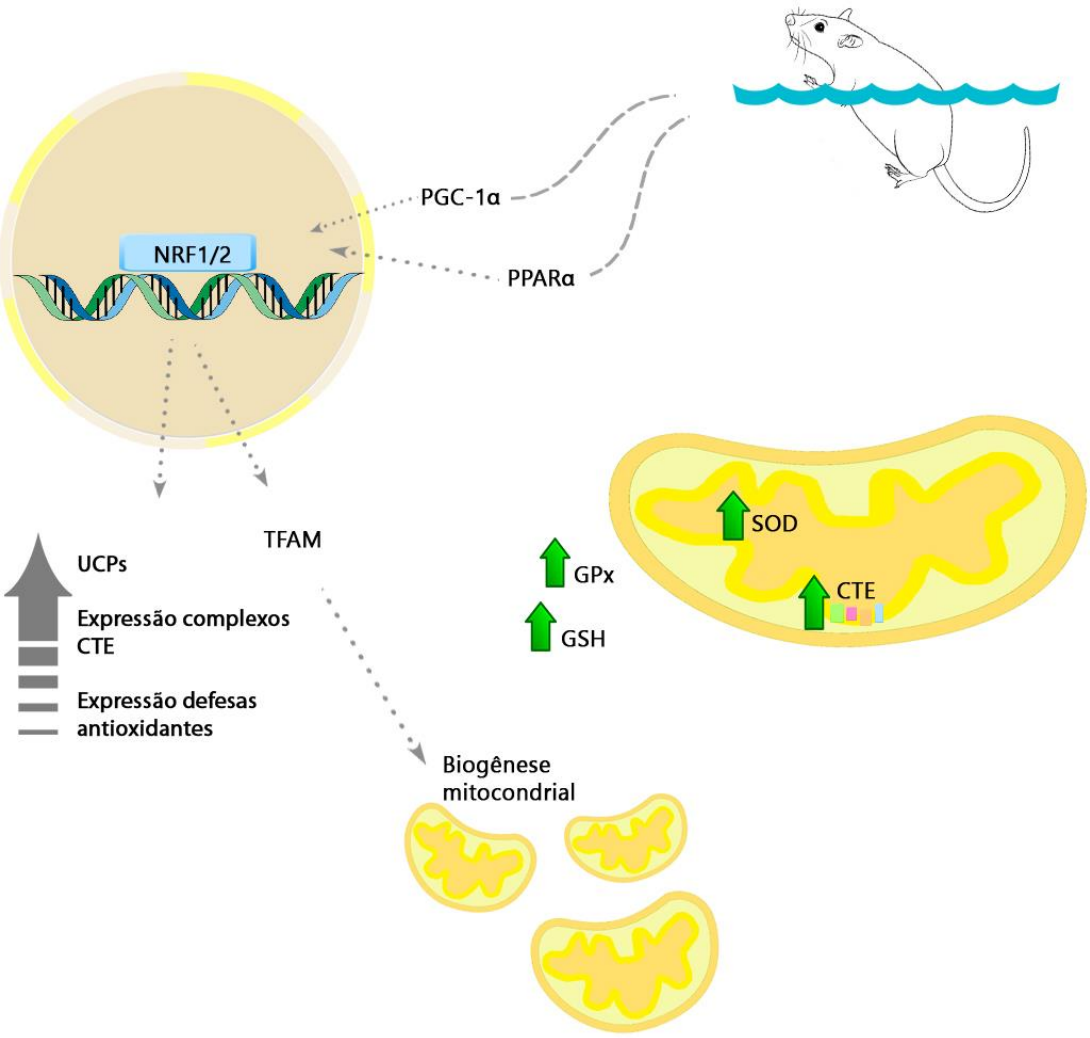

Figura 2. Efeito bioquímico do exercício de natação materno no encéfalo da prole aos sete dias de vida.

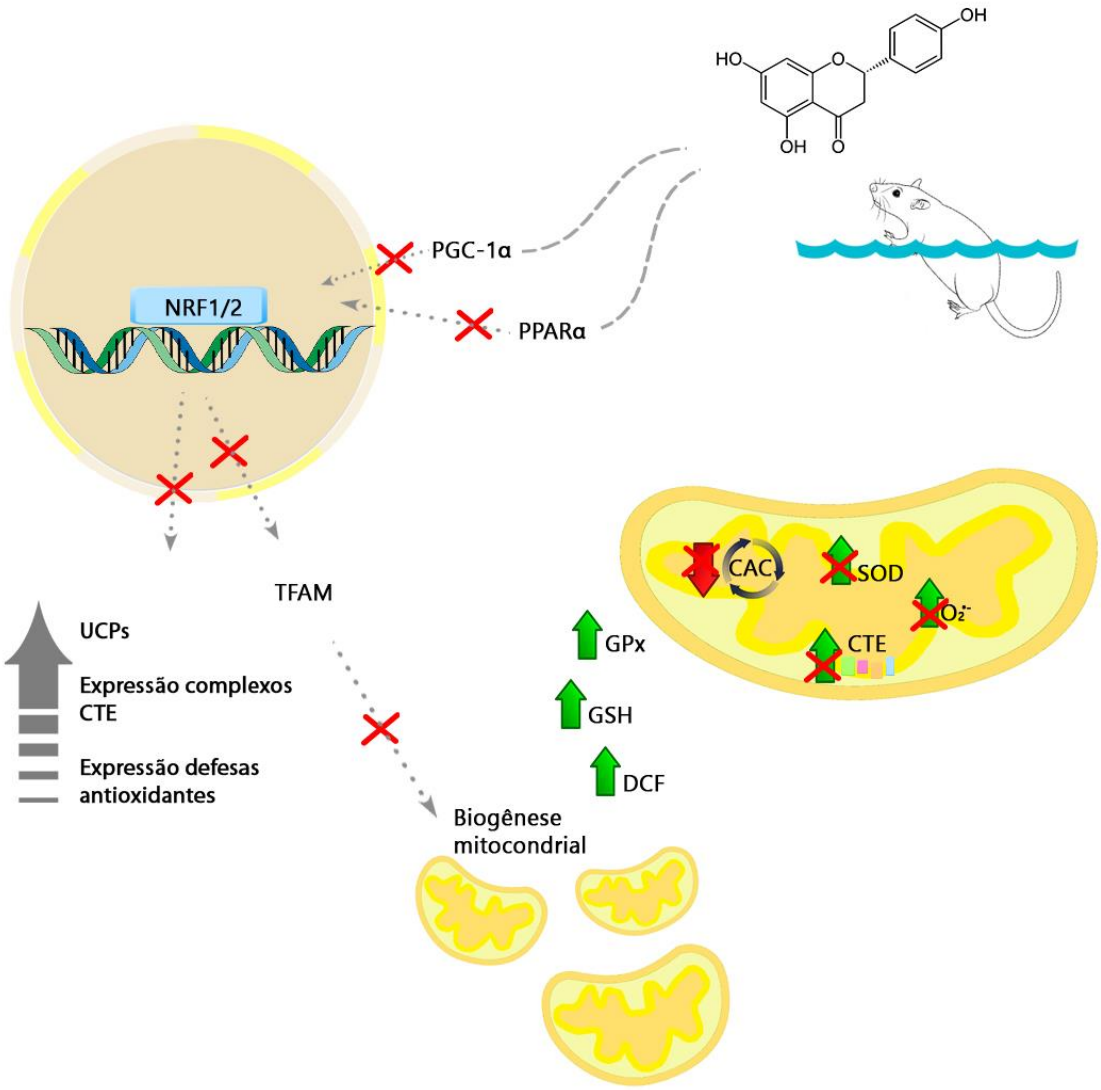

Figura 3. Efeito do exercício de natação materno aliado à suplementação com naringenina durante a gestação no encéfalo da prole aos sete dias de vida.

6. Perspectivas

As perspectivas deste trabalho visam esclarecer os mecanismos que suportam os resultados bioquímicos encontrados na prole, através da análise das vias glicolítica e de oxidação de ácidos graxos, níveis de $NAD^+/NADH^+$ formação de ATP e atividade da ATP-sintase em cerebelo, córtex parietal e hipocampo da prole de ratos Wistar submetidos ao mesmo protocolo materno.

Analisar os estágios respiratórios mitocondriais e a expressão de UCPs no mesmo modelo, a fim de esclarecer o possível desacoplamento causado pelos tratamentos isolados, e analisar as vias de sinalização induzidas por PPARα, PGC-1α, Nrf2 e TFAM, assim como marcadores de dinâmica mitocondrial – fusão e fissão – afim de esclarecer os possíveis mecanismos de indução de biogênese mitocondrial e aumento da atividade das enzimas antioxidantes e da CTE.

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Material suplementar

Concentração de superóxido mitocondrial:

A concentração de superóxido mitocondrial foi medida através da probe MitoSOX® red, da marcaI Invitrogen®, utilizando o citômetro de fluxo FACScalibur (BD Biosciences®). As amostras de tecido (100 mg) foram dissociadas em 1 mL de tampão fosfato-salina pH 7,4, contendo 1 mg% de colagenase IV e 0,5 mg% de DNase. Após a dissociação, as amostras foram filtradas, e 100 μL de cada amostra foi incubada durante 20 min à 37º C na presença do fluoróforo na concentração final de 1 μM. Após a incubação, foram avaliados 10.000 eventos por amostra através do citômetro de fluxo. As análises dos resultados foram feitas utilizando o software FlowJo®.

Resultados:

Foi encontrado um aumento na concentração de superóxido mitocondrial no cerebelo na prole aos sete dias de vida, após a suplementação materna com naringenina [$F(1,55)=5,848;p=0,0189$]. As estruturas córtex parietal [$F(1,37)=0,3772;p=0,5429$] e hipocampo [$F(1,36)=0,01284;p=0,9104$] não foram afetadas (Figura 1).

Na análise da atividade do CAC, não foi encontrada diferença significativa na atividade da IDH [$F(1,18)=0,04674;p=0,8313$], KGDH [$F(1,21)=0,7557;p=0,3945$] e MDH [$F(1,22)=0,008377;p=0,9279$] no córtex parietal da prole. Também não houve alteração no hipocampo nos mesmos

parâmetros de atividade da IDH [F(1,22)=2,044;p=0,1669], KGDH [F(1,21)=1,502;p=0,2340] e MDH [F(1,22)=0,3219;p=0,5762].

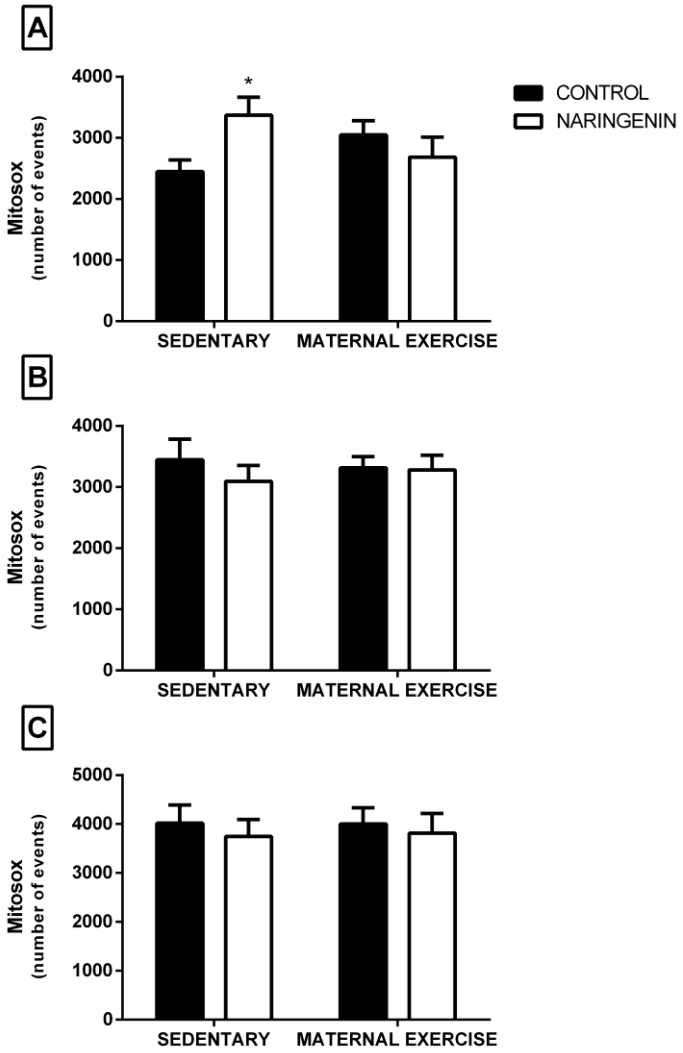

Figura 1. Efeito do exercício materno e/ou suplementação com naringenina sobre a concentração de superóxido mitocondrial em cerebelo (A), córtex parietal (B) e hipocampo (C) da prole. Os resultados estão expressos como média+SEM para *n*=10. Os dados foram analisados por ANOVA de duas vias seguida de teste de Tukey.*p<0,05

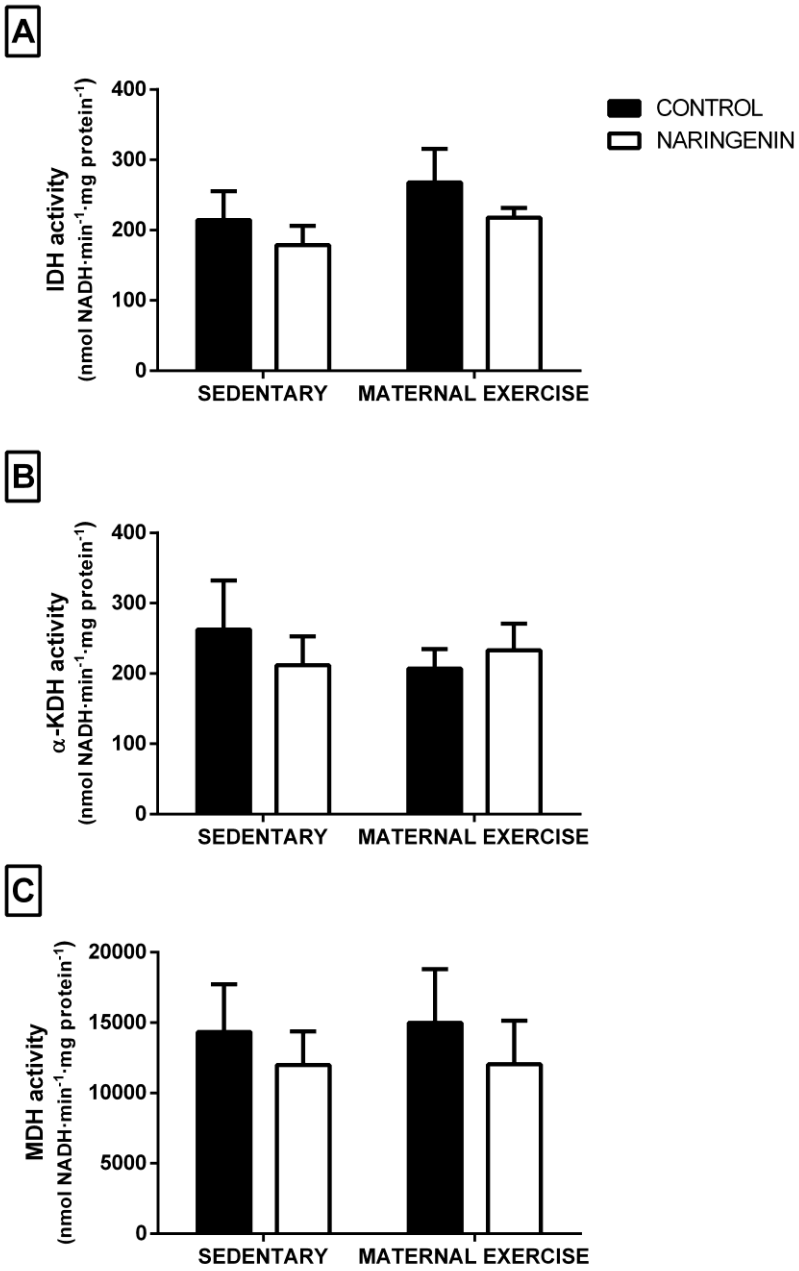

Figura 2. Efeito do exercício materno e/ou suplementação com naringenina sobre a atividade da isocitrato-desidrogenase (IDH) (A), α-cetoglutarato-desidrogenase (KGDH) (B) e malato-desidrogenase (MDH) (C) no córtex parietal da prole. Os resultados estão expressos como média+SEM para *n*=10. Os dados foram analisados por ANOVA de duas vias seguida de teste de Tukey.

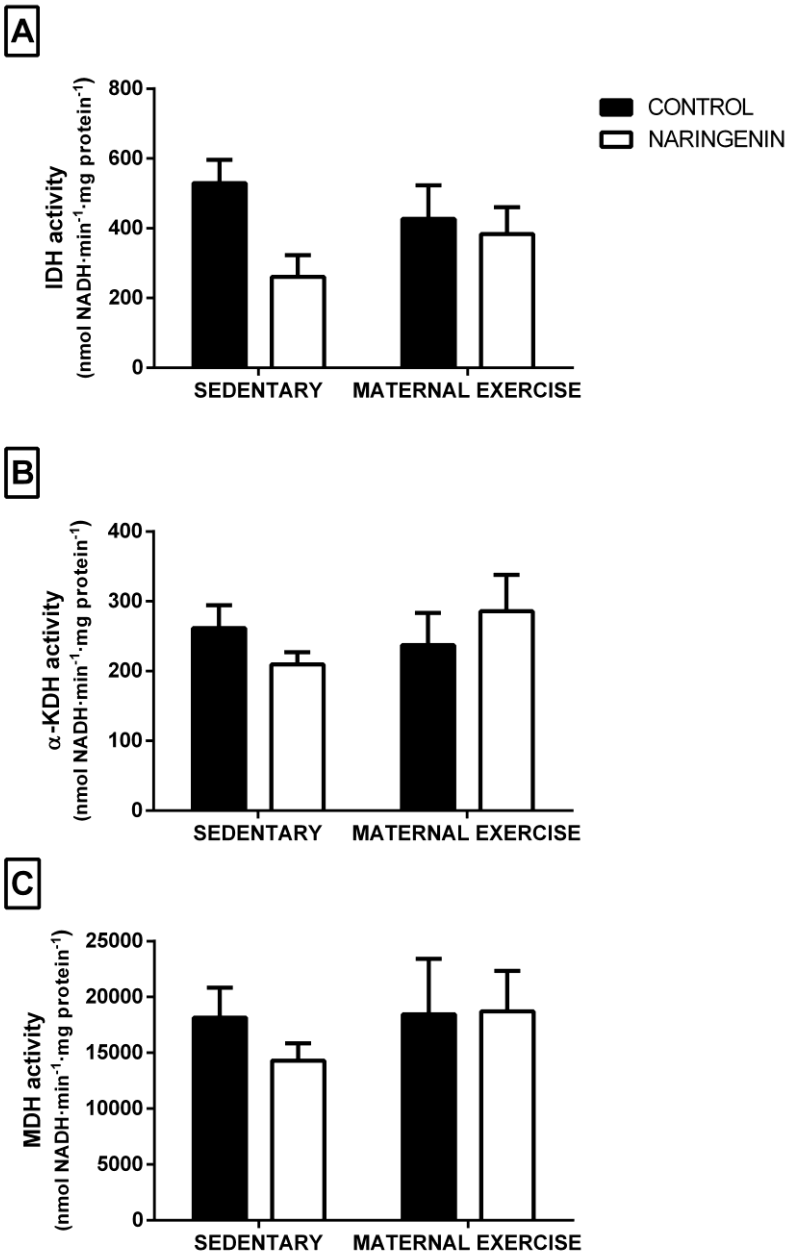

Figura 3. Efeito do exercício materno e/ou suplementação com naringenina sobre a atividade da isocitrato-desidrogenase (IDH) (A), α-cetoglutarato-desidrogenase (KGDH) (B), e malato-desidrogenase (MDH) (C) no hipocampo da prole. Os resultados estão expressos como média+SEM para *n*=10. Os dados foram analisados por ANOVA de duas vias seguida de teste de Tukey.

Anexo – Carta de aprovação da CEUA

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 26542
Título: Avaliação do efeito da naringenina sobre o estado redox e a biogênese mitocondrial em cérebro de ratos Wistar submetidos ao exercício físico materno

Pesquisadores:

Equipe UFRGS:

CRISTIANE MATTE - coordenador desde 01/04/2014
DANIELA PEREIRA STOCHER - Outra Função desde 01/04/2014
Pauline Maciel August - Aluno de Mestrado desde 01/04/2014

Equipe Externa:

Renata Dias de Fraga - Outra Função desde 01/04/2014

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 12/05/2014 - SALA MULTIUSO DA BIBLIOTECA CENTRAL, PRÉDIO DA REITORIA DA UFRGS, em seus aspectos éticos e metodológicos, para a utilização de ratos Wistar(28 fêmeas, 14 machos, e 196 filhotes de ambos os sexos), de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Quinta-Feira, 22 de Maio de 2014

STELA MARIS KUZE RATES
Coordenador da comissão de ética