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Impacto da dieta materna no perfil metabólico e comportamental de fêmeas na vida adulta

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Tese submetida ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor.

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RESUMO

Na gravidez ocorrem modificações fisiológicas no organismo materno, gerando um aumento na necessidade de nutrientes essenciais, a fim de garantir o adequado crescimento e desenvolvimetno fetal. Durante a gestação o feto é capaz de reagir a situações nutricionais adversas para o seu desenvolvimento normal e promover adaptações moleculares, celulares e bioquímicas. Fatores genéticos e ambientais relacionados com a dieta materna podem predispor a prole a uma séries de doenças importantes, como alterações metabólicas, endócrinas e cardiovasculares. Nesse contexto o objetivo desta tese foi analisar o impacto da dieta materna (restrita e hipercalórica) durante a gestação e a lactação no seu perfil metabólico. Bem como, avaliar aspectos comportamentais e metabólicos da prole fêmea quando adulta. Após confirmação de prenhez, as fêmeas de camundongos da linhagem BALB/C (n=30) foram divididas em 3 grupos (n=10 por grupo); dieta controle (CONT), restrição calórica (RD) e hipercalórica (HD). A prole fêmea foi desmamada com 21 dias e dividida aleatoriamente em dois grupos que receberam dieta controle ou restrita formando os seguintes grupos: CONT/CONT, CONT/RD, RD/CONT, RD/RD, HD/CONT, HD/RD. Os filhotes foram eutanasiados por volta dos 100 dias de vida, após a realização dos testes comportamentais do campo aberto (CA), labirinto em cruz elevado (LCE) e reconhecimento de objetos a fim de avaliar a locomoção e medo inato, ansiedade e memória de curta duração. Foram avaliadas também as concentrações séricas de leptina, insulina, glicose no sangue. No tecido hepático foi avaliado a presença de esteatose, infiltrado linfocitário e balonamento hepatocelular, além de, parâmetros de estresse oxidativo. No tecido adiposo visceral foi mensurado o infiltrado inlfamatório e o tamanho da área dos adipócitos. Já nas mães, foram avaliado no fígado as concentrações hepáticas de interleucina-6 (IL-6) e fator de necrose tumoral alfa (TNF-α), presença de esteatose hepática, infiltrado linfocitário e balonamento hepatocelular, além de, parâmetros de estresse oxidativo, e no tecido adiposo mensuramos o infiltrado inflamatório. As mães do grupo HD apresentaram alterações no fígado compatíveis com esteato-hepatite não alcoólica (EHNA) e aumento nas concentrações séricas de TNF-α quando comparado aos grupos RD e CONT. Não encontramos alterações na formação de espécies reativas de oxigênio (EROs) no fígado dos diferentes grupos de mães. No entanto, a formação de malondialdeído (MDA) e a atividade da catalase (CAT) foram maiores no grupo HD das mães quando comparadas aos grupos RD e CONT respectivamente. As mães RD apresentaram aumento do infiltrado inflamatório no tecido adiposo quando comparadas ao grupo CONT, evidenciando alterações causadas pela dieta restritiva. Já na prole observamos aumento na concentração

plasmática de glicose de filhotes de mãe HD. A prole adulta das mães RD mostrou uma diminuição nas concentrações séricas de insulina e leptina quando comparada aos outros grupos estudados. A exposição da prole a dieta restrita após o desmame promoveu um menor infiltrado inflamatório no tecido adiposo nos grupos RD/RD e HD/RD quando comparado aos outros grupos. Já o grupo HD/CONT apresentou maior nível de infiltração de macrófagos, indicando que a dieta materna associada a dieta consumida ao longo da vida afeta o metabolismo do tecido adiposo da prole adulta. Ainda nos filhotes, encontramos um efeito da dieta materna sobre o estresse oxidativo no tecido hepático, onde a atividade da superóxido dismutase (SOD) está aumentada no grupo RD/RD quando comparada com o HD/RD. Na avaliação do comportamento de ansiedade e memória não encontramos diferenças significativas entre os grupos, no entanto, a prole de mães RD exibiu uma diminuição na locomoção na área lateral no teste do CA. Concluimos que tanto a dieta materna restritiva quanto a hipercalórica causam prejuízos na saúde materna durante o período de gestação e lactação, e que ambas as dietas impactaram em parâmetros bioquímicos, no metabolismo lipídico e no comportamento no CA da prole na vida adulta, mas parecem não afetar o tecido hepático da prole. Podemos concluir também, que a dieta restrita oferecida após o desmame aos filhotes foi capaz de reverter alguns efeitos prejudiciais causados pela dieta materna desequilibrada.

Palavras-chave: gestação, lactação, restrição calórica, dieta hipercalórica, metabolismo, comportamento, estresse oxidativo.

ABSTRACT

During pregnancy, physiological changes occur in the maternal organism, generating an increase in the need for essential nutrients, in order to ensure adequate fetal growth and development. During pregnancy, the fetus is able to react to adverse nutritional situations for its normal development and to promote molecular, cellular and biochemical adaptations. Genetic and environmental factors related to maternal diet may predispose offspring to serious diseases, like metabolic, cardiovascular, and endocrine alterations. In this context, the aim of this studie was to analyze the impact of maternal diet (restricted and hypercaloric) during pregnancy and lactation on their metabolic profile. As well as, evaluating behavioral and metabolic aspects of adult female offspring. After confirmation of pregnancy, the BALB / C mice females (n = 30) were divided into 3 groups (n = 10 per group), standard diet (CONT), caloric restriction (RC) and hypercaloric (HD). The female offspring were weaned at 21 days and randomly divided into two groups that received a control or restriction diet, forming the following groups: CONT/CONT, CONT/RD, RD/CONT, RD/RD, HD/CONT, HD/RD. The offspring were euthanized around 100 days of age, after performing the open field (OF), elevated plus maze (EPM) and object recognition behavioral tests in order to assess locomotion and innate fear, anxiety and short-term memory. Serum concentrations of leptin, insulin, glucose were also evaluated. In liver tissue, we evaluated the presence of hepatic steatosis, lymphocytic infiltrate and hepatocellular ballooning, in addition to oxidative stress parameters in visceral adipose tissue we measured the inflammatory infiltrate and the size of the adipocyte area. In the dams, serum concentrations of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), presence of hepatic steatosis, lymphocytic infiltrate and hepatocellular ballooning and oxidative stress parameters were evaluated in the liver tissue. In the adipose tissue, we measured the inflammatory infiltrate. In the liver tissue of HD dams, we find alterations compatible with non-alcoholic steatohepatitis (NASH) and an increase in TNF-α serum concentrations when compared to the RD and CONT groups. We did not find changes in the formation of reactive oxygen species (ROS) in the liver among dams groups. Nevertheless, MDA formation and CAT activity was significantly increased in HD dams group when compared to CONT and RD groups respectively. RD-dams showed an increase in inflammatory infiltrate in the adipose tissue when compared to CONT group, evidencing changes caused by restrictive diet. In the offspring, we observed an increase in the glucose serum concentration in the offspring from HD dams. The offspring from RD dams showed a decrease in insulin and leptin serum concentrations when

compared to the other groups. The exposure of offspring to a restricted diet after weaning promoted a lower inflammatory infiltrate in the adipose tissue in the RD/RD and HD/RD groups when compared to the other groups. On the other hand, HD/CONT showed a higher level of macrophage infiltration, indicating that the maternal diet associated with the diet consumed throughout life affects the metabolism of adipose tissue in adulthood. Furthermore, we also verified the effect of maternal diet on oxidative stress on liver tissue, parameters such as superoxide dismutase (SOD) activity was increased in RD/RD compared to HD/RD. Evaluation of anxiety-like behavior and recognition memory showed no significant difference among groups, however, offspring from RD-fed dams exhibited a decrease in lateral area locomotion in the open field test (OF). We conclude that both restrictive and hypercaloric maternal diets, during pregnancy and lactation, cause damage to maternal health during the gestation and lactation period, and the both diets impacted biochemical parameters, lipid metabolism and behavior in the OF in the offspring in adulthood, but they do not seem to affect the liver tissue of the offspring. We can also conclude that the restricted diet offered to the offspring after weaning was able to reverse some harmful effects caused by the unbalanced maternal diet.

Keywords: gestation**,** lactation, caloric restriction, hypercaloric diet, metabolism, behavior, oxidative stress.

LISTA DE ABREVIATURAS

ATV	Área Tegmental Ventral
CA	Campo aberto
CAT	Catalase
CLS	Estruturas do tipo coroa / *Crown-like structures*
DNA	Ácido desoxirribonucleico
DA	Neurotransmissor dopamina
DHGNA	Doença hepática gordurosa não-alcoólica
EHNA	Esteato-hepatite não alcoólica
EPM	Elevated plus maze
EROs	Espécies reativas de oxigênio
GLUT 4	Transportador de glicose 4
HDL	Lipoproteína de alta densidade
HTP	Hipotálamo
IL-6	Interleucina-6
il-8	Interleucina-8
IL-10	Interleucina-10
LCE	Labirinto em cruz elevado
MDA	Malondialdeído
MCP-1	Proteína de quimioatração de monócitos
NASH	Non-alcoholic steatohepatitis
NF-κB	Fator nuclear *kappa* B
OF	Open field test
PPARa	Receptores ativados por proliferadores peroxissomais do tipo alfa
RC	Restrição calórica
SHR	Wistar espontaneamente hipertenso
SOD	Superóxido dismutase
SNC	Sistema nervoso central

TAB	Tecido adiposo branco
Th 1	T helper 1
TNF-α	Fator de necrose tumoral alfa
VLDL	Lipoproteína de densidade muito baixa

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1 REVISÃO DE LITERATURA

1.1 Efeito da dieta materna no desenvolvimento intrauterino

Durante a gravidez ocorrem modificações fisiológicas no organismo materno, gerando um aumento na necessidade de nutrientes essenciais, incluindo proteínas, carboidratos e lipídios. Isso ocorre, não só para manter a nutrição materna, mas também para garantir o adequado crescimento e desenvolvimento fetal, uma vez que a única fonte de nutrientes do concepto é constituída pelas reservas nutricionais e ingestão alimentar materna (Williams, 2001).

Durante o crescimento fetal, ocorrem diversos processos biológicos que necessitam de energia, principalmente proveniente de macronutrientes (carboidratos, lipídios e proteínas) (Bouret, 2010). Ademais, durante a gravidez ocorre um importante redirecionamento da utilização de glicose, na medida em que se observa uma alta taxa de utilização de glicose pelo concepto (Leturque *et al,* 1986). David Barker e outros pesquisadores, no final da década de 80, propuseram que alterações nutricionais, como dieta materna inadequada, durante os períodos críticos de desenvolvimento fetal, resultam em adaptações no desenvolvimento com mudanças permanentes no metabolismo e na estrutura de sistemas, órgãos e tecidos do feto, gerando consequências precoces (baixo peso ao nascimento) ou tardias (doenças na vida adulta). Seus estudos foram baseados em dados epidemiológicos de tamanho e peso ao nascimento em mais de dez mil homens colhidos na cidade de *Hertfordshire,* Inglaterra, durante os anos de 1911 e 1930 e demonstraram que o risco de doenças coronariana diminuía à medida que o peso ao nascimento era mais elevado, pois eles encontraram um aumento duas vezes maior da mortalidade por doença coronariana nos indivíduos com baixo peso ao nascer (Barker, Osmond, 1986; Barker *et al,* 1989).

Esse fenômeno ficou conhecido como a hipótese da origem fetal da doença no adulto ou “hipótese de Barker”. Diversos estudos experimentais confirmaram a “hipótese de Barker” mostrando que fatores ambientais, como por exemplo, desnutrição materna durante o desenvolvimento fetal pode influenciar o fenótipo da prole adulta. Especificamente, os insultos que ocorrem em ambientes nutricionais restritos durante a gestação ou lactação estão associados a alterações no metabolismo de lipídios, na

sensibilidade à insulina, ao desenvolvimento da resistência à insulina, diabetes mellitus, obesidade, modificações no desenvolvimento renal e hipertensão arterial na vida adulta (Barker *et al*, 1989; de Boo, Harding, 2006; Godfrey, Barker, 2001; Zandi-Nejad *et al*, 2006). Ademais, estudos realizados com seres humanos, mostraram que a restrição de alimentos durante a gestação promove, no início da vida adulta da prole, o aumento do risco de neoplasias (Innes *et al*, 2000), osteoporose (Antoniades *et al*, 2003), hipotireoidismo (Kajantie *et al*, 2006), hipertrofia cardíaca (Porrello *et al*, 2008), asma da idade adulta (Shaheen *et al*, 1999), esquizofrenia (Neugebauer, 2005), depressão (Gale, Martyn, 2004), tumor de testículo (Main *et al*, 2006), cirrose hepática (Andersen, Osler, 2004), dificuldade auditiva no adulto (Barrenãs *et al*, 2003), síndrome do ovário policístico (Ibánez *et al*, 2001) e dificuldade de aprendizagem (Strauss, 2000).

Por outro lado, também observações em seres humanos, demonstraram que o consumo em excesso de alimentos altamente calóricos pela mãe durante a gestação, pode levar ao desenvolvimento de síndrome metabólica (Varvarigou, 2010; Nüsken *et al*, 2011), obesidade (Vickers *et al*, 2000), diabetes mellitus tipo 2 (Martins, 2010), hipertensão (McMillen, Robinson, 2005) e doenças cardiovasculares (Godfrey, Barker, 2001; Velkoska *et al*, 2008) no início da vida adulta.

O termo "programação" proposto por Lucas (1991) se baseia no conceito de que existe uma resposta adaptativa dos fetos, a um ambiente intrauterino adverso. Para explicar essa programação Hales e Barker, em 1992, propuseram a hipótese do "fenótipo econômico", sugerindo que o desenvolvimento fetal é sensível ao ambiente nutricional. Com isso, aumentaria as chances de sobrevivência do feto sob condições de nutrição precárias e intermitentes o que resultaria num metabolismo pós-natal alterado (Gottlieb *et al*, 2008).

Além disso, dependendo da fase gestacional em que ocorrem as condições adversas no ambiente intrauterino, o indivíduo pode ficar propenso a desenvolver uma ou outra doença decorrente desta condição. Se as condições adversas ocorrem no início da gestação, o feto terá maior predisposição ao desenvolvimento de doenças coronarianas, na fase intermediária do desenvolvimento fetal, ocorre maior risco de problemas renais e, no final da gestação, o indivíduo estará mais propenso a apresentar baixo peso ao nascer e alterações no seu metabolismo intermediário que o levará ao desenvolvimento da obesidade e doenças relacionadas. Além do mais, a restrição de nutrientes desde o período

da pré-implantação do embrião até o seu nascimento pode desencadear intolerância à glicose na vida adulta (Marciniak *et al*, 2017).

As respostas adaptativas no metabolismo decorrentes de mudança no meio fetal podem ser programadas por meio de mecanismos epigenéticos (Gicquel *et al*, 2008; Jones, Ozanne, 2009). A regulação da expressão gênica estável mediada pelo processo epigenético, envolvendo metilação do ácido desoxirribonucleico (DNA) e modificação das histonas é o que permite ao organismo alterar o seu desenvolvimento dependendo do ambiente. Desta forma, tanto o genoma quanto o epigenoma podem influenciar o fenótipo favorecendo a plasticidade, considerada um paradigma da interação entre genes e ambiente (Lanham *et al*, 2010).

Além da influência da nutrição no desenvolvimento durante a vida intrauterina, estudos em animais, têm demonstrado a importância da nutrição após o nascimento, apresentando um papel fundamental na saúde durante a vida adulta. É no início da vida, após nascimento, que acontecem os eventos de plasticidade neural (Buckley *et al*, 2005). Assim, os efeitos da restrição nutricional intra uterina podem ser minimizados quando a alimentação materna é desbalanceada durante a gestação e torna-se equilibrada durante a lactação (Buckley *et al*, 2005).

No entanto, se a alimentação durante a lactação ainda for desbalanceada, seja ela com restrição de nutrientes ou dieta hipercalórica, esta situação poderá amplificar as alterações provocadas durante o desenvolvimento fetal (Buckley *et al*, 2005).

Entretanto, se após a deficiência ou falta de nutrientes ocorrida nos períodos gestacional e/ou de lactação houver uma disponibilidade de nutrientes exagerada, o indivíduo poderá apresentar risco aumentado do surgimento de doenças metabólicas e cardiovasculares na vida adulta (Drake, Walker, 2004; McMillen, Robinson, 2005; Leandro *et al*, 2009).

Portanto, considerando que grande parte do desenvolvimento do sistema regulador do balanço energético ocorre no período perinatal, os nutrientes advindos da dieta materna são de suma importância para o adequado desenvolvimento fetal (Lilycrop, 2011). Condições desfavoráveis como restrição alimentar ou dietas inadequadas com excessos de calorias, nesta fase de desenvolvimento, podem reprogramar o metabolismo causando alterações metabólicas que podem perpetuar-se ao longo da vida dos seus descendentes (Remmers, Delemarre-Van de Wall, 2011).

1.2 Restrição calórica

A restrição calórica (RC), a priori, é uma intervenção dietética por meio da qual se reduz o consumo de calorias, mantendo-se a proporção entre os macro e micronutrientes e evitando a desnutrição. Para tal recomenda-se o ajuste dos níveis de vitaminas e minerais, o que significa suplementação de micronutrientes na dieta a ser restringida, de forma que os animais em RC consumam a mesma quantidade de micronutrientes que os controles (Pugh *et al*, 1999). Em 1935, McCay e colaboradores publicaram pela primeira vez o efeito da RC em ratos. O estudo observou que, quando executada após a puberdade, os ratos apresentavam vida prolongada e atenuação das severas doenças crônicas.

Desde o trabalho de McCay, os protocolos de RC para roedores divergiram muito, variando de restrição de 25 a 60% de calorias. Sabe-se que, em roedores, a redução calórica de 30% a 60% menos que o *ad libitum* imediatamente após a puberdade (seis meses) causou um aumento proporcional de 30% a 60% na expectativa de vida máxima, enquanto uma redução de 44% na ingestão calórica na idade adulta (12 meses) estendeu a expectativa de vida máxima em somente 10% a 20% (Weindruch, Walford, 1982). Estudos conduzidos em camundongos atestaram que a RC aumenta a longevidade impedindo ou retardando a ocorrência das doenças crônicas como: aterosclerose, cardiomiopatia, diabetes, doenças autoimunes, câncer, Alzheimer e Parkinson (Wang *et al*, 2007; Patel *et al*, 2005).

Outras estratégias alimentares também classificadas como RC surgiram, como por exemplo, o jejum intermitente, também chamado de *every other day*, segundo o qual os animais são submetidos a ciclos alternados (24 horas) de livre acesso e não-acesso à ração (Cerqueira, Kawaltoswski, 2010).

Alguns autores como Cerqueira e Kowaltowski (2010), acreditam que a RC pode provocar desnutrição em roedores. E o jejum intermitente, na maioria dos estudos em que foi empregado, não reduziu o consumo de calorias, em contrapartida, causou estresse devido ao jejum de 24 horas, seguido de um período de hiper-alimentação compensatória (Cerqueira *et al*, 2011).

A RC tornou-se, nas últimas décadas, uma ferramenta importante no estudo dos complexos mecanismos envolvidos no envelhecimento (Colman, Anderson, 2011). Por ser capaz de prolongar a longevidade em muitas espécies, de levedura a roedores (Sohal,

Weindruch, 1996; Partridge, Gems, 2002; Speakman, Hambly, 2007), busca-se compreender seus mecanismos de ação. Muitos estudos no campo da RC trouxeram à tona vias, alteradas no envelhecimento e preservadas durante a RC, cruciais para a progressão da perda funcional celular característica do envelhecimento, que, portanto, se tornaram alvo de atenção para o desenvolvimento de protocolos de RC (Ingram *et al*, 2006; Ingram, Roth, 2011).

Algumas hipóteses sobre os mecanismos biológicos benéficos envolvidos nos efeitos da RC, propostas a partir de pesquisas com animais são: proteção contra o envelhecimento associada á deterioração do sistema imune, aumento na expressão de proteínas chaperonas, evitando erros na configuração de algumas proteínas, e assim reduzindo o risco de doenças como Parkinson e Alzheimer (Lee *et al*, 2000) e modulação na concentração de citocinas inflamatórias resultando em redução da inflamação sistêmica (Fontana, 2009).

Além dessas hipóteses, Harman (1956) propôs que o envelhecimento se deve a danos causados por radicais livres e que a RC causaria uma atenuação do dano oxidativo. Espécies reativas de oxigênio podem danificar moléculas biológicas importantes, incluindo DNA, proteínas e lipídios, alterando as funções celulares. De fato, está bem estabelecido que o RC retarda o acúmulo de moléculas danificadas por oxidação em roedores (Yu, 2006).

As EROs ativam um fator transcricional pró-inflamatório denominado NF-κB, responsável pela transcrição de proteínas pró-inflamatórias como TNF-α e inteterleucinas 1, 2 e 6 (Sohal, Weindruch, 1996; Yu, 2006). Os danos oxidativos, com a ativação de genes pró-inflamatórios, causados por essas espécies reativas de oxigênio estão fortemente relacionados ao envelhecimento e à patogênese de diversas doenças crônicas não transmissíveis, como aterosclerose, diabetes, artrite reumatoide, desordens neurodegenerativas e câncer (Raha, Robinson, 2000). O mecanismo pelo qual a restrição calórica diminui a formação das espécies reativas de oxigênio ainda não está estabelecido; no entanto, a RC parece promover redução nos danos oxidativos, como a supressão da expressão e a ativação do NF-κB. Além disso, melhora o sistema de reparo do DNA celular (Yu, 2006).

1.2.1 Dieta materna restrita e seus efeitos sobre a prole

A RC durante a vida intrauterina vem sendo associada a uma série de distúrbios na idade adulta (Ross, Desai, 2005). Dados epidemiológicos e pesquisas experimentais sobre esse assunto levaram a formulação do conceito de 'programação fetal', o que demonstrou afetar parâmetros como contagem de células, estrutura de órgãos e expressão de genes, embora os mecanismos envolvidos não sejam totalmente conhecidos (Pinheiro *et al*, 2013).

Em roedores, a RC durante a vida intrauterina acarreta prejuízos na regulação neuroendócrina, provocando hiperinsulinemia e hiperleptinemia após o nascimento, com o aparecimento de resistência a esses dois hormônios com consequente hiperfagia na vida adulta (Vickers *et al*, 2000).

Diferentes órgãos como coração, rim, pulmão e cérebro podem ser afetados pela RC materna (Desai *et al*, 2005). De fato, os filhotes nascidos de mães com restrição de alimentos demonstraram uma diminuição relativa no peso desses órgãos, apesar de um crescimento acelerado que resulta em filhotes adultos marcadamente mais pesados (Pinheiro *et al*, 2013).

Além disso, estudos descritos a partir das décadas de 1970 e 1980 passaram a associar a RC materna não só, com o retardo no crescimento intrauterino dos filhotes, mas também, com a maior predisposição para o desenvolvimento de doenças crônicas na vida adulta, em particular o diabetes mellitus tipo 2 e a obesidade (Desai *et al*, 2007).

A oferta de RC de 50% a fêmeas Wistar, quando comparado ao grupo controle, antes e durante a gestação provocou um aumento do peso corporal e diminuição da longevidade da prole masculina de segunda geração, indicando o efeito transgeracional dependente de sexo (Araminaite *et al*, 2014). Outros estudos em modelos animais, abordando os efeitos da RC durante a prenhez e a lactação, relataram ocorrer comprometimento na massa corporal das mães, com repercussões na sua prole (McMillen *et al*, 2008; Schuler *et al*, 2008), como alterações permanentes no crescimento e no metabolismo, mudanças irreversíveis no tamanho e na função de órgãos, especialmente no fígado e no pâncreas (Miñana-Solis, Escobar, 2006).

Em contrapartida, outro estudo que avaliou os efeitos da RC materna de 50% em ratas durante a gestação observou, após avaliação fetal óssea e visceral, somente redução do peso fetal, não encontrando efeitos teratogênicos (Fleeman *et al,* 2005). No mesmo sentido, Dipe (2009) encontrou diminuição do peso dos filhotes no 21° dia da gestação e da placenta das ratas submetidas a RC de 30, 45 e 60% durante a gestação, sendo esse efeito dependente da intensidade da restrição alimentar, mas não observou malformações ósseas e/ou viscerais nessas proles.

1.3 Dietas hipercalóricas

A adoção de dietas hipercalóricas (hiperlipídicas, de cafeteria, entre outras) tem sido muito utilizada como um modelo de indução de obesidade em animais de laboratório. Este modelo é extremamente útil devido à sua grande semelhança com a gênese e com as respostas metabólicas decorrentes da obesidade em humanos, ou seja, a obesidade e as consequências de um balanço energético positivo gerado por fatores ambientais, como por exemplo, o consumo em excesso de alimentos altamente calóricos e o sedentarismo (Tschop, Heiman, 2001).

O consumo em excesso de dieta hiperlipídica contribui para o desenvolvimento de diversas alterações relacionadas à obesidade e sobrepeso, sejam de origem metabólica como hiperlipidemia, diabetes tipo 2, hipercolesterolemia ou de origem cardiovasculares como hipertensão, acidente vascular encefálico, aterosclerose, infarto agudo do miocárdio, e certas formas de câncer (Engin, 2017; Ashwell, 2014). Mais recentemente, a ingestão desta dieta também foi associada a efeitos desfavoráveis no sistema nervoso central (SNC) (Zhang et al., 2008; Sack et al., 2017).

Alguns estudos tem demonstrado a eficácia da ingestão de dietas hipercalóricas ou dietas hiperlipídicas na gênese da obesidade e de suas comorbidades, principalmente em porcos (Thomas *et al,* 2020), em roedores da linhagem Sprague-Dawley (Dobrian *et al,* 2000) em Wistar e em camundongos das linhagens C57BL/6J, AKR/J, e DBA/2J (Buettner *et al,* 2012).

A literatura mostra que embora um aumento na massa corporal possa ser identificado após duas semanas de alimentação com dietas hipercalóricas, o fenótipo de obesidade induzido pela dieta se torna mais aparente após exposição por período superior

a quatro semanas (Buettner et al., 2007). Ratos Wistar tratados com dieta hipercalórica durante três meses aumentaram aproximadamente 1,4 vez a massa corporal quando comparados com animais controle (Da Silva *et al,* 2010). Outra descoberta foi que a alimentação prolongada com dieta hiperlípidica e hipercalórica induz um aumento na massa corporal de roedores, e é mais pronunciada quando a exposição se inicia após o desmame e continua por várias semanas (Cawthorn *et al*, 2012; Buettner *et al*, 2007).

Ratos submetidos à dieta hiperlipídica palatável mostraram um aumento na quantidade dos tecidos adiposos retroperitoneal e epididimal (Duarte, 2001), com tendências a distúrbios no perfil lipídico (Estadella, *et al*, 2004) e aumento nos níveis de insulina (De Schepper, *et al;* 1998; Prada *et al,* 2005) enquanto que em outras investigações não foram observadas diferenças nos níveis de insulina (López *et al*, 2003; Estadella, *et al,* 2004). Com relação à glicemia, poucos estudos (Nascimento, 2008; Barnes, *et al,* 2003), reportam aumento significativo deste parâmetro bioquímico segundo a revisão de literatura realizada por Rosini, *et al,* (2012).

Disfunções endoteliais também vem sendo investigadas a partir do modelo de indução da obesidade por dieta, visto que, a maioria dos estudos com animais submetidos a esse tratamento apresentaram importantes alterações metabólicas como aumento dos triglicerídeos, que estão relacionados com aumento na produção dos ânions superóxido e consequente diminuição da biodisponibilidade de óxido nítrico, um importante vasodilatador liberado pelo endotélio vascular (Rosini, *et al*, 2012).

A oferta das dietas hiperlípidicas e hipercalóricas pode ser dada através das dietas comercialmente produzidas para esse fim ou confeccionadas a partir dos alimentos de consumo humano. A dieta de cafeteria é composta por alimentos do consumo humano que são enriquecidos com ácidos graxos saturados e açúcares refinados em proporções acima da dieta padrão oferecidas a animais de laboratório, é uma dieta que mimetiza o *junk food*, além de ser altamente palatável a sua forma rotativa de apresentação é a que mais se aproxima do perfil da alimentação humana (Dietrich *et al*, 2007, Nascimento *et al*, 2008; Lindqvist *et al*, 2008). Estudos mostram que o consumo deste tipo de dieta leva ao aumento do ganho de peso corporal, aumento da glicemia e gordura visceral, que podem gerar lesões glomerulares, (Santos *et al*, 2010) infiltrações gordurosas no fígado (Doença Hepática Gordurosa Não-Alcoólica – DHGNA) (Moore, 2010), resistência à insulina, dentre outros problemas (Santos *et al*, 2010).

As dietas hipercalóricas comercialmente produzidas também têm a eficácia de atuar na gênese da obesidade e de suas comorbidades (Petry *et al*, 2000). Existem diversos protocolos de dietas comerciais que são formuladas em função do objetivo de cada estudo e podem ou não causar obesidade e alterações metabólicas conforme descrito por Rosini *et al.* (2012).

1.3.1 Dieta materna hipercalórica e seus efeitos sobre a prole

Existem muitos estudos em modelos animais que visam investigar a influência da nutrição materna sobre os filhotes como por exemplo; injeção intra-fetal de glicose em ovelhas durante o terço final da gestação (Muhlhausler *et al*, 2005), alimentação a base de dietas hipercalóricas (Koukkou *et al*, 1998), dietas hiperlipídicas puras ou suplementadas com leite condensado (Samuelsson, *et al*, 2008) e ainda *junk food* com alto teor de gordura ou açúcar (Bayol *et al*, 2005), conhecidas, como descrito anteriormente, de dieta de cafeteria. Em todos esses exemplos de modelos de nutrição materna, os efeitos prejudiciais como: hiperfagia, alta adiposidade corporal, elevados níveis de triacilgliceróis, glicemia anormal e sensibilidade reduzida à insulina são alguns dos sinais mais observados nos filhotes nascidos de mães que receberam estas dietas (Ainge *et al*, 2011).

Shalev *et al*, (2010) investigaram os efeitos da administração de dieta altamente palatável durante a gestação e a lactação de ratas e sua interação com a exposição à mesma dieta após o desmame. Os autores mostraram que a exposição a essas dietas aumentou o risco de obesidade em ratos na puberdade, sendo acompanhada por adaptações nos sistemas de recompensa dopaminérgicos mesolímbicos.

Filhotes de ratas alimentadas com dieta hiperlipídica durante a prenhez e lactação apresentaram puberdade precoce, hiperleptinemia, hiperinsulinemia e obesidade quando adultos, independente da dieta pós-desmame (Howie *et al*, 2009*).* Os descendentes de camundongos, cujas mães foram submetidas à dieta hiperlipídica durante a gestação e lactação apresentaram aumento no peso corporal e na gordura visceral, hipertrofia dos adipócitos e resistência à insulina, sem associação com resistência central à leptina (Volpato *et al*, 2012).

As consequências provocadas por uma alimentação com dieta hipercalórica/ hiperlipídica estão também bem estabelecidas nas funções cardiovasculares (Ascherio, 2002), sendo que a disfunção endotelial é mais severa em animais provenientes de mães que receberam dieta hiperlipídica durante a lactação (Khan *et al*, 2005). Além disso, Murabayashi e colaboradores (2013) relataram que filhotes de camundongos descendentes de mães que receberam dieta hiperlipídica antes e durante a gestação apresentaram resistência à insulina, acompanhado de aumento na expressão do gene TNF-α, e diminuição do GLUT4 no tecido adiposo, essas mudanças podem estar relacionadas a um aumento no risco de obesidade e distúrbios metabólicos quando adulto.

Modelos animais de roedores alimentados com dieta hipercalórica composta por alto teor de açúcares ou lipídios vem sendo amplamente utilizados para avaliar os efeitos da nutrição materna sobre parâmetros metabólicos, comportamentais entre outros nos seus descendentes.

1.4 Integração metabólica durante a restrição alimentar

Durante a RC ocorre uma importante alteração fisiológica que é iniciada com a redução da concentração de glicose no sangue ocasionada pela baixa ingestão de energia proveniente da dieta. Isto levará a uma diminuição da produção de insulina pelas células ß do pâncreas e, consequentemente, a uma diminuição do depósito de tecido adiposo, principalmente do tecido adiposo branco (TAB) (Koubova, 2003). A alteração do depósito de gordura, principalmente o TAB, pode modificar a secreção dos hormônios e citocinas liberando uma menor concentração de TNF-α e maior concentração de adiponectina melhorando a sensibilidade à insulina em diversos tecidos, como o hepático e o muscular. Essas mudanças endócrino-metabólicas podem estar envolvidas com o aumento da expectativa de vida, como já relatado (Combs *et al*, 2003; Wu, 2003).

Porém, durante a gestação, se ocorre déficit de nutrientes, a resposta metabólica imediata do feto é o catabolismo, que consome seus próprios substratos para angariar energia, conduzindo a um incorreto desenvolvimento. Ocorre redistribuição do fluxo sanguíneo, com redução da circulação nos tecidos como o músculo, o pâncreas e o rim, e aumento nos órgãos nobres, como o cérebro e o coração (Baschatt *et al*, 2001; Dubiel *et al*, 2003).

Os insultos que ocorrem em ambientes nutricionais restritos durante a gestação ou lactação estão associados a alterações no metabolismo de lipídios, na sensibilidade à insulina, ao desenvolvimento da resistência à insulina, diabetes mellitus, obesidade, modificações no desenvolvimento renal e hipertensão arterial na vida adulta (Barker, *et al*, 1989; Godfrey, Barker, 2001; Zandi-Nejad *et al*, 2006).

Em ratos, a RC durante a gestação e lactação provocou aumento de gordura retroperitoneal e perigonadal (Armitage *et al*, 2004), do colesterol de alta densidade (HDL-colesterol), do colesterol total e de lipídios totais, reduziu os níveis de triglicerídeos plasmáticos, bem como levou ao acúmulo de triglicerídeo hepático, explicado pela síntese reduzida de apolipoproteína hepática que forma a lipoproteína de muito baixa densidade (*Very Low Density Lipoprotein* - VLDL), responsável pelo transporte de triglicerídeos no plasma (Berleze, 2005). Ainda em ratos cujas mães receberam dietas com restrição calórica durante a gestação, foram observadas alterações no metabolismo hepático da glicose, no metabolismo do colesterol, na resistência à insulina e no desenvolvimento renal (Godfrey, Barker, 2001). A RC no período gestacional, pode afetar o crescimento do fígado e, consequentemente, provocar distúrbios no metabolismo lipídico e de glicose, com alterações permanentes em duas enzimas hepáticas: a fosfoenolpiruvato carboxilase e a glicocinase, envolvidas na síntese e quebra da glicose, respectivamente (Barker, 1989).

Franco *et al*, (2002) avaliou ratos Wistar espontaneamente hipertensos (SHR) nos quais as fêmeas foram alimentadas com dieta padrão ou 50% de restrição durante todo o período gestacional e demonstraram que a RC agrava a hipertensão e a disfunção endotelial, juntamente com um comprometimento do desenvolvimento renal nos descendentes machos e fêmeas.

Segundo Leandro e colaboradores (2012) a restrição de proteína durante a gestação e lactação pode provocar alterações no tipo de fibra muscular esquelética (predispondo á formação de fibras de contração rápida ou glicolíticas) e essas alterações morfológicas e bioquímicas podem influenciar no desenvolvimento da resistência à insulina em ratos machos quando adultos. Outro estudo com restrição proteica durante a gestação em ratos, observou uma diminuição persistente na metilação do DNA e maior expressão de alguns genes hepáticos como o receptor de glicocorticóides e receptores ativados por proliferadores peroxissomais do tipo alfa (PPARalfa) na prole (Gicquel *et al*, 2008). A restrição alimentar materna moderada com redução de 30% na ingestão *ad*

libitum, em porquinhos-da-índia resultou em intolerância à glicose em adultos descendentes masculinos e a insulina plasmática elevada foi observada em descendentes de machos adultos de porquinhos-da-índia submetidos a redução de 15% da ingestão *ad libitum* (Kind *et al,* 2003).

Já a RC de 50% comparada a dieta padrão durante a gestação até o desmame em ratos Wistar prejudica o desenvolvimento mas não a proliferação de células beta. A ingestão de dieta balanceada subsequente pela prole é seguida pelo aumento da proliferação de células beta, mas isso não é suficiente para restaurar completamente a massa de células beta (Garofano *et al,* 1998).

1.5 Integração metabólica durante a dieta hipercalórica

Com o excesso de consumo de dietas hipercalóricas/hiperlipídicas, muitas vezes ocasionando a obesidade, os adipócitos se hipertrofiam e não armazenam energia de forma eficiente, as células imunes se desregulam, e em alguns casos adquirem um fenótipo pró-inflamatório com efeitos deletérios para o organismo. Além da hipertrofia dos adipócitos, os vasos sanguíneos se tornam rarefeitos no tecido adiposo levando a hipóxia e estresse oxidativo. Este evento induz a morte celular dos adipócitos causando elevação da produção de mediadores inflamatórios derivados do adipócito como a leptina, resistina e outros como a proteína de quimioatração de monócitos (MCP-1), que induz o recrutamento, ativação e proliferação de macrófagos. Os macrófagos assumem um fenótipo pró-inflamatório (M1), ocorre o aumento de linfócitos T helper 1 (Th1) e linfócitos citotóxicos $CD8^+$ (Exley *et al,* 2014). Estes macrófagos fagocitam os adipócitos em processo de morte ou mortos, formando uma estrutura chamada "*crow-like structures"* (CLS), que apresenta uma morfologia de um anel de macrófagos e outras células do sistema imunológico que cercam estes adipócitos. Esta formação parece ser um processo adaptativo para limitar a liberação de lipídios tóxicos ou eliminar os detritos celulares quando os adipócitos sofrem morte celular por meio de fagocitose (Brestoff, Artis, 2015). Porém, os macrófagos ativados com fenótipo pró-inflamatório secretam TNF-α, IL-6, interleucina-8 (IL-8), cuja atividade leva à resistência à insulina. Em resposta, são ativados os macrófagos com fenótipo anti-inflamatório (M2), que aumentam a liberação de citocinas anti-inflamatórias e interleucina-10 (IL-10) para proteger os

adipócitos dos riscos de um processo inflamatório exacerbado (Lumeng *et al*, 2007). Tanto o número de macrófagos, quanto a mudança de fenótipo de pró para anti-inflamatório/reparador (M2) são afetados pela perda de peso (Lumeng *et al*, 2007) (Figura 1).

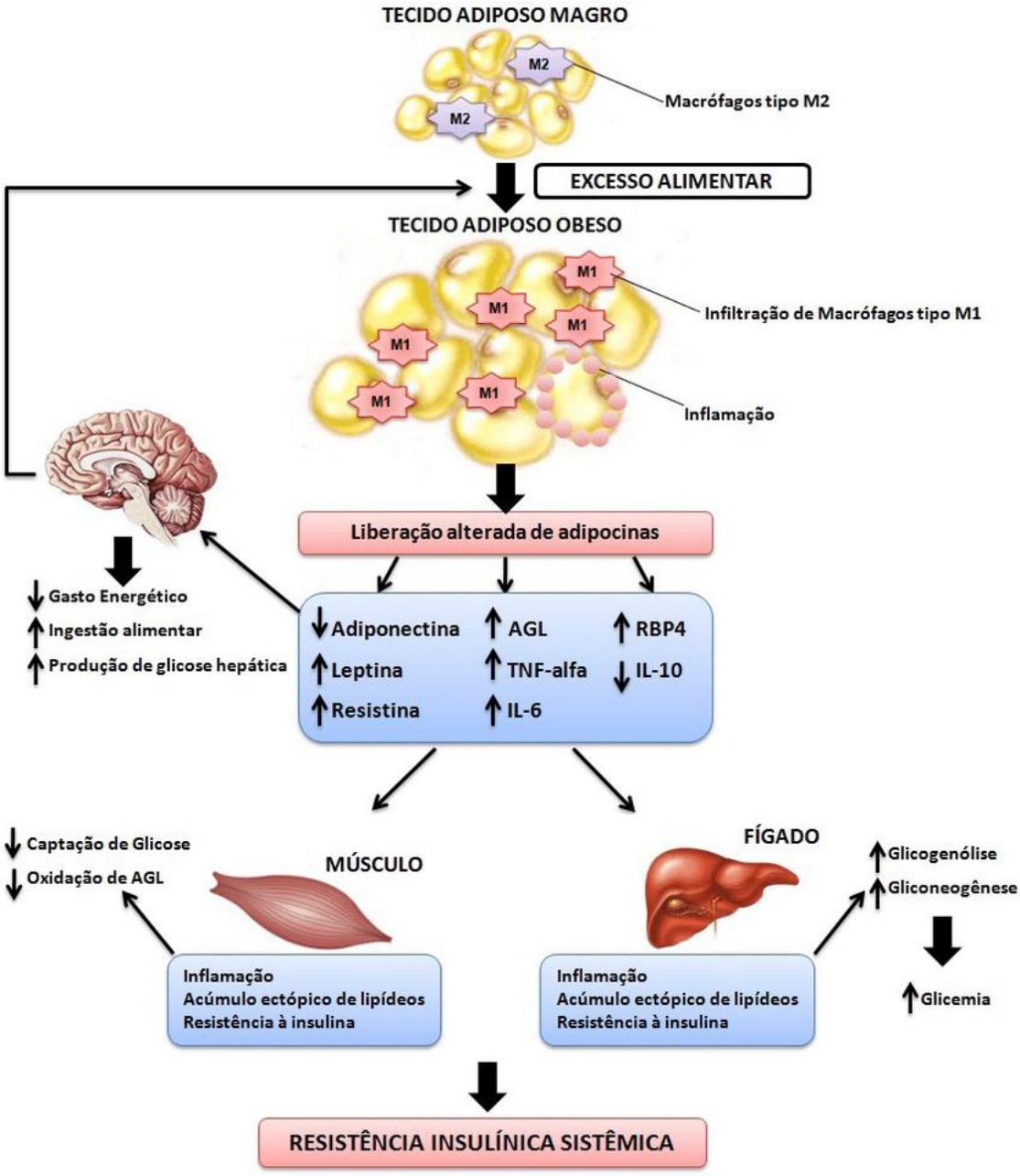

Figura 1: Esquema do processo inflamatório no tecido adiposo causado pelo excesso alimentar. Figura adaptada de Galic e colaboradores (Kershaw, Flier, 2004).

Ademais, Bulamarqui *et al*, (2011), chamam a atenção para o aumento do consumo de dietas hipercalóricas e hiperlipídicas nas últimas décadas e a ligação dessa

mudança com o aumento do aparecimento da DHGNA. Os autores demonstraram que o consumo de dieta hiperlipídica, rica em gordura poli-insaturada, promove esteatose hepática e incremento de peso, reduz os níveis séricos de triglicerídeos, colesterol total e lipoproteína de alta densidade (*High Density Lipoproteins*- HDL) em ratos Wistar, após 23 semanas de experimento quando comparados ao grupo que recebeu dieta padrão. A DHGNA é considerada uma manifestação hepática da síndrome metabólica, cujo principal componente é a resistência à insulina seguida de hiperinsulinemia, (Guidorizzi *et al,* 2005; Moscatiello *et al*, 2007), caracterizada pelo aumento da captação, síntese e acúmulo de ácidos graxos nos hepatócitos, levando a lipogênese e fígado gorduroso (Rinella, 2015). Além disso, os defeitos de oxidação mitocondrial podem aumentar a síntese de ácidos graxos em detrimento da secreção de triacilglicerol, contribuindo para a esteatose hepática (Minehira *et al*, 2008). A hiperinsulinemia também induz o estresse oxidativo com a consequente peroxidação de lipídios na membrana dos hepatócitos e produção de citocinas, principalmente o TNF-α (Minehira *et al*, 2008).

1.6 Aspectos do desenvolvimento comportamental nas dietas restritas e hipercalóricas

A atividade locomotora e o comportamento do tipo ansioso da prole podem ser modulados pelo consumo alimentar materno durante a gestação e a lactação (Ramirez-Lopez *et al*, 2016).

A ingestão de alimentos ricos em gordura e açúcar, tanto em humanos quanto em animais, aumenta drasticamente a síntese e secreção de dopamina em áreas envolvidas com o sistema de recompensa (Ryczko e Dubuc, 2017).

Nosso grupo de pesquisa demonstrou que mães que receberam dietas de restrição e hipercalória durante a gestação e lactação exploraram mais o CA do que mães do grupo controle. Foi encontrada uma correlação positiva entre a duração da locomoção total no CA e a expressão do gene Slc6a3/ Dat1 na área tegmental ventral (ATV) dessas mães. Já no hipotálamo (HTP) encontramos uma correlação negativa de locomoção e níveis de nRNA Drd4, e uma correlação positiva com a expressão do gene Th. Ou seja, uma expressão reduzida do receptor e aumentada da enzima de síntese de dopamina estão relacionadas ao aumento da locomoção das mães no CA. Portanto, a hiperatividade

desses animais quando expostos ao teste do CA pode ser explicada pela modulação do sistema dopaminérgico (Feistauer *et al*, 2020).

Trabalho prévio (Bilbo, Tsang, 2010) demonstrou que ratas expostas cronicamente a dietas hipercalóricas durante o período pré e pós-natal provoca aumento do comportamento do tipo ansioso no teste do LCE e no teste do CA nos descendentes durante a vida adulta.

Além disso, ratos machos tratados desde o período pós-natal com dietas hipercalóricas por 8 semanas demonstraram no CA redução no número de cruzamentos dos quadrantes e percorreram distâncias menores tanto no CA quanto nos braços fechados no teste do LCE quando comparados ao grupo que recebeu dieta controle (White *et al*, 2009).

Já em filhotes de mães que receberam dieta de cafeteria antes do acasalamento, observou-se que a obesidade materna reduziu significativamente a atividade locomotora dos filhotes machos e fêmeas no teste do LCE. Já no grupo que recebeu dieta de cafeteria durante a lactação foi observado um efeito ansiolítico nos filhotes machos, tanto pelo aumento de entradas quanto tempo gasto nos braços abertos do LCE, além de apresentarem menor latência para entrar no centro do CA (Wright *et al*, 2011).

Diversos estudos tentam explicar as diferenças comportamentais encontradas em animais alimentados com dieta rica em gordura e que nasceram de mães alimentadas com dieta hiperlipídica/hipercalórica durante a gestação e lactação, uma hipótese plausível seria o aumento da ativação glial e do estresse oxidativo no córtex cerebral (White *et al*. 2009).

Ramirez-Lopez *et al*, (2016) demonstraram que ratos Wistar machos de mães que receberam 20% de restrição calórica durante o período pré-concepcional e gestacional apresentaram comportamentos do tipo ansioso quando adultos, uma vez que permaneciam menos tempo nos braços abertos e mais tempo nos braços fechados no LCE quando comparados aos machos descendentes de mães que receberam a dieta controle.

Em contrapartida, Valadares *et al*, (2006) investigaram os efeitos da restrição proteica materna (6%) durante a lactação e o desmame no desempenho dos filhotes nas tarefas que avaliavam aprendizagem e memória. Os resultados mostraram prejuízos na memória operacional no labirinto aquático de Morris, porém esse déficit diminuiu seis meses depois, sugerindo que a recuperação nutricional foi eficiente para reverter as alterações

causadas pela restrição protéica nos descendentes. No entanto, a tarefa de memória no teste de reconhecimento de objetos foi prejudicada independente do período. Já, Akitaki *et al*, (2015) demonstraram que a restrição calórica durante a gravidez leva a um pior desempenho da prole no teste de reconhecimento de objetos. Entretanto, essa alteração foi vista apenas com 3 semanas de idade, quando o teste foi realizado novamente, com esses camundongos já adultos, não apresentaram diferenças significativas. Esses achados mostraram que a restrição proteica, em estágios iniciais da vida do animal, produz alterações estruturais, neuroquímicas e funcionais no SNC podendo causar prejuízos no desenvolvimento cognitivo e comportamental.

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2 JUSTIFICATIVA

A literatura ao longo dos últimos anos vem buscando elucidar os efeitos da nutrição materna nas desordens metabólicas e comportamentais em seus descendentes. Está bem estabelecido que a dieta influencia a liberação de hormônios relacionados ao balanço energético e ao apetite nas mães e sua prole, porém, são escassos os estudos que avaliam os efeitos de dietas hipercalórica e restritiva. A dieta de restrição calórica vem sendo estudada por apresentar diversos benefícios, no entando ainda precisamos esclarecer se ela promove reparação sobre alterações metabólicas já ocorridas. Ou seja, nossa hipótese é que a dieta restrita oferecida à prole após o desmame possa ter efeito benéfico sendo capaz de proteger alterações metabólica já descritas na literatura.

3 OBJETIVOS

3.1 Objetivo geral

Analisar o impacto da dieta materna (restrita e hipercalórica) durante a gestação e a lactação no seu perfil metabólico. Bem como, avaliar aspectos comportamentais e metabólicos da prole fêmea quando adulta.

3.2 Objetivos específicos:

Nas mães:

- Avaliar as concentrações séricas de IL-6 e TNF- α (artigo 2);
- Analisar a morfologia do tecido hepático (artigo 2);
- Avaliar o infiltrado inflamatório no tecido adiposo (artigo 2);
- Analisar parâmetros de estresse oxidativo no tecido hepático (artigo 2).

Na prole fêmea adulta:

- Mensurar as concentrações plasmáticas de insulina, leptina e glicose (artigo 1);
- Analisar a morfologia do tecido hepático (artigo 1);
- Estudar os comportamentos do tipo ansioso, medo inato, locomoção e memória de curta duração (artigo 1);
- Avaliar o infiltrado inflamatório no tecido adiposo (artigo 2);
- Analisar parâmetros de estresse oxidativo no tecido hepático (artigo 2).

4 ARTIGOS

4.1 Artigo 1: *Maternal feeding associated to post-weaning diet affects metabolic and behavioral parameters in female offspring*

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ABSTRACT

Genetic and environmental factors related to maternal diet may predispose offspring to serious diseases. However, consequences of a maternal diet intervention during gestation and lactation, and its association with caloric restriction after weaning on the progeny are not completely known. In this context, the goal of the present study was to investigate how different maternal diets, control (CONT), hypercaloric (HD) or restrictive (RD) diets during gestation and lactation, may affect the metabolism and behavior of the offspring that was also submitted to RD. Experimental groups were abbreviated accordingly maternal/offspring diets: CONT/CONT, CONT/RD, RD/CONT, RD/RD, HD/CONT, HD/RD. Our results showed that glucose serum concentration is increased in mice from dams fed a HD. However, offspring from RD-fed dams showed lower insulin and leptin levels than the other groups, indicating a maternal diet effect. Moreover, animals from RD/CONT group showed a higher adipocyte area in comparison to both HD/CONT and CON/CONT. Offspring from RD-fed dams exhibited a decrease in lateral area locomotion in the open field test. Evaluation of anxiety-like behavior and recognition memory showed no significant difference among groups. Thus, maternal RD provides a beneficial response in metabolic parameters, but its effects on behavior is not completely clarified.

1. Introduction

It is well known a variety of conditions such as unbalanced maternal nutrition, exposure to environmental insults, infection or stress occurring during fetal development can lead to health dysfunctions in the progeny [1,2]. In the past decade, there was an increase in the incidence of maternal obesity during pregnancy, which can be explained by lifestyle changes (high-caloric food intake and reduced physical activity). Studies with rodent models have demonstrated an association between hypercaloric diet exposition during gestation, and a higher risk to development of adverse outcomes in the offspring, for instance, congenital anomalies, and of developing obesity and metabolic syndrome [2–5]. On the other hand, maternal undernutrition during the periconceptional period and pregnancy also causes metabolic dysfunction resulting in increased adiposity, reduced fetal capacity to modulate muscle glucose uptake, and alterations in fetal pancreatic function and insulin signaling [1,6,7].

Caloric restriction in adulthood has been described as a protective approach which contributes to promote longevity by providing antioxidant and anti-inflammatory pathways activation [8–10], improvement of glucose metabolism [11] and remodeling of adipose tissue [12]. However, it is poorly known whether caloric restriction during gestation and lactation is beneficial or detrimental for the development of the offspring.

Since fetal programing has been widely associated with nutritional interventions, we aimed to investigate whether behavior and metabolism of the progeny may be affected by different maternal diets (hypercaloric or restrictive diets) during gestation and lactation. Since the

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benefits of caloric restriction are still controversial, we also evaluated if caloric restriction in the offspring can be protective and able to reverse an altered metabolic programing caused by ingestion of hypercaloric diet by the dams. Thus, we administered three different diets during gestation and lactation: control, restrictive diet and hypercaloric diet. Then, we investigated metabolic and behavioral parameters on the offspring following control and restrictive diet.

2. Methods

2.1. Animals

Ten male and 30 female BALB/c albino mice (60 days old, weighing between 18 and 24 g) were obtained from the Animal Housing Facility of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) and properly maintained under controlled temperature (22 ± 2 °C), humidity (55 ± 5%) and luminosity (12 h light/dark cycle; lights on at 6 a.m.) conditions. All procedures were performed accordingly to the ethical rules established by the guidelines of the *Brazilian Society for Neuroscience and Behavior* and the *Guide for the Care and Use of Laboratory Animals* of the Institute for Laboratory Animal Research. The Institutional Animal Care and Use Committee of UFCSPA (#388/15) approved this study and all efforts were made in order to minimize the number of animals used and its suffering.

2.2. Experimental procedures

The adult females were divided into three groups (n = 10 per group) and individually housed with water *ad libitum*. The control group (CONT) received standard mice chow (Nuvital, Curitiba, Brazil) *ad libitum*, with a total energy content of 3.4 kcal/g (63% carbohydrate, 26% protein, 11% fat); the restrictive diet group (RD) had a 30% lower feed availability, adjusted according to the consumption of CONT group; and the hypercaloric diet group (HD) received a special chow (Pragsoluções Biociências, Jaú, Brazil) *ad libitum*, with total energy content of 4.9 kcal/g (40.3% carbohydrate, 11.3% protein, 48.3% fat).

Females received the diets during 25 days for adaptation, after that, they were housed with males for mating during 7 days, in a ratio of 3 females:1male. The mating was confirmed by checking if the copulatory plug was present in the vaginal smear. When pregnancy was confirmed, females were housed in separate cages until delivery. If pregnancy was not confirmed, females were re-housed with males. On the first post-partum day (PPD), the litters were standardized to 6 pups (3 males and 3 females, whenever possible).

2.2.1. Offspring

After weaning (21 days old), the female offspring of CONT, RD and HD dams were randomly divided into two groups: fed with standard chow (CONT) or a restrictive diet (RD). Then, six experimental groups were generated: CONT/CONT (maternal standard chow/offspring standard chow), CONT/RD (maternal standard chow/offspring restriction), RD/CONT (maternal restriction/ offspring standard chow), RD/RD (maternal restriction/offspring restriction) HD/CONT (maternal hypercaloric/offspring standard chow), and HD/RD (maternal hypercaloric/offspring restriction). The offspring diet (CONT or RD) was maintained along all their lives. The behavioral test was assessed at 80 days old and euthanasia followed by tissue and blood collection was performed at around 100 days old.

2.3. Behavioral tests

Around 70 days of age, the estrous cycle of the offspring was assessed, by vaginal smear, in order to submit all animals to the behavioral tests in the diestrus period. The behavioral tests were performed and recorded during the light cycle (from 08:00 to 11:30). First the animals were submitted to the open field test, one week later they were analyzed in the elevated plus maze and after another week of interval they were submitted to the novel object recognition test. After each animal submitted to the specific assay, the apparatus was washed with alcohol 50% and dried, in order to avoid any smell recognition. The recordings were analyzed using "*The Observer*" software (Noldus®, Holland).

2.3.1. Open field test (OF)

The test was performed in a wooden box with 100 cm^2 square of open-field, surrounded by walls 30 cm high, whose floor was divided into 25 squares marked on the floor, being 16 in the lateral zone and 9 in the central zone of the arena. The experimental procedure consisted of placing the animal in a corner of the open field (randomly chosen) and monitoring its movement. Then, parameters such as frequency and duration in the lateral or central zone of the arena were measured. The innate fear behavior was verified by the evaluation of the frequency of locomotion in the central zone of the OF [13,14].

2.3.2. Elevated plus maze test (EPM)

The EPM consists of an elevated apparatus above the floor (50 cm), with two open arms (25 × 5 cm) across from each other and perpendicular to other two closed arms (25 × 5 cm, with walls 13 cm high), with a central open area platform. The animals were individually tested, being placed in the center zone with its head facing to the open arm of the apparatus, to asses: the percentage of entries into the open arms [100 × open/(open + enclosed)], the percentage of time spent in the open arms [100 × open/(open + enclosed)], and the number of head-dipping, as previously described in literature [14,15].

2.3.3. Novel object recognition test (NOR)

Animals were habituated in a plastic box (30.5 cm × 19.8 cm × 13.4 cm) during 10 min. On the next day, they were individually introduced into the same arena containing two identical objects that they could freely explore for 5 min. Exploration was defined by the amount of time spent actively sniffing or interacting with the object at a distance no > 2 cm. Three hours later, mice were placed back into the same arena containing two objects, one of them being the same previously presented (familiar object), and the other being a new and unknown (novel object). This test session evaluated short-term memory. Recognition index was calculated by time spent exploring the new object/total exploration time multiplied by 100 [16].

2.4. Tissue and blood collection

Around 100 days old, the females were anesthetized and euthanized with ketamine (90 mg/kg, DOPALEN, Vetbrands, São Paulo, Brasil) and xilazyne (10 mg/kg, ANASEDAN, Ceva, São Paulo, Brasil), for the collect of biological material. The trunk blood was collected in sterile tubes, centrifuged for 10 min, at 1500*g* and 4 °C. Serum was separated and stored at −80 °C for posterior analysis.

Hepatic and visceral adipose tissues (from stomach, spleen, pancreas, small and large intestines, and reproductive tract) were dissected, weighed and formalin-fixed for histological analysis.

2.4.1. Serum analysis

Insulin and leptin serum concentrations were measured in duplicate using mouse-specific ELISA commercial kits (SIGMA-ALDRICH™ Saint Louis, MO, USA), according to the manufacturer. Glucose level was determined using standard enzymatic method with an automatic analyzer (BS120 Chemistry Analyzer, Mindray, Shenzhen, China).

2.4.2. Histological analysis and scoring system definition

After tissue collection, samples were fixed in 4% buffered paraformaldehyde for at least 24 h, histologically processed with ethanol/xylene overnight embedded in paraffin. Tissue sections of 4 μm were stained with hematoxylin-eosin. For morphometric analysis of adipose

tissue, micrographs of three independent fields of histological section were obtained with a 20× objective, using light microscopy (Olympus BX-61, Japan) for each animal. Images were treated using the open free access Fiji software [17] to better define all limits of adipocytes, and the area of each cell was measured using Image Pro Plus 6.0 software (IPP6 – Media Cybernetics, Silver Spring, MD, USA) in a blinded manner to the experimental groups. Around 1000 to 1500 cells were assessed in each experienced group, followed by an exclusion filtering of adipocytes presenting area below 350 μm^2, once they may be a mixture of adipocytes and stromal cells [18]. The average area ± standard deviation was firstly calculated among filtered adipose cells of each animal, followed by an average calculation among all average area of animals of each tested group.

The hepatic tissues were analyzed manually and classified according a semi-quantitative analysis by an experienced pathologist, blinded to experimental groups, using light microscopy (Olympus BX-61, Japan). In accordance with the histological features, they were evaluated about three broad categories: steatosis, inflammatory lymphocyte infiltration and ballooning. The evaluation system was based on [19].

2.5. Statistical analysis

Data were expressed as mean ± S.E.M. The Shapiro-Wilk test was used to assess data normality. The results were analyzed by two-way (ANOVA), followed by Bonferroni *post hoc* test. Statistical analyses were made using GraphPad Prism 6, (La Jolla, USA). In all cases, differences were considered significant when $p < 0.05$.

3. Results

An effect of maternal diet was found on the glucose serum concentration [$F_{(2,24)} = 13.64$; $p = 0.001$, Fig. 1A], indicating that maternal HD induces a hyperglycemic profile in the offspring, irrespective of its diet pattern (CONT or RD). There was an increase in glucose concentration in HD/CONT when compared to CONT/CONT ($p < 0.01$) and RD/CONT ($p < 0.05$). HD/RD glycemia was also higher than CONT/RD ($p < 0.01$) and RD/RD ($p < 0.01$).

Fig. 1B shows the insulin levels in the serum of CONT and RD-fed offspring. An effect of maternal diet was observed on insulin levels [$F_{(2,27)} = 6.76$; $p = 0.004$]. Post-hoc test showed that HD/RD had a higher insulin serum concentration than RD/RD ($p < 0.05$).

Leptin serum concentration (Fig. 1C) showed a maternal diet [$F_{(2,27)} = 9.07$; $p = 0.001$] and also an offspring diet [$F_{(1,27)} = 5.75$; $p = 0.02$] effect. Leptin was increased in HD/CONT in comparison to CONT/CONT ($p < 0.05$) and RD/CONT ($p < 0.001$). RD-fed offspring showed an increase in leptin levels in CONT/RD compared to RD/RD ($p < 0.01$).

In the histological analysis of the adipose tissue (Fig. 2), it was found a maternal diet [$F_{(2,66)} = 3.83$; $p = 0.02$] and an offspring diet [$F_{(1,66)} = 8.32$; $p = 0.005$] effect on the adipocyte area. RD/CONT showed a higher adipocyte area in comparison with HD/CONT ($p < 0.05$) and CON/CONT ($p < 0.001$). HD/CONT was also higher than CONT/CONT ($p < 0.05$).

On the other hand, histological analysis of the liver did not show pathological hepatic alterations such as steatosis, inflammatory lymphocyte infiltration and ballooning (data not shown).

In the evaluation of the locomotor activity and innate fear behavior in the OF test, it was observed a maternal diet effect on the frequency of locomotion in the lateral area [$F_{(2,64)} = 10.75$; $p < 0.0001$, Fig. 3A]. In the post-hoc test, we found a decrease in this frequency in RD/CONT compared to CONT/CONT ($p < 0.05$) and HD/CONT ($p < 0.001$). RD/RD also showed a decrease in lateral locomotion in comparison to HD/RD ($p < 0.05$). Regarding frequency of locomotion in the central area, there was also an effect of maternal diet [$F_{(2,68)} = 4.61$; $p = 0.013$, Fig. 3B], showing that RD-fed dams had decreased this parameter. However, no differences between groups were found in the post-hoc test.

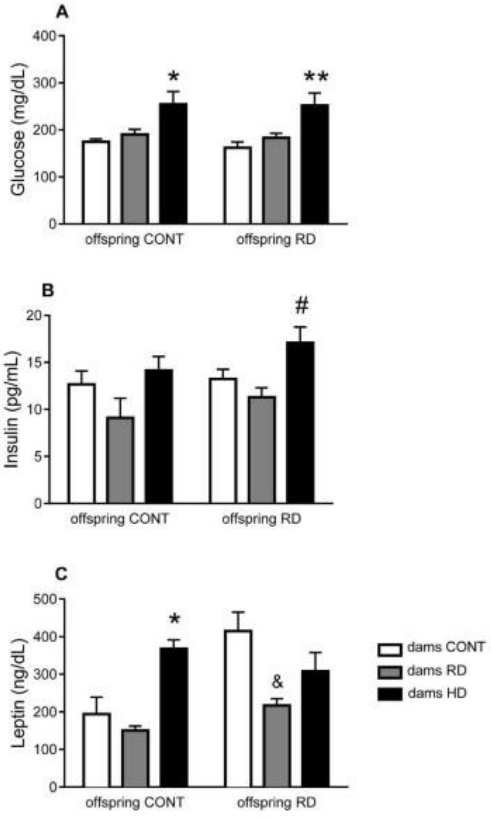

Fig. 1. Metabolic markers. (A) Glucose serum concentration (mg/dL) in offspring CONT/CONT (n = 6), RD/CONT (n = 6), HD/CONT (n = 6), CONT/RD (n = 4), RD/RD (n = 5) and HD/RD (n = 5). (B) Insulin serum concentration (pg/mL) in offspring CONT/CONT (n = 6), RD/CONT (n = 6), HD/CONT (n = 7), CONT/RD (n = 6), RD/RD (n = 6) and HD/RD (n = 7); (C) Leptin serum concentration (ng/dL) in offspring CONT/CONT (n = 6), RD/CONT (n = 7), HD/CONT (n = 8), CONT/RD (n = 7), RD/RD (n = 8) and HD/RD (n = 6). Data expressed as mean ± SEM ($p < .05$). * indicate significance in relation to CONT/CONT and RD/CONT; ** indicate significance in relation to CONT/RD and RD/RD; # indicate significance in relation to RD/RD; & indicate significance in relation to CONT/RD.

Table 1 shows the results of anxiety-like behavior and recognition memory, evaluated using EPM and NOR tests, respectively. No significant difference was found in the percentage of entries into the open arms and in the percentage of time spent in the open arms, as well as, in the number of head-dipping in all groups. Besides, data from the NOR also showed no significant differences in the recognition index at 3 h after habituation among the analyzed offspring groups.

4. Discussion

Previous studies using animal models have reported that consumption of a high fat diet during pregnancy and lactation may cause adverse metabolic consequences in the progeny (males and females),

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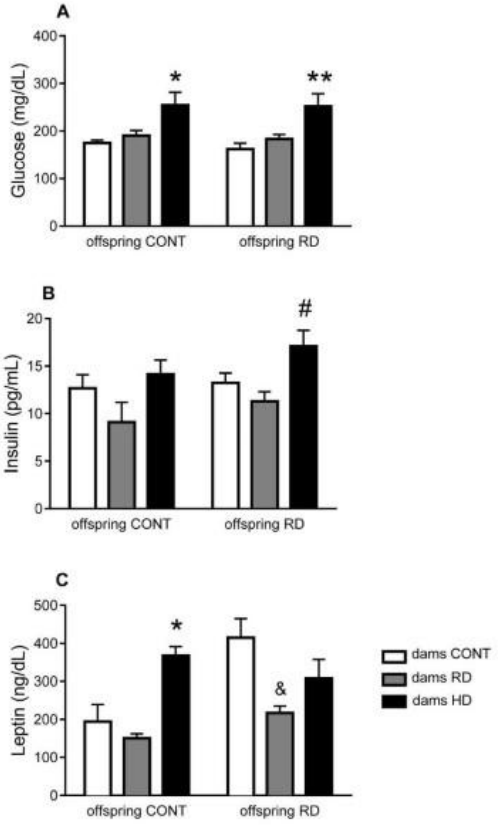

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4. Discussion

Previous studies using animal models have reported that consumption of a high fat diet during pregnancy and lactation may cause adverse metabolic consequences in the progeny (males and females),

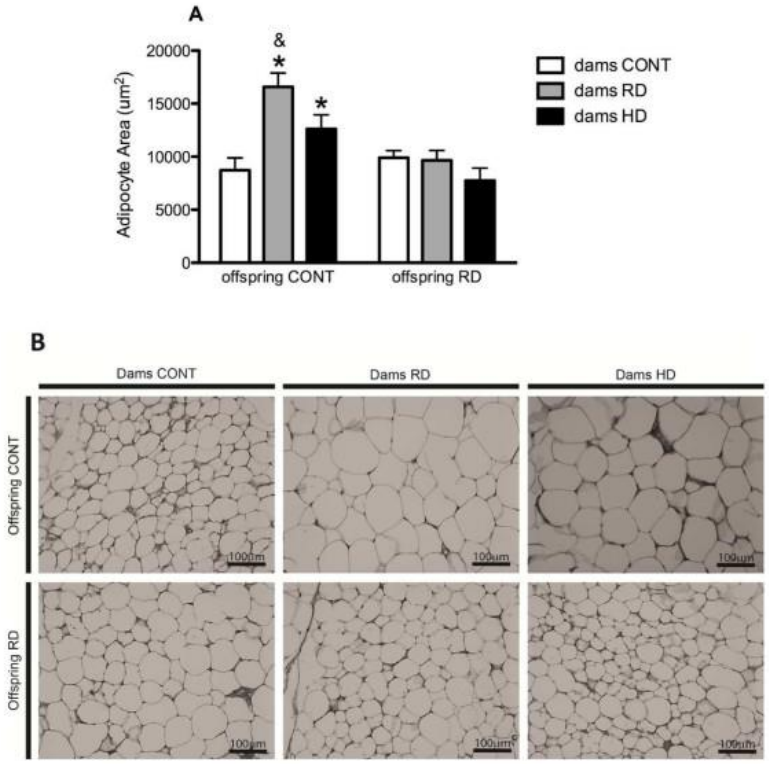

Fig. 2. Morphometric analysis of adipose tissue. (A) Adipocyte area (μm^2) was measured in offspring CONT/CONT (n = 6), RD/CONT (n = 7), HD/CONT (n = 6), CONT/RD (n = 6), RD/RD (n = 5) and HD/RD (n = 6). (B) Representative micrographs of adipose tissue from each experimental group. Data expressed as mean ± SEM. * indicate significance in relation to CONT/CONT; & indicate significance in relation to HD/CONT.

which can arise from weaning to adulthood [20,21]. In the present study, we showed that glycemia and insulin levels in the offspring are increased by the maternal HD. In addition, the RD feeding intervention in the offspring starting soon after weaning was not sufficient to improve these parameters in the adulthood. On the other hand, leptin levels and adipocyte size are influenced for both maternal and offspring diets. Behavioral assessment showed again a maternal diet effect, in this case, maternal RD exposure led to lower locomotion activity and increased innate fear. Thus, despite many studies have reported the effects of maternal diet on the offspring physiology, here we also submitted the progeny to a diet intervention, *i.e.* RD.

Caloric restriction is able to reduce body weight and ameliorates insulin and glucose levels in male mice and human beings [11,22]. The main outcome of caloric restriction is the increase in longevity, and this anti-aging effect is associated with enhanced insulin sensitivity [11,23]. Several molecular mechanisms might explain the beneficial effects of RD on aging, including a decrease in free radical production and in the inflammatory processes, induction of cytoprotective responses to cellular stress, and stimulation of growth factors production [11,24]. However, in the present study, RD was not able to revert the HD maternal imprints on glucose and insulin plasma levels. These findings corroborate with similar studies showing that the progeny of dams fed obesogenic diets during pregnancy and lactation showed insulin resistance and glucose intolerance [3,4,25]. It is worth to mention that these studies did not evaluate RD in the offspring. Thus, we corroborate with previous findings showing that metabolism is highly susceptible to intrauterine programming, which reinforces the idea that epigenetic modifications exert a robust effect on the fetal environment, influencing the adult phenotype in multiple generations [3,26,27].

Leptin is an anorexigenic hormone produced by adipose tissue, thus its blood levels are decreased in food-restricted animals and usually increased when the adipose depots are elevated [28]. In the current study, leptin levels were influenced by both maternal and offspring diets, but it is clearly shown that offspring from RD-fed dams had a decreased leptin level. It was previously demonstrated that offspring from dams that received junk food showed higher leptin and insulin concentrations compared with their chow-fed counterparts [29]. Here, we not only addressed the maternal impact of the HD but also the capacity of RD-fed offspring to revert the epigenetic influence of maternal HD. In this context, we showed that HD/CONT offspring had a higher leptin concentration than CONT/CONT and RD/CONT, which highlights the importance of this programming to modulate metabolic responses of the progeny. A similar finding was showed by Gali et al. (2018) that evaluated in male the effect of maternal high-fat diet immediately after weaning. They demonstrated a hypermethylation in the anorexigenic Pomc gene in the hypothalamus, which interferes in leptin

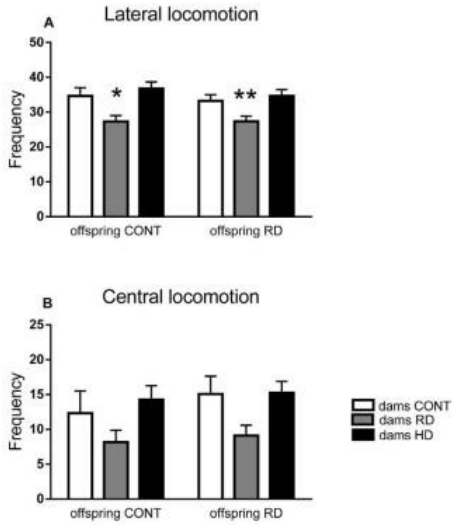

Fig. 3. Locomotor activity in the open field test. (A) Frequency in lateral locomotion was analyzed in offspring CONT/CONT (n = 12), RD/CONT (n = 13), HD/CONT (n = 14), CONT/RD (n = 9), RD/RD (n = 9) and HD/RD (n = 14). (B) Frequency in central locomotion. Data expressed as mean ± SEM. * indicates significance in relation to CONT/CONT and HD/CONT; ** indicates significance in relation to HD/RD.

signaling. Thus, the increase in leptin levels is not correlated with a satiety response. These results reinforce that maternal perinatal environment programs long-term changes in metabolic homeostasis [30].

In the present study, histological analysis of the adipose tissue showed a significant increase in the adipocyte area of RD/CONT and HD/CONT offspring compared to CONT/CONT group, showing that maternal diet interventions affect adipocyte area in the adulthood. Narita et al. (2018) showed in male Wistar rats that caloric restriction has a different impact on adipose tissue stores distributed throughout the body, including adipocyte size, lipid metabolism, signaling pathways, adipocytokine secretion profile. Differently from the study cited, we analyzed the visceral adipose tissues of the females [12]. A possible explanation is that not only HD leads to increased adiposity in the offspring, but maternal undernutrition during the periconceptional period and pregnancy, also results in increased adiposity in the progeny as described elsewhere [1,6,7]. However, when offspring was submitted to RD, adipocyte area did not change irrespective of the maternal diet. Taken together, these findings show that adipocyte area is influenced by maternal diet, but RD in the offspring is able to overcome the effect of hyper or hypocaloric nutrition during pregnancy and lactation.

It is known that HD promotes liver dysfunction such as Nash (for review see [31]). Thus, in the present study, the lack of hepatic pathological findings may be related to the fact that the offspring did not received HD, and hepatic dysfunction is less influenced by epigenetic mechanisms in comparison to other metabolic parameters. It was previously demonstrated that caloric restriction protects the liver from oxidative stress and inflammation [32]. However, caloric restriction during pregnancy does not cause hepatic steatosis in the male Wistar offspring [33] which is in accordance with our results.

It is well demonstrated that obesity promotes cognitive decline and it is associated with the development of psychiatric disorders, such as depression [34]. On the other hand, caloric restriction ameliorates neurological function [9,35]. Substantial evidences indicate that diet-induced obesity leads to deficits in learning and memory processes in male rodents [36,37]. Almeida-Suhett et al. (2017) found that high-fat diet-fed mice presented an impairment in working memory. Here, we used object recognition test to assess recognition memory. Neither maternal or offspring diet intervention interfered in recognition memory. However, a previous study demonstrated that caloric restriction during pregnancy leads to a worse performance of the offspring in the object recognition test. Nevertheless, it was seen only at 3 weeks of age, and adult mice did not show significant differences, which is in accordance with our results [38]. Thus, even the absence/lack of memory impairment seen in adult mice does not exclude the possibility of a detrimental effect of the maternal diet on cognition in animals of different ages.

Based on the need to conserve energy, it would be expected that caloric restriction promotes a reduction in the physical activity of animals. Unexpectedly, many studies have reported that caloric restriction actually causes increased levels of activity [39,40]. However, we have found a maternal diet effect on the locomotor activity in the OF test, and the offspring of the RD-fed dams showed decreased locomotion in the lateral and central area of the apparatus. Even 1 week of caloric restriction prior to conception caused a decrease in the time spent in the central zone of the OF, showing an anxiogenic effect on adult male offspring [41] which is in agreement with our data. It was also shown that adult male offspring of dams subjected to RD exhibited increased fear reactivity to a predator odor, with no alteration in anxiety-like behavior as measured in the EPM and OF [42]. We found similar results in the EPM, with no differences between groups. It is interesting to note that paternal diet can also modify the behavioral responses in the adulthood. The offspring of males Wistar that received RD exhibited a reduction in anxiety-like behavior, as evidenced by the increase in time spent in the open arms of the EPM and a higher frequency of entries into the central area of the OF [43].

Table 1
Anxiety-like behavior and recognition memory in adult female offspring.

	CONT/CONT	RD/CONT	HD/CONT	CONT/RD	RD/RD	HD/RD
Elevated Pluz Maze						
% time spent into the open arms	43.00 ± 8.69	46.00 ± 7.04	41.10 ± 7.79	57.10 ± 8.88	44.62 ± 5.21	52.90 ± 8.34
% entries into the open arms	47.88 ± 1.70	44.78 ± 3.12	45.10 ± 2.83	49.50 ± 3.47	50.10 ± 5.05	48.83 ± 2.20
Number head-dipping	15.14 ± 2.95	9.70 ± 1.41	8.90 ± 1.66	9.6 ± 2.00	14.75 ± 1.91	10.78 ± 1.47
Object Recognition						
Recognition index (3 h)	63.67 ± 3.02	62.00 ± 3.77	62.46 ± 1.49	56.10 ± 3.89	57.63 ± 3.03	62.90 ± 2.33

Data expressed as mean ± S.E.M.
Two-way ANOVA, followed by Bonferroni post-hoc test.
Abbreviations: CONT/CONT (maternal standard chow/offspring standard chow), CONT/RD (maternal standard chow/offspring restriction), RD/CONT (maternal restriction/ offspring standard chow), RD/RD (maternal restriction/offspring restriction) HD/CONT (maternal hypercaloric/offspring standard chow), and HD/RD (maternal hypercaloric/offspring restriction).

Considering the findings in this study, we conclude that the maternal diet, during gestation and lactation, influences metabolic and behavioral responses of the offspring in a long-term. In addition, maternal RD appears to exert a protective metabolic effect. However, in the OF test the locomotion activity was decreased in the offspring of RD-fed dams. Thus, despite RD provides a beneficial response in metabolic parameters, the effects of RD on behavior need more investigation.

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4.2 Artigo 2: *Maternal diet causes inflammation of adipose tissue and oxidative stress of liver tissue in mothers and female offspring*

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ABSTRACT

During pregnancy, physiological changes occur in the maternal organism, generating an increase in the need for essential nutrients, in order to ensure adequate fetal growth and development. During pregnancy, the fetus is able to react to adverse nutritional situations for its normal development and to promote molecular, cellular and biochemical adaptations. In this context, the goal of the present study was to analyses how different maternal diets, standard (CONT), hypercaloric (HD) and restrictive (RD), offered during gestation and lactation periods affects dams and their female offspring, assessing the histochemical characteristics of the liver and adipose tissue related to inflammation and the oxidative stress. The female offspring were weaned at 21 days and randomly divided into two groups that received a standard or restriction diet, forming the following groups: CONT/CONT, CONT/RD, RD/CONT, RD/RD, HD/CONT and HD/RD. Our results of histological evaluation of dams' liver tissue demonstrated that HD dams group presented diagnosis of non-alcoholic steatohepatitis (NASH) and increase in tumor necrosis factor alpha (TNF-α) concentrations when compared to the RD and CONT dams, indicating pro-inflammatory state. In relation to reactive oxygen species (ROS) formation, we did not observe any difference; however, malondialdehyde (MDA) formation and catalase (CAT) activity were higher in the liver of HD dams when compared to the CONT and RD group respectively. In the adipose tissue, RD-dams showed an increase in inflammatory infiltrate when compared to CONT group, evidencing changes caused by restrictive diet. In the offspring that received RD throughout their lives, we verified a decrease in the inflammatory infiltrate in relation to the offspring that were fed a standard diet. On the other hand, HD/CONT showed a higher level of macrophage infiltration, indicating that the maternal diet associated with the diet consumed throughout life affects the metabolism of adipose tissue in adulthood.

Furthermore, we also verified the effect of maternal diet on stress parameters such as superoxide dismutase (SOD) activity was increased in RD/RD compared to HD/RD.

In summary, we demonstrate that both a hypercaloric diet and caloric restriction, during pregnancy and lactation, alter the adipose and liver tissues of mothers. Furthermore, maternal diet negatively impacts the parameters studied in the offspring's adipose tissue but does not cause any liver damage in these female in adult life.

Keywords: gestation, caloric restriction, hypercaloric diet, oxidative stress, inflammation, adipose tissue, liver.

1 INTRODUCTION

During pregnancy, physiological changes occur in the maternal organism, generating an increase in the need for essential nutrients, in order to ensure adequate fetal growth and development. During pregnancy, the fetus is able to react to adverse nutritional situations for its normal development and to promote molecular, cellular and biochemical adaptations. Prenatal nutritional programming has been analyzed experimentally in a number of species using a range of techniques to manipulate the fetal nutrient supply (Armitage et al, 2004). Nowadays, it is already known that nutrition during fetal life affects metabolic, cardiovascular, and endocrine function in the mature individual (Ousey et al, 2008). This led to the concept that tissues are programmed in utero during critical periods of development by the availability of nutrients (Hoet & Hanson, 1999).

Previous studies (Gali et al, 2018; Almeida-Suhett et al, 2019) with rodents demonstrated that the consumption of hypercaloric diets during pregnancy and lactation causes an increased risk of developing diseases in the offspring, such as metabolic syndromes, congenital abnormalities, and obesity.

In contrast, maternal undernutrition during the whole of pregnancy, or even for discrete periods of gestation, has been shown to affect adversely pancreatic endocrine function, glucose tolerance and insulin sensitivity in the adult offspring of rats and guinea pigs (Bloomfield et al, 2003; Gardner et al, 2005).

Dipe (2009) demonstrated in Wistar rats that food restriction induced a decrease in maternal gestational body weight gain. Despite, there were no malformations, the food restriction also promotes lower birth weight and significant changes in the immune system of the offspring (Dipe, 2009).

The adipose tissue and liver is an essential organ of inflammatory events in obesity. In addition to containing, several cell types, all contributing to the inflammatory response during obesity (Van der Heijden et al, 2015). The adipose tissue is responsible for regulating fat mass and nutrient homeostasis. The adipocytes mediate the inflammatory response through the secretion of cytokines, adipokines and chemokines that enhance the recruitment of immune cells, especially macrophages to the adipose tissue. These adipose tissue macrophages once activated can spread the inflammatory state and interfere with insulin sensitivity in insulin target cells (Van Der Heijden et al, 2015).

Although, excessive nutrient intake over a long period of time results in metabolic inflammation associated with adipocyte dysfunction, expansion of metabolic inflammation in obesity is not restricted to the adipose tissue. The liver is also a strong contributor to the development of this type of inflammation (Bloomfield et al, 2003; Gardner et al, 2005).

In contrast, studies show an association between protein restriction (during critical periods of development) and morphological changes in the liver, changes in triglycerides and changes in the activity of fatty acid metabolism enzymes (Simões Alves et al, 2019). Study previous (Simões Alves et al, 2019) demonstrated that one of the main causes of metabolic disorders, such as non-alcoholic fatty liver disease (NAFLD), hepatic steatosis and type 2 diabetes, is associated with mitochondrial dysfunction and oxidative stress.

In this context, we investigated the consequences of the restriction or excess of nutrients, during pregnancy and lactation, as well as monitoring the offspring who also had a restriction of nutrients throughout their lives. Thus, the aim of this study was to analyze the effects of the diet interventions in the dams and their offspring, assessing the histochemical characteristics of the liver and adipose tissue related to inflammation, and the oxidative stress.

2 METHODS

2.1 Animals

Ten male and 30 female BALB/c albino mice (60 days old, weighing between 18-24 g) were obtained from the Animal Housing Facility of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) and properly maintained under controlled temperature (22 ± 2 °C), humidity (55 ± 5%) and luminosity (12 h light/dark cycle; lights on at 6 a.m.) conditions. All procedures were performed accordingly to the ethical rules established by the guidelines of the *Brazilian Society for Neuroscience and Behavior* and the *Guide for the Care and Use of Laboratory Animals* of the Institute for Laboratory Animal Research. The Institutional Animal Care and Use Committee of UFCSPA (#388/15) approved this study and all efforts were made in order to minimize the number of animals used and its suffering.

2.2 Experiment 1: Analyses in the dams

Adult females were separated in three different groups (n=10 per group) and individually housed with water *ad libitum*. To the control group (CONT) was administrated a standard mice chow (Nuvital, Curitiba, Brazil) *ad libitum*, with a total energy content of 3.4 kcal/g (63% carbohydrate, 26% protein, 11% fat); the restrictive diet group (RD) had 30% reduction in the standard chow amount, compared according to the consumption of CONT group; and the hypercaloric diet group (HD) was fed with a special chow (Pragsoluções Biociências, Jaú, Brazil) *ad libitum*, with total energy content of 4.9 kcal/g (40.3% carbohydrate, 11.3% protein, 48.3% fat).

Diet adaptation lasted 25 days for the female mice, after they were housed with males for mating for 7 days, in a ratio of 3 females: 1 male. Pregnancy was confirmed

checking the copulatory plug in the vaginal smear. When pregnant they were housed in separate cages until delivery. If pregnancy was not confirmed, they were re-housed with males. On the first postpartum day (PPD), litters were standardized at 6 pups (3 males and 3 females, whenever possible).

2.2.1 Tissue collection

After weaning, the dams were anesthetized and euthanized with ketamine (90 mg/kg, Dopalen®, Vetbrands, São Paulo, Brasil) and xilazyne (10 mg/kg, Anasedan®, Ceva, São Paulo, Brasil). Visceral adipose and hepatic tissues were collected for further analysis.

2.2.2 Histological analysis and scoring system definition

In the liver, four-micrometer sections of formalin-fixed and paraffin-embedded tissue slices were stained with hematoxylin-eosin. A pathologist, blinded to experimental protocol, analyzed all liver fragments using light microscopy. The histological features were grouped into three broad categories: steatosis, inflammatory lymphocyte infiltration and ballooning. These features were used for diagnostic categorization of NASH according to a scoring system described by Kleiner et al. (2005).

2.2.3 Immunohistochemistry for F4/80

The adipose tissue sections were incubated with the primary antibody Rat anti Mouse F4/80 (Monoclonal - Clone Cl:A3-1) (MCA497; AbD Serotec); and later with the secondary antibody Chicken Anti-Rat IgG H&L (HRP) (ab112448; Abcam). Immunoreaction was developed using a solution of DAB (3,3′-diaminobenzidine tetrahydrochloride; Sigma-Aldrich), and slides counterstained with hematoxylin.

Microscopic analysis was performed in 5 fields for macrophage counting, and the staining intensity was assessed in three categories: absent, weak, or strong.

2.2.4 Oxidative stress parameters

Liver tissues were vigorously vortexed in Tris-HCl lysis buffer (10 mM; pH 7.4 with PMSF 1%) and then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatants were used for 2′,7′-dichlorofluorescein (DCF) and for thiobarbituric acid-reactive substances (TBARS) assays, and for the activities of SOD and CAT.

2.2.4.1 DCFH-DA measurement

This method is based on the deacetylation of the DCFH-DA probe and its subsequent oxidation by reactive oxygen species into a highly fluorescent compound DCF. For the DCHF-DA assay, phosphate-buffered saline (PBS; pH 7.4), 500 μM DCFH-DA and tissue suspension (total volume 200 μL) were incubated in 96-well dark-plates at 37 °C for 30 min. Fluorescence was measured with a SpectraMax M2e Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The excitation and emission wavelengths were 490 nm and 525 nm, respectively. All experiments were carrying out in a dark room to prevent oxidation of the DCHF-DA.

2.2.4.2 Thiobarbituric acid-reactive substances assay

To analyze the MDA concentration in the samples, the tissue homogenate was combined with TCA-TBA-HCl and mixed thoroughly, then, the mixture was heated at 100 for 15 min. After cooling, the precipitate was removed by centrifugation at 1000 rpm for 5 min. The of the supernatant was measured at 532 nm.

2.2.4.3 Superoxide dismutase activity

After treatment, cells were lysed by using RIPPA buffer with protease inhibitor and then protein content was measure by Folin phenol method (Lowry et al, 1951). The SOD activity was determined according to the method of Misra and Fridovich (1972) and the results were expressed as units of SOD per mg of protein. Briefly, to 10 µL of cell suspension was added 170 µL of 50 mM glycine buffer (pH 10.2) and 10 µL of 10 mM catalase, following by 10 µL of 2mM epinephrine. Then, the absorbance was immediately recorded every 36 s for 15 min at 480 nm in SpectraMax M2 e Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California). One unit of SOD is defined as the amount of enzyme that inhibits the rate of adrenochrome formation in 50%.

2.2.4.4 Catalase activity

The CAT activity was determined by the hydrogen peroxide (H_2O_2) decomposition rate, according to Aebi (1984) and the results were expressed as unites of CAT per mg of protein. Briefly, 40 µL of protein extract was added to 150 µL of potassium phosphate buffer (KH_2PO_4 50 mM, $Na_2HPO_4$50 mM, pH 7.0). Then, 10 µL of H_2O_2 (25 mM) was added and the absorbance was immediately recorded every 30 s for 5 min, using SpectraMax M2e Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California).

2.2.5 Enzyme-linked immunosorbent assay

Liver concentrations of interleukin 6 (IL-6) and TNF-α were analyzed by ELISA (Sigma-Aldrich), according to the manufacturer's instructions.

2.3 Experiment 2: Analyses in the offspring

After weaning, the female offspring of CONT, RD and HD dams was randomly divided into two groups: one fed with standard chow (CONT), and the other had a restrictive diet (RD). Thus, six experimental groups were generated: CONT/CONT (maternal standard chow/offspring standard chow), CONT/RD (maternal standard chow/offspring restriction), RD/CONT (maternal restriction/ offspring standard chow), RD/RD (maternal restriction/offspring restriction) HD/CONT (maternal hypercaloric/offspring standard chow), and HD/RD (maternal hypercaloric/offspring restriction). The offspring diet (CONT or RD) was maintained along all their lives. The euthanasia, followed by tissue collection, was performed when they were 100 days old using the mother protocol.

2.3.1 Immunohistochemistry for F4/80

In the adipose tissue, immunohistochemistry for F4/80 was performed in the same way as previously described for the dams.

2.3.2 Oxidative stress parameters

Liver tissue was processed in the same way as previously described for the dams, and the supernatants were used for DCF and for TBARS assays, and for the activities of SOD and catalase.

2.4 Statistical analysis

Data were expressed as mean ± S.E.M. The Shapiro-Wilk test was used to assess data normality. Results were compared by one-way ANOVA in the dams, and by two-way ANOVA for the offspring, followed by Tukey's multiple comparison test. Statistical

analyses were made using GraphPad Prism 6, (La Jolla, USA). In all cases, differences were considered significant when $p< 0.05$.

3 RESULTS

Histological evaluation of dams' liver tissue demonstrated the presence of low lobular inflammatory infiltrate, hepatocellular ballooning and mild steatosis in HD-fed mice (Figure 1A). Our findings are graded as 1 for inflammatory infiltrate, 2 for hepatocyte ballooning and 2 for steatosis. According Kleiner's system (Kleiner et al, 2005), scores ≥ 5 are correlated with a diagnosis of NASH, which is the case for HD-fed dams. CONT and RD groups did not show features of liver injury. The ELISA assay in the liver of the dams (Figure 1B) shows that IL-6 had no differences among the groups. There is an increase in TNF-α in HD dams compared to CONT and RD (p=0.046).

In the liver tissue of the dams, we did not find changes in ROS formation among groups as measured by DCF assay (Figure 2A). Nevertheless, MDA formation was higher in HD in comparison to RD group (Figure 2B). SOD activity decreased in RD mice compared to CONT group ($F(2,17) =5.337$; $p=0.01$) (Figure 2C). In contrast, CAT activity was significantly increased in HD when compared to CONT ($F(2,14) =4.391$; $p=0.04$) (Figure 2D). In addition, we analyzed the SOD/CAT ratio (Figure 2E). We found a decreased SOD/CAT ratio in the RD and HD groups in comparison to the CONT group ($F(2,15) =13.53$; $p=0.0007$).

The results for the offspring's livers demonstrate differences in the oxidative stress parameters. There was a maternal diet effect ($F(2,12)$ p=4.407), and Figure 3C shows that SOD activity was increased in RD/RD compared to HD/RD (p=0.036). There were no differences in the other parameters.

Figure 4 shows the immunohistochemistry for F4/80 in the dam's adipose tissue, it is demonstrated that RD dams showed a significant increase in the macrophage population in the adipose tissue when compared to CONT dams. The analysis of macrophage population from adipose tissue of the offspring is shown in Figure 5, and we verified interaction between the dam's and offspring diets. The HD/CONT showed increased macrophage infiltrate in the adipose tissue when compared to CONT/CONT and RD/CONT. The RD/RD show a decrase in macrophage infiltrate in adipose tissue when compared to CONT/RD.

4 DISCUSSION

Gestation is a period when oxidative stress is naturally increased. Also, obesity is characterized by an imbalance in the oxidant-antioxidant parameters (Fleeman, et al, 2005). When pregnancy is accompanied by obesity, the oxidative stress can develop uncontrollably, a condition that may affect negatively the mother and offspring health. However, caloric restriction can generate a protective function, as it enhances cellular autophagy, reduces inflammation and oxidative stress, and consequently, rises longevity (López-Lluch & Navas, 2016).

It is known that HD promotes liver dysfunction such as Nash (for review see Kucera et al, 2014), as it was previously demonstrated that caloric restriction protects the liver from oxidative stress and inflammation (Mohammadi et al, 2014). As prior described, oxidative stress can be involved in the development of metabolic disorders, such NAFLD, steatosis and type 2 diabetes, associated with mitochondrial dysfunction (Taylor et al, 2007).

In our findings HD-fed dam's hepatic tissue had low lobular inflammatory infiltrate, hepatocellular ballooning and mild steatosis, according to Kleiner's system (Kleiner et al, 2005), presenting scores correlated with a diagnosis of NASH. Furthermore, an increase in TNF-α in the liver of HD dams, but not in the IL-6, indicate pro-inflammatory state as a result of hyipercaloric diet. Moreover, we did not find changes in ROS formation in liver of the different groups of dams. However, MDA formation, and CAT activity were higher in HD group of the dams when compared to CONT and RD group respectively. Also, HD and RD groups showed a reduced SOD/CAT ratio in the liver.

Previously it was reported that the consumption of a hypercaloric diet increases fatty acids accumulation in the liver, which favors lipid peroxidation, contributing to the alteration in the oxidative-antioxidant balance (Fowden & Forhead, 2004). ROS and lipid peroxidation products disturb the respiratory chain in hepatocytes, leading to mitochondrial dysfunction that could cause apoptosis or necrosis, depending on the energy state of the cell (Taylor et al, 2007). In addition, the decrease in the antioxidant enzymes activity in the liver has been observed following high-fat diet and may be related to their inactivation elicited by the diet (Noeman et al, 2011). The increased CAT activity may occur to restrain oxidative stress, which may explain the absence of ROS generation measured by DCF assay, considering that our study was endpoint. However, we found an increased lipid peroxidation in HD-fed dams, and it was evidenced the development of liver injury in this group, which showed the hallmarks of NASH, such as inflammatory infiltrate, hepatocyte ballooning and steatosis. These findings reinforce the impairment of the hepatic function due to maternal HD. In addition, it was previously shown that maternal high-fat diet leads to an increased expression of pro-fibrogenic genes in the liver of the offspring (Wankhade et al, 2017). The presence of fibrosis or cirrhosis is associated

to an increased risk for the development of hepatocellular carcinoma, which is the most common type of liver cancer (Marengo et al, 2016).

On the other hand, caloric restriction may promote a detrimental effect on the antioxidant defenses of lactating mothers in the liver evidenced by the alteration of SOD/CAT ratio. Thus, caloric restriction does not seem to be a proper protective measure in the pre-gestational, pregnancy and lactation periods. The special need for nutrient intake seems to be a much more important factor in these periods (Fleeman, et al, 2005). In the liver, there was no oxidative stress, as evaluated by MDA formation and DCF assay in RD group, but indeed the reduced SOD/CAT ratio indicates that the hepatic tissue may be more susceptible to oxidative damage.

Despite HD-fed dam's preset liver inflammation and redox unbalance, offspring's liver did not present changes in oxidative stress parameters. These findings corroborate to our previous study that demonstrated no inflammatory evidence in offspring's liver (Fisch et al, 2019). It is known that high-fat diet cause liver inflammation and oxidative (Kucera et al, 2014; Mohammadi et al, 2014), but our results suggest that dams' diet did not affect hepatic tissue, at least not permanently, when offspring's diet is not hypercaloric.

High consumption of dietary fat contributes to obesity, which induces a permanent state of inflammation via the generation of white adipose tissue that secretes proinflammatory factors **(**Muñoz & Costa, 2013). Although, prolonged nutrient overload results in metabolic inflammation associated with adipocyte expansion and dysfunction, metabolic inflammation in obesity is not restricted to the adipose tissue; the liver is also a strong contributor to the development of this type of inflammation (Bloomfield et al, 2003; Gardner et al, 2005).

Health benefits of caloric restriction are known, extending lifespan and reducing the incidence of metabolic disorders. One of the hallmarks of caloric restriction is the significant reduction of white adipose tissue, and consequently metabolic changes in this tissue (Fabbiano et al, 2016). In our study, RD-dams showed an increase in inflammatory infiltrate in the adipose tissue when compared to CONT group, evidencing changes caused by restrictive diet. Different studies have demonstrated association of caloric restriction to browning of white adipose tissue and increased of M2 macrophage infiltration (Fabbiano et al, 2016; Miller at al, 2017; Corrales et al., 2018). Fabbiano et al. (2016) suggest that energy scarcity could be the physiological sign driving the browning of the white fat. Considering these, it is possible that RD led to adipose tissue browning in dams. However, more studies are necessary to identify the macrophages and other markers in adipose tissue of RD dams to confirm this hypothesis. We also observed an increase of inflammatory infiltrate in the adipose tissue of offspring from CONT dams exposed to RD, suggesting the same profile, increased M2 macrophage infiltrate. Besides, offspring RD exposure seems to be beneficial once RD/RD and HD/RD show lower inflammatory infiltrate than RD/CONT and HD/CONT. Still, HD/CONT presents the higher level of macrophage infiltration, indicating that exposure to intrauterine adverse environment impact offspring adipose tissue metabolism, corroborating to other studies that demonstrated increased inflammatory markers in adipose tissue of offspring when mothers received obesogenic diet (Umekawa et al, 2015; Dos Santos, et al., 2020).

Considering the findings in this study, we conclude that both a hypercaloric diet and caloric restriction, during pregnancy and lactation, alter the adipose and liver tissues of mothers. Furthermore, the mothers' unbalanced diet promote changes in the inflammatory profile of the offspring' adipose tissue, but they do not cause liver damage.

Acknowledgements

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FIGURES LEGENDS

Figure 1. Histological evaluation and ELISA assay in the liver of the dams: (A) representative hematoxylin-eosin staining of liver sections of control (CONT), caloric restriction (RD) and hypercaloric diet (HD)-fed dams (white arrows show ballooning-B and steatosis-S; scale bar=50μm); (B) enzyme-linked immunosorbent assay (ELISA) for interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-alpha) in the liver of CONT, RD and HD-fed dams. Data are expressed as mean ± SEM, compared by the one-way ANOVA, followed by Tukey's multiple comparison test. *p<0.05.

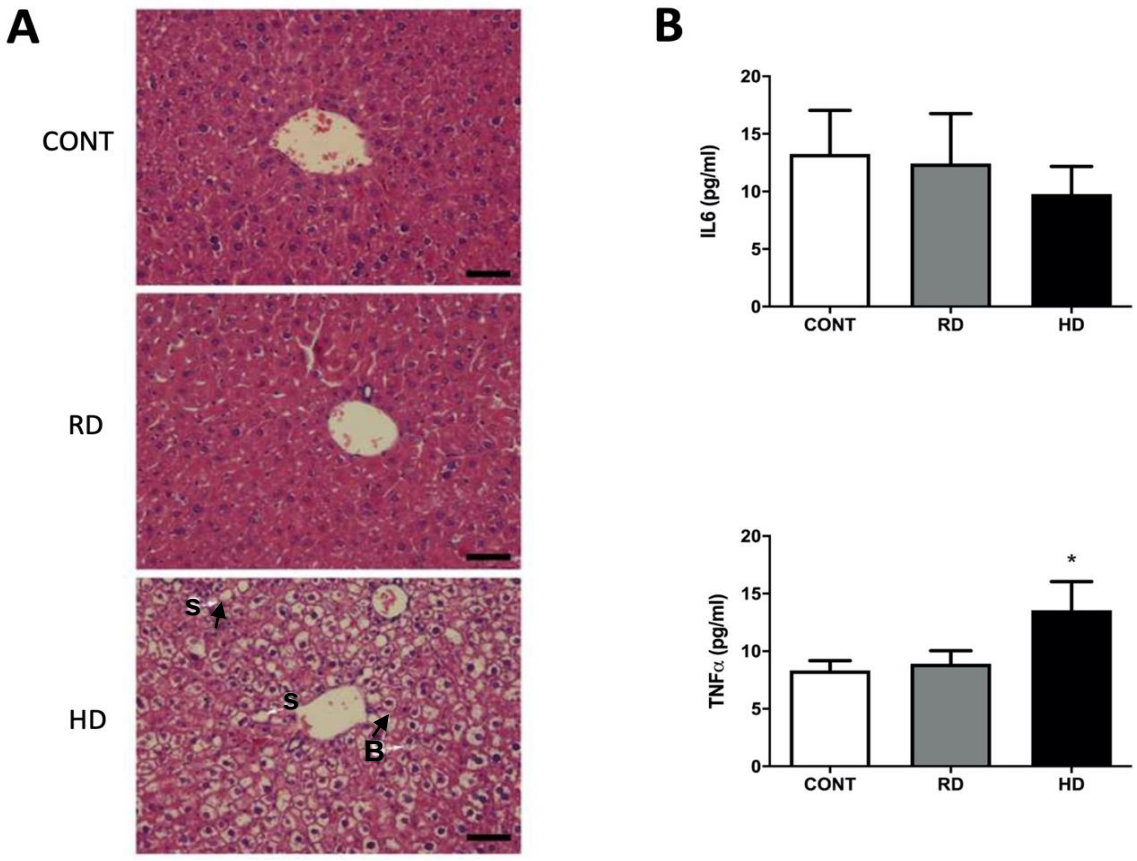
Figure 2. Oxidative stress parameters in the liver of the dams: 2′,7′-dihydrodichlorofluorescein (DCF) oxidation assay (A), malondialdehyde (MDA) formation (B), superoxide dismutase (SOD) (C) and catalase (CAT) (D) activities, and SOD/CAT ratio (E) of control (CONT), caloric restriction (RD) and hypercaloric diet (HD)-fed dams. Data are expressed as mean ± SEM, compared by the one-way ANOVA, followed by Tukey's multiple comparison test. *p<0.05 when compared to CONT; #p<0.05 when compared to RD.

Figure 3. Oxidative stress parameters in the liver of the offspring: 2′,7′-dihydrodichlorofluorescein (DCF) oxidation assay (A), malondialdehyde (MDA) formation (B), superoxide dismutase (SOD) (C) and catalase (CAT) (D) activities, and SOD/CAT ratio (E) of the following female offspring groups: CONT/CONT (maternal standard chow/offspring standard chow), CONT/RD (maternal standard chow/offspring restriction), RD/CONT (maternal restriction/ offspring standard chow), RD/RD (maternal restriction/offspring restriction) HD/CONT (maternal hypercaloric/offspring standard chow), and HD/RD (maternal hypercaloric/offspring restriction). Data are expressed as mean ± SEM, compared by the two-way ANOVA, followed by Tukey's multiple comparison test. *p<0.05.

Figure 4. Immunohistochemical analysis of macrophage population from adipose tissue of adult female mice. (A) Representative images of F4/80 staining in control (CONT), restrictive diet (RD) and hypercaloric diet (HD) groups at magnification 40x (1 – 3) and 100x (4 – 6), respectively (EVOS FL Auto 2 microscope, Thermo Fisher Scientific). (B) The data represent the average of F4/80 counts ± standard deviation of five samples per group and were analyzed by one-way ANOVA. *p<0.05 when compared to CONT.

Figure 5. Immunohistochemical analysis of macrophage population from adipose tissue of offspring CONT/CONT (maternal standard diet/offspring standard diet), CONT/RD (maternal standard diet/offspring restriction), RD/CONT (maternal restriction/offspring standard diet), RD/RD (maternal restriction/offspring restriction), HD/CONT (maternal hypercaloric/offspring standard diet), HD/RD (maternal hypercaloric/offspring restriction). (A) Representative images of F4/80 staining in offspring groups at magnification 40x and 100x (EVOS FL Auto 2 microscope, Thermo Fisher Scientific). (B) The data represent the average of F4/80 counts ± standard deviation of five samples per group and were analyzed by one-way ANOVA. * and # p<0.05 when compared to HD/CONT. & p<0.05 when compared to CONT/RD.

Figure 1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CONT
RD
HD
S
S
B
B
IL6 (pg/ml)
20
15
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CONT
RD
HD
TNFα (pg/ml)
20
15
10
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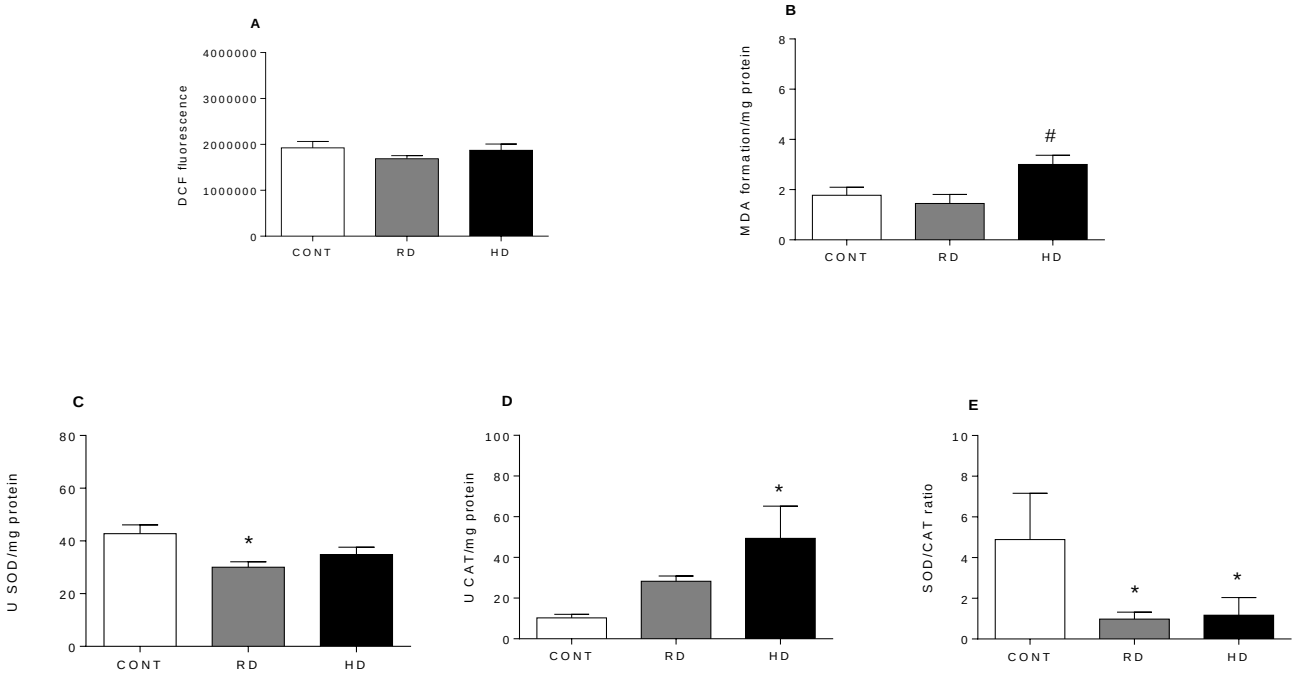
*
CONT
RD
HD

Figure 2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CONT
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U SOD/mg protein
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SOD/CAT ratio
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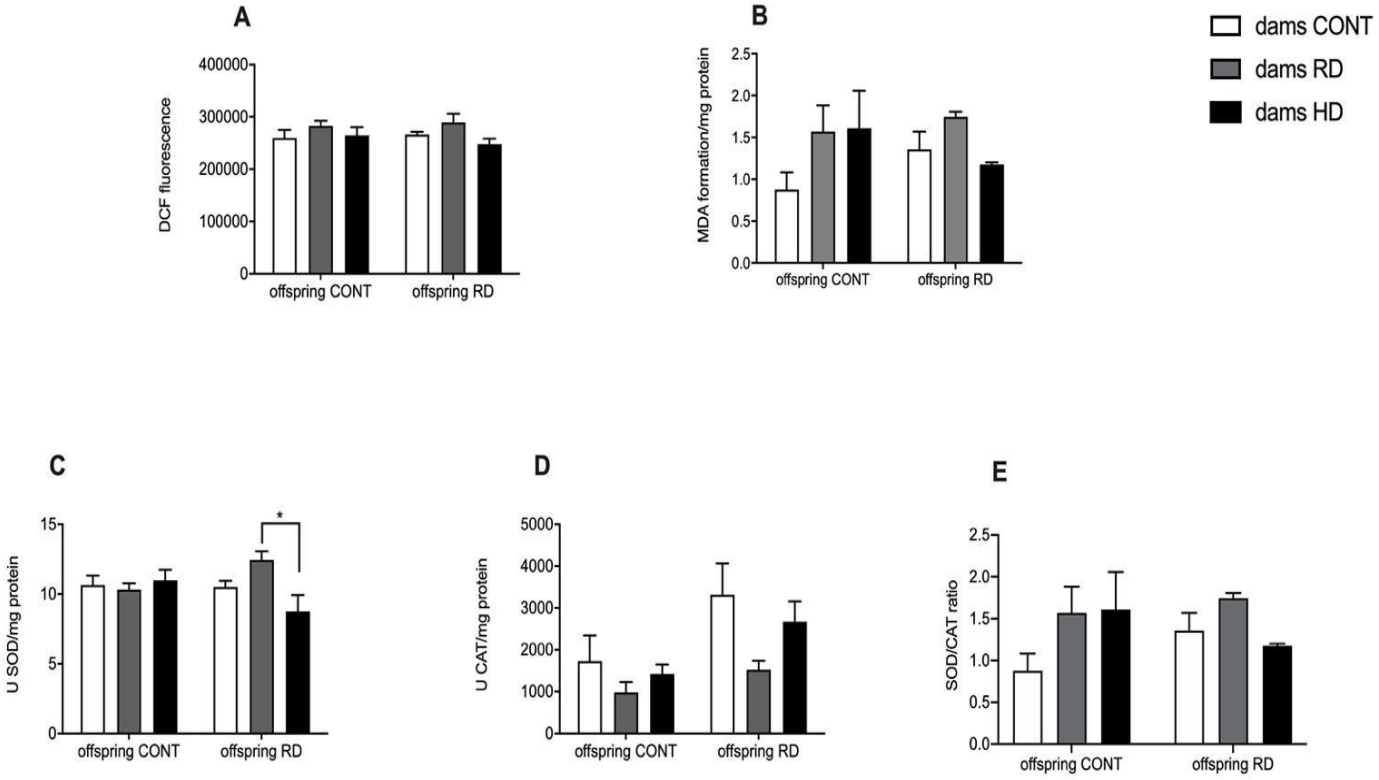
Figure 3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offspring CONT
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B
MDA formation/mg protein
2.5
2.0
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1.0
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offspring CONT
offspring RD
dams CONT
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dams HD
C
U SOD/mg protein
15
10
5
0
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offspring CONT
offspring RD
D
U CAT/mg protein
5000
4000
3000
2000
1000
0
offspring CONT
offspring RD
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SOD/CAT ratio
2.5
2.0
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offspring CONT
offspring RD

Figure 4

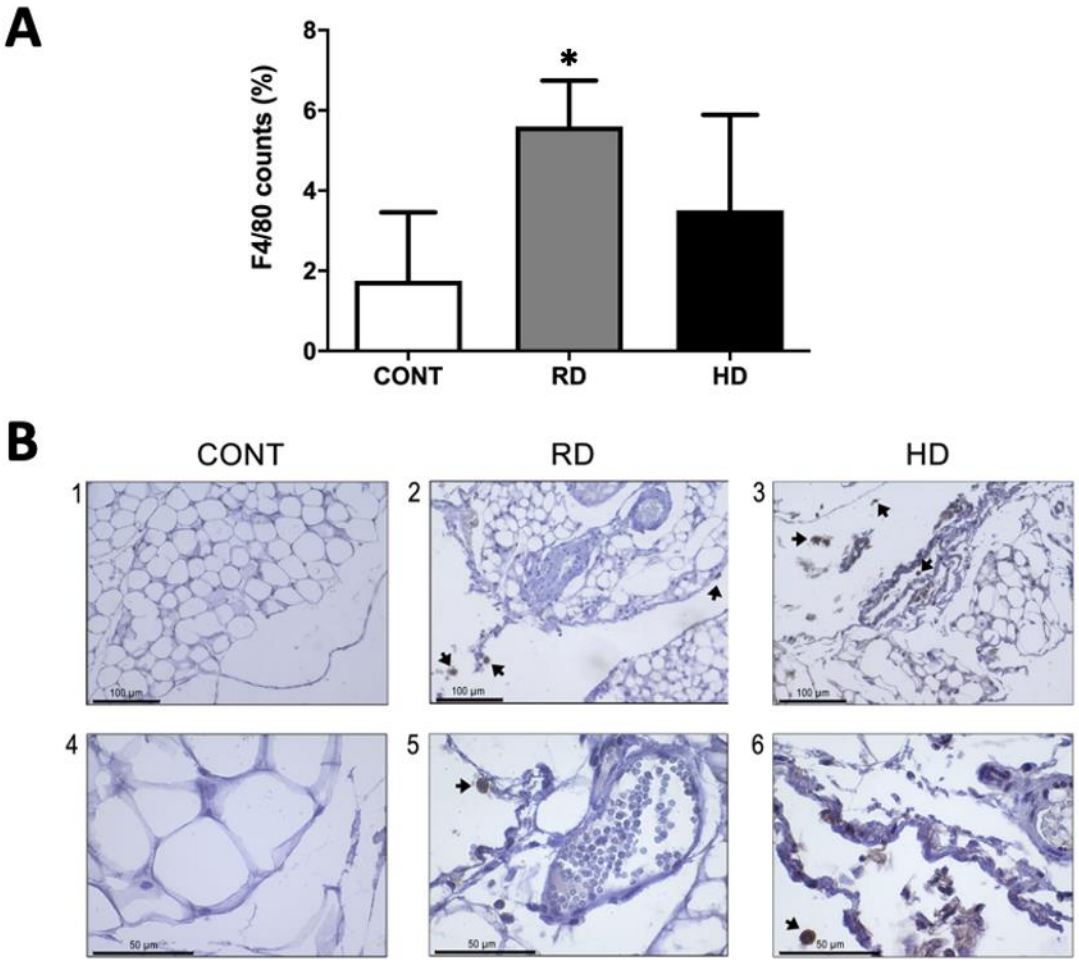
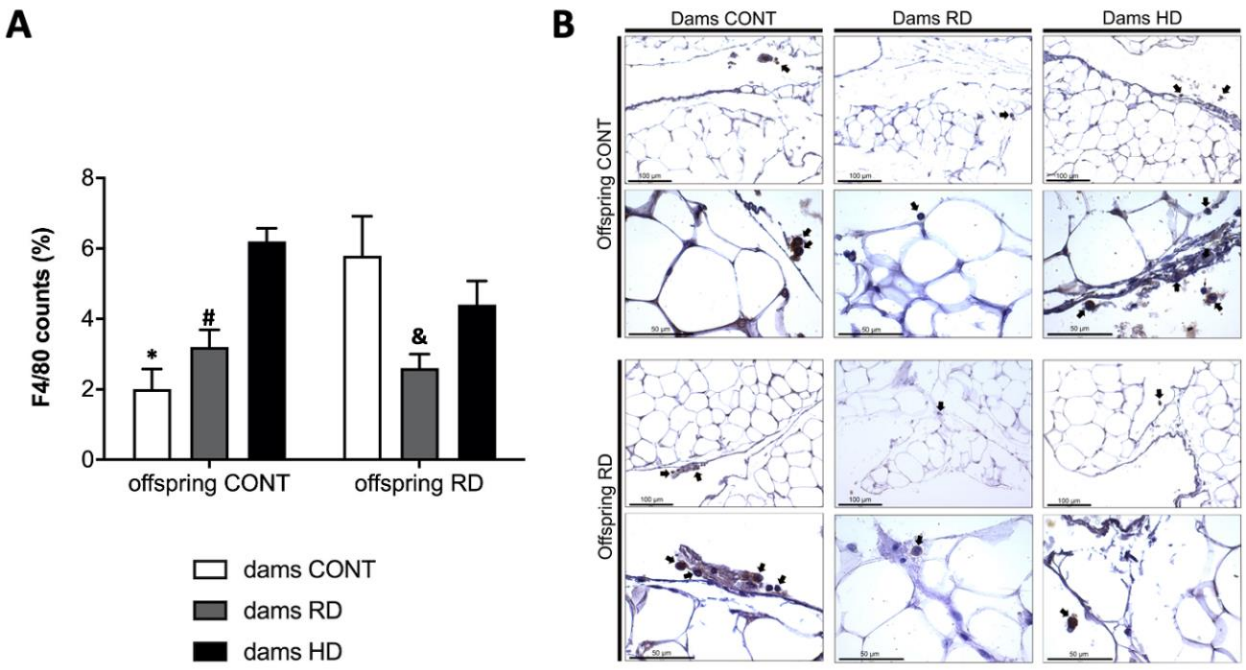


Figure 5


A
F4/80 counts (%)
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&
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Dams CONT
Dams RD
Dams HD
Offspring CONT
Offspring RD

5 CONCLUSÕES

A partir dos resultados deste estudo podemos concluir que:

- mães alimentadas com dieta de restrição calórica, ao longo da gestação e da lactação, apresentaram aumento do perfil inflamatório no tecido adiposo, sem nenhuma alteração no tecido hepático;

- mães alimentadas com dieta hipercalórica, ao longo da gestação e da lactação, desenvolveram NASH no tecido hepático, sem nenhuma alteração no tecido adiposo;

- ambas dietas (restrição e hipercalórica) consumida pelas genitoras provocaram respostas ao estresse oxidativos desenvolvido pelo tecido hepático;

- ambas dietas (restrição e hipercalórica) consumida pelas genitoras influenciaram o tamanho do adipócito da prole na vida adulta;

- fêmeas que foram submetidas a dieta restrita após o desmame foram capazes de reverter parâmetros nocivos da dieta desequilibrada das mães, como reversão do tamanho de area do adipócito, regulação da concentração sérica de leptina e diminuição do infiltrado inflamatório no tecido adiposo;

Já nas filhas controle descendentes de mães hipercalóricas observamos um efeito da dieta materna materna associada a dieta consumida ao longo da vida no perfil inflamatório do tecido adiposo, onde este grupo apresentou um aumento no infiltrado de macrófagos neste tecido;

- a locomoção avaliada no teste de campo aberto mostrou-se diminuída na prole que recebeu tanto a dieta controle quanto a restritiva das mães alimentadas com dieta restrita;

- encontramos um efeito da dieta materna onde a atividade da SOD está aumentada no tecido hepático no grupo RD/RD comparado ao HD/RD.

- a dieta hipercalórica alterou o perfil inflamatório do tecido adiposo dos filhotes que receberam dieta controle, além provocar alterações metabólicas, tais como aumento da concentrações séricas de glicose, insulina e leptina.

- ambas dietas maternas, hipercalórica e restrita, não causaram dano no tecido hepático da prole quando adulta.

Em resumo, a dieta restrita oferecida aos filhotes após o desmame foi capaz de promover efeitos benéficos na prole adulta revertendo as ações metabólicas desfavoráveis da dieta materna desequilibrada. Desta forma, a atenção com a nutrição materna deve ser mais valorizada e discutida, até mesmo deve ser melhor divulgada, pois impacta tanto na vida intrauterina como ao longo de vida adulta do animal, além de impactar diretamente na saúde da mãe.

ANEXO A – PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

CEUA –COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 171/15

2) DATA DO PARECER: 09/03/2016 Parecer 388/15

3) TÍTULO DO PROJETO:

Análise comportamental, bioquímica e epigenética do efeito da nutrição materna em modelo animal.

4) PESQUISADOR RESPONSÁVEL:

Márcia Giovenardi

5) RESUMO DO PROJETO:

O projeto visa analisar parâmetros comportamentais, bioquímicos e epigenéticos dos sistemas dopaminérgico e serotonérgico em camundongos expostos a diferentes dietas (restrição calórica-DR, normal-DC e hipercalórica-DHC). Protocolo 1: camundongas serão acasaladas na proporção de 3:1 e iniciarão a dieta quando confirmada a prenhez (esfregaço vaginal), sendo avaliadas no 1º e 10º dia pós-natal, quanto a massa corporal e no 7º dia pós-natal quanto ao comportamento maternal, 8º dia – ansiedade (labirinto em cruz) e 9º dia, quanto a locomoção (campo aberto). Estas serão avaliadas também, quanto a massa corporal nos dias 3, 9, 15 e 21 pós-natal e eutanasiadas por decaptação no 22º dia, para coleta de sangue troncular para avaliação bioquímica (glicemia, colesterol total, triglicerídeos, HDL, LDL e colesterol) e regiões cerebrais para análise da expressão de genes envolvidos nos sistemas dopaminérgico e serotonérgico. Protocolo 2: as ninhadas serão padronizadas ao nascimento, em oito filhotes cada e, 3 machos e 3 fêmeas de cada ninhada serão submetidos as respectivas dietas (independentemente da dieta materna). Será verificado na prole: massa corporal nos dias

3, 9, 15 e 21 pós-natal, 55º dia - ansiedade (labirinto em cruz) e 60º dia - locomoção (campo aberto), sendo as fêmeas acompanhadas quanto ao ciclo estral (3 ciclos regulares) e testadas em diestro. A eutanásia será realizada em torno de 65 dias pós-natal, da mesma forma e com avaliação das mesmas variáveis descritas para as mães.

6) OBJETIVOS DO PROJETO:

Geral: Analisar parâmetros comportamentais, bioquímicos e epigenéticos dos sistemas dopaminérgico e serotonérgico em camundongos expostos a diferentes dietas (restrição calórica-DR, normal-DC e hipercalórica-DHC).

Específicos:

- analisar o efeito das diferentes dietas nos comportamentos maternal, de ansiedade e de locomoção das genitoras;

- analisar o efeito das diferentes dietas na expressão de genes dos sistemas dopaminérgico e serotoninérgico das genitoras;

- analisar o efeito das diferentes dietas nos comportamentos de ansiedade e locomoção da prole;

- analisar o efeito das diferentes dietas na expressão de genes dos sistemas dopaminérgico e serotonérgico da prole;

Identificar padrão de metilação nos genes que possuam modificação da expressão de genes do sistema dopaminérgico da prole;

- identificar padrão de metilação nos genes que possuam modificação da expressão de genes do sistema serotoninérgico da prole;

- analisar o efeito de diferentes dietas no perfil lipídico da genitora e da prole.

7) FINALIDADE DO PROJETO:

[ ] Ensino [X] Pesquisa

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:

Título [X] Adequado [ ] Comentários

Introdução	[X]	Adequada	[ ]	Comentários
Objetivos	[X]	Adequados	[ ]	Comentários
Relevância e Justificativa	[X]	Adequados	[ ]	Comentários
Materiais e Métodos	[X]	Adequados	[ ]	Comentários

Detalhar na metodologia:

- o cálculo do tamanho da amostra foi apresentado e referenciado;

- o procedimento de acasalamento e adequado a espécie em consonância com os objetivos da pesquisa.

- a justificativa para a decaptação foi apresentada e referenciada.

Cronograma para execução da pesquisa	[X]	Adequado	[ ]	Comentários
Orçamento e fonte financiadora	[X]	Adequados	[ ]	Comentários
Referências Bibliográficas	[X]	Adequadas	[ ]	Comentários

9) O PROJETO ESTÁ ADEQUADO À LEGISLAÇÃO VIGENTE:

[x] Sim [ ] Não

10) INFORMAÇÕES RELATIVAS AOS ANIMAIS:

Grau de dor/estresse: **B** [ ] **C** [X] **D** [ ] **E** [ ]

Justifique:

Espécie: Camundongo **Número Amostral:** 15/grupo

Redução Amostral: [ ] Sim [x] Não

Justifique:

Substituição de Metodologia: [ ] Sim [X] Não

Se achar necessário, justifique e sugira uma nova metodologia:

Aprimoramento da Metodologia: [ ] Sim [X] Não

Se achar necessário, justifique e sugira aprimoramentos da metodologia:

Acomodação e manutenção dos animais: [x] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Manipulação dos animais: [X] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Analgesia dos animais (se aplicável): [X] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com analgésico substituto

Anestesia dos animais (se aplicável): [X] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com anestésico substituto:

Eutanásia dos animais (se aplicável): [X] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com metodologia substituta:

Local de Realização (Biotério/Labotarório): Biotério da UFCSPA, Laboratório de Fisiologia comportamental e metabólica e o Laboratório de Biologia Molecular.

Outra instituição. Qual?

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade
Não referida	Camundongos	Macho	15
Não referida	Camundongas	Fêmeas	45

12) RECOMENDAÇÃO:

[X] Aprovado

[ ] Com Pendência

[ ] Não aprovado

Data de início 10/03/2016 Data de Término 30/12/2019

Comentários gerais sobre o projeto:

O tema é relevante, o uso dos animais é justificado por necessitar avaliação genética, metabólica e comportamental *in vivo*. O projeto está adequado para ser desenvolvido na forma apresentada.