Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

EFEITO DOS ÁCIDOS GRAXOS *TRANS* SOB MARCADORES INFLAMATÓRIOS, BIOQUÍMICOS, COMPORTAMENTAIS E DO SISTEMA NERVOSO CENTRAL EM RATOS ADULTOS WISTAR.

TESE DE DOUTORADO

Rafael Longhi

Porto Alegre, março de 2016

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(Orientador)

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas: Bioquímica, como requisito parcial para a obtenção do título de Doutor em Ciências Biológicas – Bioquímica.

Porto Alegre, março de 2016

"Sem dados, você é apenas uma pessoa qualquer sem opinião"

William Edward Deming

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A todos, meu sincero, MUITO OBRIGADO!!

SUMÁRIO

APRESENTAÇÃO

Esta tese está organizada nas seguintes partes: Introdução, Objetivos, Capítulos (1 a 2, referente aos artigos científicos), Discussão, Conclusões, Perspectivas e Bibliografia.

A Introdução apresenta o embasamento teórico, que nos levou a formular a proposta de trabalho. O Objetivo geral e os objetivos específicos estão dispostos no corpo da tese, e, especificamente, dentro de cada capítulo. Os capítulos contêm os artigos científicos, os quais foram organizados como resposta aos objetivos propostos. Todos os trabalhos foram desenvolvidos no Departamento de Bioquímica – ICBS – UFRGS; e Laboratório de Análises Clínicas – LABMED, Santa Maria – RS.

A seção Discussão contém uma interpretação geral dos resultados obtidos nos diferentes trabalhos. Os tópicos seguintes, Conclusões e Perspectivas, abordam as conclusões gerais da tese, bem como, possibilidades de futuros trabalhos a partir dos resultados descritos. A seção Bibliografia lista as referências citadas na Introdução e Discussão. As referências utilizadas nos diferentes artigos estão listadas ao final de cada trabalho. Os resultados desta tese de doutorado estão apresentados sob a forma de artigos científicos. As seções Materiais e Métodos, Resultados, Discussão e Referências bibliográficas encontram-se nos próprios artigos.

LISTA DE ABREVIATURAS

AE – Ácidos Elaídico

ALT – Alanina Aminotransferase

AST – Aspartato Aminotransferase

AGCL – Ácidos Graxos de Cadeia Longa

AGCM – Ácidos Graxos de Cadeia Média

AGE – Ácidos Graxos Essenciais

AGI – Ácidos Graxos Insaturados

AGS – Ácidos Graxos Saturados

AGT – Ácidos Graxos *Trans*

AGTM – Ácidos Graxos *Trans* Monoinsaturados

AGTP – Ácidos Graxos *Trans* Poliinsaturados

AVE – Acidente Vascular Encefálico

BHE – Barreira Hematoencefálica

DA – Doença de Alzheimer

DCF – Diclorofluorescina

DCNT – Doenças Crônicas Não-Transmissíveis

DMII – Diabetes *Mellitus* do Tipo II

DP – Doença de Parkinson

EM – Esclerose Múltipla

ERO – Espécies Reativas de Oxigênio

FDA – Foods and Drugs Administration (Administração de Drogas e Alimentos)

HDL-c – Lipoproteína de Alta Densidade

IL1 – Interleucina 1

IL1β – Interleucina 1 beta

IL6 – Interleucina 6

IL33 – Interleucina 33

LCAD – Acil CoA Desidrogenase de Cadeia Longa

LDL-a – Anticorpo de Liproteína de Baixa Densidade

LDL-c – Lipoproteína de Baixa Densidade

LDLOx – Lipoproteína Oxidada de Baixa Densidade

LPS – Lipopolissacaerídeo de *E. Coli*

MTG – Mitotracker Green

MTR – Mitotracker Red

NAFLD – Non-alcoholic fatty liver disease (Esteatose Hepática Não-Alcoólica)

n3 – Ácidos Graxos Ômega 3

n6 – Ácidos Graxos Ômega 6

OMS – Organização Mundial da Saúde

SBC – Sociedade Brasileira de Cardiologia

SNC – Sistema Nervoso Central

TGF-β – Fator Transformador do Crescimento Beta

TNF- α – Fator de Necrose Tumoral Alfa

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PARTE I

RESUMO

Dados recentes mostram que os Ácidos Graxos *Trans* (AGT) são uma classe de lipídios os quais estão relacionados a malefícios a saúde humana. AGT são ácidos graxos insaturados que contém ao menos uma ligação dupla não conjugada na cofiguração *trans*, gerando uma gordura de característica mais linear. A presença de uma ligação dupla *trans* na cadeia dos ácidos graxos resulta em um menor ângulo de ligação, em comparação as ligações duplas *cis*, resultando em uma conformação mais similar aos ácidos graxos saturados do que aos insaturados. Diversos estudos têm identificado uma associação entre a ingestão de AGT e maior risco a doenças neurodegenerativas, cardiovasculares e efeitos pró inflamatórios. Neste estudo, avaliamos os efeitos dos ácidos graxos *trans* sob marcadores inflamatórios, bioquímicos, comportamentais e do sistema nervoso central em ratos adultos Wistar. Nossos resultados demonstraram que AGT induzem um aumento na produção de citocinas pró inflamatórias (IL1, IL6, IL10 e TNFα) em ambas concentrações normo e hiperlipídicas tanto no líquido cerebroespinhal quanto no plasma. Com relação aos marcadores oxidativos, AGT elevaram as concentrações destes marcadores mais do que as respectivas dietas com banha de porco suportando um possível componente inflamatório nos AGT independente da concentração da dieta. Nos marcadores cerebrais, a suplementação com AGT causou uma redução na massa e potencial de membrana mitocondrial no córtex, elevou marcadores inflamatórios e oxidativos em córtex e hipocampo e gerou prejuízos a *performance* dos ratos Wistar no Campo Aberto. Este estudo representa uma nova ferramenta para entender os mecanismos bioquímicos dos AGT em marcadores séricos, do líquido cerebroespinhal e do sistema nervoso central.

De uma forma geral, esses achados sugerem que a composição lipídica é mais importante do que a quantidade consumida em termos de AGT e ácidos graxos *cis*.

ABSTRACT

Recent data regarding *Trans* Fatty Acids (TFAs) have implicated this lipid as being particularly deleterious to human health. TFAs are unsaturated fatty acids that contain at least one non conjugated double bond in the *trans* configuration, resulting in a more linear shape. The presence of a *trans* double bond in a fatty acid chain results in a smaller bond angle, or kink, than in a *cis* double bond, resulting in a fatty acid chain conformation that is more similar to a saturated fatty acid than to an unsaturated fatty acid. Several studies have identified an association between *trans* fat intake and a risk of neurodiseases, cardiometabolic disease and pro-inflammatory effects. In this study, were evaluated the *trans* fatty acids on inflammatory, biochemical and behavioral parameters and central nervous system of adult Wistar rats. Our results has demonstrated that TFAs induce the production of proinflammatory cytokines (IL1, IL6, IL10 and TNFα) in animal models fed normo- and hyperlipidic diets in both cerebrospinal liquid and blood. In relation to oxidative parameters, the TFA diet elevated the CSF concentration of these parameters more than the *Cis* Fatty Acid diet, supporting a possible inflammatory component to the PHSO diet that is independent of concentration. In the brain parameters, dietary supplementation with AGT caused a reduction in mitochondrial mass and membrane potential in the cortex, impaired inflammatory and oxidative parameters in the cortex and hippocampus and resulted in alterations in the open field task performance of Wistar rats. This study represents a new approach to understand the biochemical mechanisms of TFA in serum parameters, cerebrospinal fluid and center nervous system. Overall, these findings suggest that fat composition is more important than the quantity of fat consumed in terms of dietary *cis* and *trans* fatty acids.

1. INTRODUÇÃO

Na década de 60, a partir de campanhas de saúde pública direcionadas a diminuir o consumo de gorduras animais, a indústria alimentícia inseriu quantidades significativas de óleos vegetais parcialmente hidrogenados para serem usados no processamento de alimentos, dessa forma, iniciou-se o consumo de ácidos graxos *trans* (AGT)(Downs, Thow et al. 2013). Com o passar do tempo, evidências começaram a mostrar que o aumento no consumo de gorduras parcialmente hidrogenadas (ricas em AGT) estavam relacionadas com diversas comorbidades, como doenças cardiovasculares, inflamação sistêmica, dislipidemias, disfunção endotelial e mais recentemente doenças neurodegenerativas(Mozaffarian, Abdollahi et al. 2007; Barnard, Bunner et al. 2014). A partir dessas pesquisas, profissionais e órgãos governamentais começaram a recomendar a redução no seu consumo, inclusive requerendo a identificação nos rótulos à presença desta gordura (Hunter, 2006).

Independentemente das preocupações com a saúde pública, a indústria continua inserindo os AGT em seus produtos devidas as seguintes vantagens comerciais: (i) assegura sua estrutura e textura; (ii) aumenta o tempo de prateleira do alimento (*shelf-life*); (iii) mantém por mais tempo o sabor nos alimentos; (iv) diminui a capacidade oxidativa do alimento; (vi) aumenta sua estabilidade durante estocagem a temperatura ambiente; (vii) aumenta sua estabilidade durante fritura e (viii) assegura sua estabilidade em emulsão (Menaa, Menaa, Tréton, et al. 2013).

1.1. Hidrogenação dos Ácidos Graxos Insaturados

O processo de hidrogenação foi descoberto pelo químico francês, ganhador do prêmio Nobel, Paul Sabatier, o qual usa-se o níquel como ferramenta de catálise e mais recentemente o químico alemão Wilhelm Normann desenvolveu um processo de hidrogenação usando gás hidrogênio. Esse foi o processo que, com o passar do tempo, foi introduzido às gorduras vegetais para a produção de lipídios parcialmente hidrogenados (Eckel, Borra, et al. 2007). Durante o processo, o hidrogênio é borbulhado através da gordura a altas temperaturas na ausência de oxigênio e na presença do níquel como catalisador (Priego-Capote, Ruiz-Jiménez, et al. 2007), dessa forma AGT são produzidos quando o óleo, líquido a temperatura ambiente, é convertido a gordura sólida através do referido processo químico (Bhardwaj, Passi, et al. 2011).

O óleo de soja é um dos mais empregados para a hidrogenação, é composto por ácidos graxos saturados (AGS) e insaturados (AGI), sendo os principais saturados o ácido palmítico (C16:0 em torno de 10 a 12%) e o esteárico (C18:0 em torno de 3 a 5%) e os principais insaturados são o ácido oleico (C18:1 em torno de 24%), o linoleico pertencente a família ômega-6 (n6) (C18:2; 54%,) e o linolênico pertencente a família ômega-3 (n3) (C18:3, 8.0%,) (Bellaloui, Mengistu, et al. 2013). Pela presença de duas ligações duplas isoladas, o ácido linoleico é o mais suscetível a oxidação ou degradação durante o aquecimento, gerando acúmulo de AGT em óleos comestíveis (Liu, Inbaraj, et al. 2007; Li, Ha, et al. 2012). Importante salientar que tanto o ácido linoleico quanto o linolênico são considerados ácidos graxos essenciais (AGE), ou seja, nosso organismo não produz, então devemos adquiri-los pela dieta.

Durante hidrogenação do óleo de soja, a ligação dupla dos AGI linoleico e linolênico podem ser deslocados para uma nova forma de isômeros *cis* ou *trans* não presentes nos óleos

vegetais, criando um isômero geométrico, o ácido vacênico (11-trans-C18:1). Já o ácido oleico passa por uma isomerização geométrica durante sua hidrogenação tornando-se o ácido elaídico (AE) (9-trans-C18:1), dessa forma, o ácido oleico "natural" é transformado em um ácido "não natural". (Kummerow, 2009).

Atualmente, além do processo supracitado, processamentos térmicos durante refinamento ou fritura de óleos vegetais também são capazes de produzir AGT, como segue:

1. Refinamento: óleos comestíveis são refinados para a retirada de certas impurezas naturalmente presentes como ácidos graxos livres, fosfolipídios, carboidratos, proteínas e seus produtos de degradação, os quais alteram a cor, sabor e aroma. Durante refinamento, os óleos vegetais são aquecidos entre 60^0C e 100^0C, em seguida submetido à desodorização (remoção de substâncias que dão ao produto odor desagradável) o qual melhora as características sensoriais do óleo, mas durante esse processo a temperatura fica em torno de 180^0C a 270^0C, iniciando da formação de AGT e reduzindo os teores do ácido linolênico (Bhardwaj, Passi, et al. 2011).

2. Fritura: ocorre em temperatura em torno de 150^0C a 190^0C ou mais, durante esse processo, ocorrem reações químicas que incluem oxidação, hidrólise, isomerização, polimerização e ciclização, tendo como resultado a produção de diversos produtos como ácidos graxos livres, mono e diacilgliceróis, monômeros oxidados, dímeros e polímeros, os quais são incorporados ao alimento e responsáveis pelo aroma, aparência e seu sabor. Atualmente, estudos com espectroscopia tem mostrado a perda das ligações duplas *cis* com consequente aumento das ligações *trans*, desse modo confirmando a presença de AGT durante esse processo (Bhardwaj, Passi, et al. 2011).

Os processamentos químicos supracitados produzem principalmente o ácido elaídico (Remig, Fada, et al. 2010).

Segundo Martin et al., (2008), a hidrogenação dos óleos vegetais ou a biohidrogenação (processo natural que ocorre durante a digestão de ruminantes), formam predominantemente ácidos graxos *trans* moninsaturados (AGTM), grupo de ácidos graxos com apenas uma insaturação a qual necessariamente é na forma *trans*, enquanto que os ácidos graxos *trans* poliinsaturados (AGTP), pertencem a essa classe de isômeros e similarmente aos seus homólogos *cis*, possuem 2 ou mais insaturações, podendo ou não ser todas na forma *trans*, estes produzidos pelos tratamentos de desodorização e/ou fritura.

1.1.1. Estrutura Química e Metabolismo dos Ácidos Graxos Trans

Ácido Elaídico (AE) é uma gordura monoinsaturada contendo 18 carbonos em sua cadeia, com uma ligação dupla na configuração *trans* na posição 9 (9-*trans*-C18:1, ácido 9-trans-octadecenóico) (Fig.1) (Nielsen, Krogager et al. 2013).

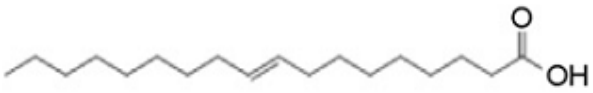

Figura 1. Estrutura do ácido Elaídico (Modificado de Bhardwaj, S, Passi, S. J, Misra, A. 2011).

DeLany, et al., (2000), destaca em sua pesquisa com humanos que existe uma tendência geral de que ácidos graxos de cadeia longa (AGCL), entre 14 e 22 carbonos na cadeia, sejam oxidados mais lentamente em comparação aos ácidos graxos de cadeia média (AGCM) (entre 8 e 14 carbonos) e AGI são oxidados mais rapidamente do que AGS, os quais têm decréscimo da oxidação com o aumento de sua cadeia.

Especificamente em relação ao AE, o artigo mostra que apesar de haver uma relação linear entre oxidação e número de ligações duplas, o AE, em comparação ao seu isômero ácido oleico, teve maior pico de oxidação (Fig. 2). Em parte essa informação pode explicar possíveis diferenças no ganho de peso em modelos animais e também possíveis relações à obesidade humana.

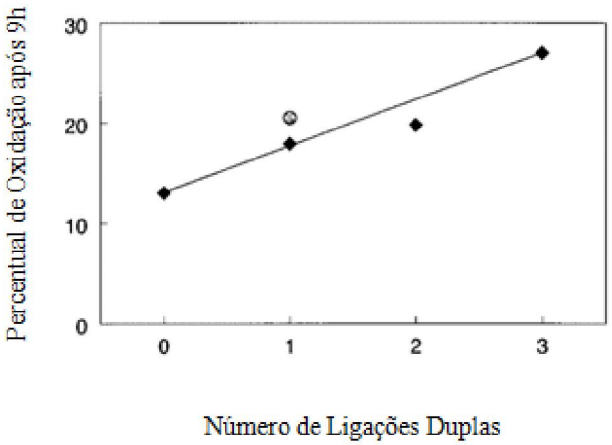

Figura 2. Relação entre oxidação e número de ligações duplas nos ácidos graxos de 18 carbonos. AGT é o círculo preenchido (Modificado de DeLany, Windhauser, 2000).

Ligações duplas em AGI podem ser tanto na conformação *cis* (onde os átomos de hidrogênio estão no mesmo lado da cadeia) ou *trans* (átomos de hidrogênio estão em lados opostos da cadeia). De uma forma geral, a conformação *cis* é a que mais ocorre nas gorduras dietéticas. A presença de uma ligação dupla *trans* em uma cadeia resulta em menor ângulo de ligação do que uma ligação dupla de conformação *cis*, resultando em uma conformação mais similar aos AGS do que aos AGI. Se por um lado, a presença de uma ligação *cis* impede que as cadeias se alinhem gerando uma característica de gordura líquida a temperatura ambiente, por outro, o maior alinhamento da ligação *trans* resulta em uma gordura mais viscosa (sólida), a temperatura ambiente (Lichtenstein, 2014).

Com relação ao seu metabolismo, gorduras com ligações na conformação *trans* possuem características diferenciadas e dúvidas na literatura. Segundo Robert et al., (2005), a degradação de AGI, contendo ligações duplas na conformação *trans,* produz o intermediário 2-*trans*-4-*trans*-dienol-CoA, o qual pode ser futuramente metabolizado via 2 rotas distintas, na via redutase-dependente, 2-*trans*-4-*trans*-dienol-CoA é substrato para o 2,4-dienol-CoA redutase, gerando 3-*trans*-enoil-CoA, o qual é convertido pela Δ3, Δ2-enoil-CoA isomerase a 2-*trans*-enoil-CoA o qual é novamente inserido a β-oxidação; e uma outra via alternativa também tem sido descrita por meio da 2-*trans*,4-*trans*-dienol-CoA a qual pode ser diretamente metabolizada pela atividade da enzima multifuncional enoil-CoA hidratase II para 3-hidroxi,4-*trans*-enoil-CoA, esta então podendo ser degradada na β-oxidação (Robert, Marchesini et al. 2005).

O autor supracitado cita a β-oxidação de forma generalista a todos lipídios com conformação *trans*, Yu et al., (2004) e cita uma possível β-oxidação incompleta, especificamente do AE o qual é parcialmente convertido a ácido 5-*trans*-tetradecenóico, gerando um incomum extravasamento deste intermediário durante sua β-oxidação. Em seu artigo, o autor comenta que a β-oxidação do AE pode ser um processo atípico, pelo fato do substrato ou parte dele (5-*trans*-tetradecenoil-CoA) ser incompletamente degradado e essa degradação incompleta pode estar relacionada à diminuição da eficiência catalítica da acil-Coa desidrogenase de cadeia longa (LCAD) em ligações duplas *trans* (Yu, Liang, et al. 2004).

1.2. Fontes Dietéticas e Consumo de Ácidos Graxos Trans

A Organização Mundial da Saúde (OMS) implementou em 2003, a partir de novos dados e pesquisas, estratégias para redução significante ou virtual eliminação dos ácidos graxos *trans* produzidos industrialmente dentro de sua "*Global Strategy on Diet, Physical Activity and Health*", citando diversas conclusões e recomendações para eliminação desta

gordura produzida industrialmente (Uauy, Aro, et al. 2009). Seguindo essa premissa, também em 2003, a Food and Drug Administration (FDA) determinou a fabricantes de alimentos a inserção do conteúdo de AGT no painel de informações nutricionais dos alimentos e suplementos dietéticos a partir de 01 de janeiro de 2006, subsequentemente, legislações começaram e banir ou restringir esse produto na alimentação nos Estados Unidos da América (Lefevre, Mensink, et al. 2012).

Aqui no Brasil, a Sociedade Brasileira de Cardiologia (SBC), em suas Diretrizes de 2013, fala da recomendação de Agências de Saúde para a diminuição do consumo desses ácidos graxos pela população mundial e cita as políticas públicas de redução deste consumo e as obrigações da indústria de alimentos em alterar a fonte de gordura utilizada (SBC, 2013).

AGT são naturalmente encontrados em baixos níveis em carnes e produtos lácteos pelo fato de ocorrer hidrogenação bacteriana no estômago dos ruminantes, entretanto a maior fonte de AGT na dieta são alimentos que contém a fração industrial desta gordura, além dos óleos parcialmente hidrogenados (Klonoff, 2007). O conteúdo de AGT industrial gira em torno de 60% do teor lipídico dietético (Tab. 1), enquanto que a fração *trans* proveniente das carnes e produtos lácteos não ultrapassa os 5% do conteúdo lipídico da dieta (Dhaka, Gulia, et al. 2011).

Tabela 1. Percentual de ácidos graxos *trans* ingeridos na dieta (Modificado de Dhaka, Gulia, et al. 2011.).

Grupo Alimentar	Percentual de AGT consumido
Bolos, biscoitos, pães, *cream cracker*	40
Produtos animais	21
Margarina	7
Batata Frita	8
Batata frita chips, pipoca	5
Cereais matinais	5

Ratificando os dados supracitados, Kris-Etherton et al., (2012), mostra dados referentes a mais de 16.000 indivíduos, a partir dos 3 anos de idade nos EUA, em relação ao consumo de AGT. Seus resultados apontam para uma ingestão de AGT associada com o aumento total da gordura dietética (26.7–37.6 % da energia total), gordura saturada (7,6-10,5% da energia total) e calorias para os pesquisados com mais de 20 anos. As maiores fontes foram bolos, biscoitos, tortas e pastéis.

Chardigny et al., (2008), avaliou o consumo de alimentos que contém AGT (na forma natural e industrializada) em sua composição de 40 sujeitos (19 homens e 21 mulheres) e como resultado encontrou aumento tanto de lipoproteínas de alta densidade (HDL-c) quanto lipoproteínas de baixa densidade (LDL-c) em mulheres e queda de HDL-c com AGT de fontes industriais em homens, mas terminam a pesquisa citando dificuldades em desenhar uma conclusão sobre os possíveis efeitos deletérios de fontes *trans* da dieta.

Alguns autores sugerem que o consumo de AGT proveniente dos ruminantes oferecem benefícios à saúde, enquanto outros reivindicam que esses benefícios não estão totalmente

esclarecidos pela literatura científica. Entretanto há um consenso geral em relação aos efeitos nocivos à saúde com o consumo de AGT industrial(Brouwer, Wanders, et al. 2010). Como ainda há dúvidas na literatura, faz-se necessária a continuidade nas pesquisas.

1.3. Riscos à Saúde Associados aos Ácidos Graxos Trans

Triagens clínicas têm mostrado que o excesso de AGT na dieta (4 a 6% da energia total) aumentam LDL-c e diminuem as concentrações de HDL-c, além de inúmeros estudos epidemiológicos mostrando uma clara evidência de que o consumo de altas quantidades de AGT industrializados, aumenta o risco cardiovascular (Ochiai, Fujii, et al. 2013).

Os efeitos do consumo de AGT sobre fatores de risco cardiovascular foram avaliados em uma revisão feita por Mozaffarian et al., (2009), tanto em triagens controladas quanto em estudos observacionais. Seus resultados apontam efeitos adversos em lipídios (↑LDL-c e ↓HDL-c), marcadores inflamatórios (↑ Fator de Necrose Tumoral-α (TNF- α), ↑ Interleucina-6 (IL6)) e disfunção endotelial, tendo os efeitos de AGT mais proeminentes em comparação ao seu análogo *cis* e piora na sensibilidade à insulina em comparação aos AGS quando avaliados sujeitos com predisposição à resistência. Por outro lado, Aronis et al., (2012) conclui em sua revisão sistemática (vale salientar que foram apenas 7 triagens) que a ingestão de AGT não resulta em modificações nas concentrações de insulina, glicose e triglicerídeos, apenas altera o perfil lipídico de LDL-c e HDL-c.

Em modelos animais, Chen et al., (2011) mostrou que uma dieta hiperlipídica com AGT gera lesões ateroescleróticas e a supressão do Fator Transformador do Crescimento Beta (TGF-β), o qual segundo o mesmo autor é fator de risco para ateroesclerose. Sua teoria baseia-se na premissa de uma possível afinidade entre AGT e o colesterol, com isso dietas ricas em AGT podem causar uma lesão pela supressão do TGF-β nas células vasculares pela incorporação desses ácidos graxos à membrana fosfolipídica, com resultante aumento na

integração do colesterol à membrana. Em humanos, Han et al., (2002) oferece a 19 participantes gordura vegetal hidrogenada por 32 dias e conclui que dietas ricas em gordura hidrogenada aumentam a produção de citocinas pró inflamatórias (TNF-α e IL6) as quais estão associadas com a patofisiologia da ateroesclerose.

Eventos cardiovasculares e metabólicos são comumente associados à inflamação sistêmica ou localizada, e AGT têm sido relacionados a eventos pró-inflamatórios em diversos estudos, dessa forma, podem agir em qualquer processo inflamatório, inclusive o intestinal (Okada, Tsuzuki, et al. 2013). Em outro estudo conduzido por Okada et al., (2013), camundongos que foram alimentados com dietas entre 1 e 3% de AGT e colite induzida por dextran sulfato de sódio tiveram exacerbação em sua inflamação colônica pela maior expressão de citocinas proinflamatórias na mucosa inflamada.

Outra disfunção metabólica de grande importância, o Diabetes tipo II (DMII), também possui associações e evidências experimentais frente ao consumo de AGT, entretanto tais conclusões ainda são limitadas (Dorfman, Laurent, et al. 2009). Corroborando, Thompson et al., (2011), cita como fraco o risco entre AGT e resistência à insulina e/ou desenvolvimento de DMII em ambos estudos com animais e humanos. Por fim, Risérus (2006), conclui que há potencial efeito adverso dos AGT sobre o metabolismo da glicose, mas não há dados suficientes para relacionar a desequilíbrios no metabolismo da insulina em pessoas saudáveis, apenas em sujeitos que já estejam com a patologia instalada.

Em relação ao tecido hepático, Shao e Ford (2014), demonstraram que o AE potencialmente induz a atividade da SRE, um dos maiores fatores de transcrição que regulam biossíntese de ácidos graxos e colesterol. Interessante citar que seu análogo *cis*, o ácido oleico, não gera essa alteração segundo os autores. Ratificando os dados acima, Krogager, et al., (2015) mostra em suas investigações em cultura de células que o AE afeta metabolismo do

colesterol em hepatócitos (HepG2-SF) ao contrário do ácido vacênico, a porção *trans* de ocorrência natural.

Para concluir, em relação à obesidade, dados de cinco anos de acompanhamento sugerem que o aumento no consumo de AGT prejudicam estratégias de perda de peso, tanto em homens quanto em mulheres, com risco aumentado ao ganho para as mulheres (Chajès, Biessy, et al. 2015).

1.3.1. Ação no Sistema Nervoso Central

Em uma pesquisa que acompanhou idosos com mais de 65 anos por mais de três anos, dos 815 pesquisados, 131 desenvolveram doença de Alzheimer (DA). Nesta revisão foi concluído que a ingestão de gordura hidrogenada aumenta o risco para DA (Morris, Evans, et al. 2003). Corroborando com o supracitado, Banard et al., (2014) indica uma relação entre AGS e AGT ao aumento no risco de desordens cognitivas.

In vitro, experimentos de Grimm et al., (2012), claramente mostraram que AGT em comparação a conformação *cis*, aumentaram a produção, oligomerização e agregação de peptídeos β-amilóide, principais componentes das placas senis, as quais são características da DA. Em modelos animais, gorduras vegetais hidrogenadas foram oferecidas a ratos recém nascidos e como resultado, houve um maior incorporação de AGT no córtex, hipocampo e estriado, além de intensificar eventos oxidativos no cérebro, possibilitando uma possível hiperlocomoção a qual serve como marcador de risco para eventos neurológicos (Trevizol, Roversi, et al. 2013). Teixeira et al., (2012) mostrou em recente trabalho uma pequena mas significante incorporação de AGT no cérebro de ratos adultos alimentados durante 60 semanas com gordura vegetal hidrogenada, o qual favoreceu o desenvolvimento de desordens de movimento. Já de Souza et al., (2012), usou ratos descendentes de mães que consumiram gordura vegetal hidrogenada (grupo *trans*) para avaliar memória espacial e aversiva e em seus

resultados a autora cita que AGT foram incorporados em pequenas quantidades no hipocampo, não afetando memória aversiva mas afetando memória espacial.

Em revisão sobre a relação entre AGT e acidente vascular encefálico (AVE) feita ao longo de sete anos, os autores encontraram forte relação entre seu consumo e o aumento no risco de AVE, para cada 2g/dia no aumento de AGT na dieta, o risco para AVE sobe para 14% em homens (Kiage, J. N, Merrill, et al., 2014).

Por fim, AGT estão associados com todas as causas de mortalidade, provavelmente pelo aumento dos níveis de AGT industrial na dieta, conclusão de uma requintada revisão com 50 artigos feita por de Souza et al., (2015).

2. Objetivos

2.1. Objetivo geral

Investigar o efeito dos ácidos graxos *trans* sob marcadores inflamatórios, bioquímicos, comportamentais e do sistema nervoso central em ratos adultos Wistar.

2.2. Objetivos Específicos

2.2.1. Investigar os efeitos de dietas enriquecidas com gordura parcialmente hidrogenada em ratos adultos Wistar utilizando marcadores séricos e do líquido cerebroespinhal.

- Avaliar como os ácidos graxos *cis* e *trans* influenciam no ganho de peso.
- Investigar a resposta inflamatória sérica e no líquido cerebroespinhal dos ácidos graxos *trans*.

- Analisar a diferença entre os tratamentos no perfil lipídico sérico e no líquido cerebroespinhal.

- Analisar o tecido hepático, focando no conteúdo lipídico e atividade de enzimas antioxidantes.

2.2.2. Investigar os efeitos de dietas enriquecidas com gordura parcialmente hidrogenada em ratos adultos Wistar utilizando tarefas comportamentais e tecido cerebral.

- Avaliar como os ácidos graxos dietéticos *cis* e *trans* afetam a *performance* em tarefas comportamentais.

- Investigar a resposta inflamatória dos ácidos graxos *trans* em córtex e hipocampo.

- Analisar a diferença entre massa mitocondrial e potencial de membrana mitocondrial no tecido cerebral com os diferentes tratamentos dietéticos

PARTE 2

3. Artigo I

Effect of a *Trans* Fatty Acid-Enriched Diet on Biochemical and Inflammatory Parameters in Wistar Rats

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ORIGINAL CONTRIBUTION

Effect of a *trans* fatty acid-enriched diet on biochemical and inflammatory parameters in Wistar rats

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Abstract

Purpose Recent data regarding *trans* fatty acids (TFAs) have implicated these lipids as particularly deleterious to human health, causing systemic inflammation, endothelial dysfunction and possibly inflammation in the central nervous system (CNS). We aimed to clarify the impact of partially hydrogenated soybean oil (PHSO) with different TFA concentrations on cerebrospinal fluid (CSF), serum and hepatic parameters in adult Wistar rats.

Methods Wistar rats ($n = 15$/group) were fed either a normolipidic diet or a hyperlipidic diet for 90 days. The normolipidic and hyperlipidic diets had the same ingredients except for fat compositions, concentrations and calories. We used lard in the *cis* fatty acid group and PHSO in the *trans* fatty acid group. The intervention groups were as follows: (1) low lard (LL), (2) high lard (HL), (3) low partially hydrogenated soybean oil (LPHSO) and (4) high partially hydrogenated soybean oil (HPHSO). Body weight, lipid profiles and the inflammatory responses in the CSF, serum and liver tissue were analyzed.

Results Surprisingly, with the PHSO diet we observed a worse metabolic response that was associated with oxidative stress in hepatic tissue as well as impaired serum and CSF fluid parameters at both PHSO concentrations. In many analyses, there were no significant differences between the LPHSO and HPHSO diets.

Conclusions Dietary supplementation with PHSO impaired inflammatory parameters in CSF and blood, induced insulin resistance, altered lipid profiles and caused hepatic damage. Overall, these findings suggest that fat composition is more important than the quantity of fat consumed in terms of *cis* and *trans* fatty acid diets.

Keywords Obesity · Hydrogenated vegetable oils · Proinflammatory · Brain function

Abbreviations

AD	Alzheimer's disease
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMI	Body mass index
CVD	Cardiovascular disease
CAT	Catalase
CNS	Central nervous system
CSF	Cerebrospinal fluid
DIO	Diet-induced obesity
GPx	Glutathione peroxidase
HDL	High-density lipoprotein cholesterol
HFD	High-fat diet
HL	High lard
HPHSO	High partially hydrogenated soybean oil
IL1	Interleukin 1
IL6	Interleukin 6
IL10	Interleukin 10
LDL	Low-density lipoprotein cholesterol
LL	Low lard
LPHSO	Low partially hydrogenated soybean oil
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
OxLDL	Oxidized low-density lipoprotein
PHSO	Partially hydrogenated soybean oil

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SOD	Superoxide dismutase
TFA	*Trans* fatty acid
TNF-α	Tumor necrosis factor alpha

Introduction

Recent data regarding *trans* fatty acids (TFAs) have implicated this lipid as being particularly deleterious to human health [1]. Industrial and natural fats contain similar species of TFA but in different proportions; elaidic acid (9-trans-C18:1) is more prevalent in industrial sources, and vaccenic acid is predominant (11-trans-C18:1) in milk and meat from ruminant animals [2]. Chardigny et al. [3] commented in their research that epidemiological studies have shown a positive association between cardiovascular disease (CVD) risk and TFA intake from industrial sources, but have not shown a correlation between CVD risk and TFA from natural sources.

Major biochemical problems, including an increased serum ratio of low-density lipoprotein cholesterol (LDL)/high-density lipoprotein cholesterol (HDL) and alterations to the structure and fluidity of the phospholipid bilayer, which lead to an increased risk of coronary heart disease, have been reported with TFA consumption from dietary sources [4–8].

According to the American College of Cardiology, global cardiometabolic risk has a specific relationship to serum lipoprotein abnormalities, including disruption of glucose homeostasis, an increase in LDL/HDL ratios and increased triglycerides in plasma, which contribute to insulin resistance, weight gain, obesity and metabolic syndrome [9]. These metabolic and cardiovascular diseases are commonly associated with systemic or localized inflammation, and TFAs have been shown to have proinflammatory effects [10].

Substantial literature reports have suggested that TFA may increase lipoprotein A and C-reactive protein serum concentrations, insulin resistance, visceral adiposity and inflammatory factors, leading to metabolic syndrome and diabetes mellitus, although these data are not conclusive and correspond to relatively high TFA intakes [11–13]. Furthermore, the increased cardiometabolic risk associated with TFA consumption has been attributed to TFA-induced systemic inflammation and endothelial dysfunction [14]. Several studies in the field of neuroinflammation have focused on cerebrospinal fluid (CSF) because it plays a critical role in many physiological processes [15], including obesity, and has been associated with alterations in brain structure and function and cognitive deficits [16] as well as conditions such as bacterial infection, multiple sclerosis and neuroimmune conditions [17]. According to some studies, TFA intake may increase the risk of these conditions, including Alzheimer's disease (AD) [18].

Specifically in rodents, a chronic caloric oversupply by a high-fat diet (HFD) induces body weight gain, fat mass accumulation and insulin resistance, but the intensity of these changes depends on the type of dietary fat [19]. Researchers have suggested that not only total fat intake but also dietary fatty acid composition plays an important role in the development of obesity, type two diabetes mellitus and CVDs [20]. Obesity alters the metabolic and endocrine functions of adipose tissue and leads to an increased release of fatty acids, hormones and proinflammatory molecules that contribute to the onset of obesity-related complications [21].

Due to observations that have correlated the magnitude of physiological effects from dietary TFA with biochemical values, the aims of this study were to: (1) evaluate how *cis* and *trans* fatty acids influence weight gain in rats, (2) investigate the inflammatory response of TFAs in serum and CSF, (3) analyze the difference between the treatments in regards to lipid profiles in serum and CSF and (4) analyze hepatic tissue, focusing on lipid contents and antioxidant enzyme activities.

Materials and methods

Animal treatment

Sixty adult male Wistar rats were divided into four groups with 15 rats each (60 days old, weighing 250–350 g) and were exposed to a 12-h light/dark cycle (lights on at 7:00 AM) at a constant temperature of 22 ± 1 °C. The rats were housed in plastic cages (5 per cage) with water and food ad libitum following characteristic diets (explained below). All behavioral procedures were conducted between 9:00 AM and 5:00 PM. Animals were maintained in accordance with the Brazilian Society for Neuroscience and Behavior's recommendations for animal care. All protocols followed the Ethical Committee of the Federal University of Rio Grande do Sul.

Diet

In our experiments, we used two types of fat: (1) lard (*cis* fatty acid group) and (2) partially hydrogenated soybean oil (PHSO) (*trans* fatty acid group). The TFA levels in original soybean oil have been shown to range between 0.8 and 2.6 % [22]. Here, after industrial hydrogenation processes, the new TFA content was 57.3 %, according to the manufacturer (Table 2). The normolipidic and hyperlipidic intervention groups were (1) low lard (LL), (2) high lard (HL), (3) low partially hydrogenated soybean oil (LPHSO)

Table 1 Composition in g/kg of LL, HL, LPHSO and HPHSO diets

Composition	LL	HL	LPHSO	HPHSO
Starch	575	250	575	250
Soy protein isolate[a]	269	264	269	264
Sucrose	50	50	50	50
Vitamin mix[b]	10	10	10	10
Mineral salt mix[c]	20	20	20	20
DL-Methionin[d]	3	3	3	3
DL-Lysin[e]	3	3	3	3
Soy oil	10	10	10	10
Lard (TFA content in grams)[f]	60 (0.28)[g]	390 (1.44)[g]	–	–
Partially hydrogenated soybean oil (TFA content in grams)	–	–	60 (34.38)[h]	390 (223.4)[h]

Salt and vitamin composition are according to Horwitz (1980)

[a] Soy protein isolate, purity 97 % (from Solae, Esteio, Brazil)

[b] Vitamin mixture: mg/100 g of diet (from Roche, São Paulo, Brazil): vitamin A (retinyl acetate), 4; vitamin D (cholecalciferol), 0.5; vitamin E (DL-a-tocopheryl acetate), 10; menadione, 0.5; choline, 200; PABA, 10; inositol, 10; niacin (nicotinic acid), 4; pantothenic acid (calcium D-pantothenate), 4; riboflavin, 0.8; thiamin (thiamine hydrochloride), 0.5; pyridoxine (pyridoxine hydrochloride), 0.5; folic acid, 0.2; biotin [D-(+)-biotin], 0.04; vitamin B12, 0.003

[c] Mineral salt mixture: mg/100 g of diet (from Roche, SãoPaulo, Brazil): NaCl, 557; KI, 3.2; KH_2PO_4, 1556; $MgSO_4$, 229; $CaCO_3$, 1526; $FeSO_4$–$7H_2O$, 108; $MnSO_4$–H_2O, 16; $ZnSO_4$–$7H_2O$, 2.2; $CuSO_4$–$5H_2O$, 1.9; CoCl–$6H_2O$, 0.09

[d] DL-Methionin (from Merk, Rio de Janeiro, Brazil)

[e] DL-Lysine (from Merk, Rio de Janeiro, Brazil)

[f] Lard (from Sadia, São Paulo, Brazil)

[g] (C16:1 9t + C18:1 9t + C18:1 11t) × g/100. Values in Table 2

[h] C18:1 9t × g/100. Values in Table 2

Table 2 Fatty acid composition of the partially hydrogenated soybean oil and lard diet (dos Santos et al. [23])

Fatty acids composition in PHSO diet	% Total fatty acids	Fatty acids composition in lard diet	% Total fatty acids
C4	0.0	C14	1.14
C6	0.0	C16	22.7
C8	0.1	C16:1 9t	0.29
C10	0.1	C16:1 9c	1.66
C12	1.6	C17	0.37
C14	0.7	C17:1 10c	0.25
C16	10.8	C18	14.33
C16:1	0.1	C18:1 9t	0.09
C18	17.6	C18:1 11t	0.10
C18:1c	9.0	C18:1 9c	36.38
C18:2c	0.4	C18:1 11c	2.36
C18:3c	0.0	C18:2 9c, 12c (w6)	16.87
C20	0.5	C18:3 6c, 9c,12c (w6)	0.05
C20:1	0.0	C18:3 9c,12c,15c (w3)	1.67
C22	0.5	C19	0.09
C22:1	0.0	C19:1 7c	0.08
C24	0.0	C20	0.28
C18:1t	57.3	C20:2 11c,14c (w6)	0.61
C18:2t	1.4	C22:4 7c,10c,13c,16c	0.26
C18:3t	0.0		
Total TFA	57.3	Total TFA	0.48

Total TFA: lard diet: 0.48 (C16:19t = 0.29; C18:1t = 0.09; C18:1 11t = 0.10) PHSO diet: 57.3 (C18:1t)

and (4) high partially hydrogenated soybean oil (HPHSO). Diets were prepared according to AIN-93 by adding vitamins and mineral salts to the mixtures to ensure that the daily needs of the animals were fulfilled (Tables 1, 2). The fatty acid compositions of lard were according to dos Santos et al. [23]. The TFA content in lard and the PHSO diets were (values in grams): LL (0.28), HL (1.44), LPHSO (34.38) and HPHSO (223.4). The calories (calculated based on diet compositions, see Table 1) of each diet were 4230 kcal (low-fat diet) and 5880 kcal (high-fat diet). Pellets were produced every 48 h and stored in a commercial refrigerator.

Cerebrospinal fluid

After 90 days of dietary treatments, the rats were anesthetized with xylazine (10 mg/kg body weight, i.p.) and ketamine (100 mg/kg body weight, i.p.) and were placed in a stereotaxic apparatus. The CSF was collected (100–150 µL) by directly puncturing the cisterna magna with an insulin syringe (27 gauge × 1/2-inch length). Individual samples with visible blood contamination were discarded. All samples were centrifuged at 10,000*g* at 4 °C in an Eppendorf centrifuge for 10 min to obtain cell-free supernatants, which were stored in single tubes at −70 °C according to the literature [24].

Cerebrospinal fluid analyses

Oxidized LDL (OxLDL) was also determined by capture ELISA according to the manufacturer's instructions (Mercodia AB, Uppsala, Sweden), as described in the literature [25]. OxLDL autoantibodies (anti-OxLDL) were determined using ELISA, as described previously [26]. The HDL of the animals (data not show) was calculated using the Friedewald algorithm [27]. Levels were expressed as mg/dL.

The cytokine levels were measured using ELISA for interleukin 1 (IL1), interleukin 6 (IL6), interleukin 10 (IL10) and tumor necrosis factor alpha (TNF-α) (ELISA kits were purchased from R&D Systems, Minneapolis, MN, USA). Levels were expressed as pg/mL [28].

Serum parameters

After CSF collection, rats were killed by decapitation after 12 h of fasting. The blood was collected, and the serum was obtained after centrifugation (1000*g* for 10 min at 4 °C). Serum was stored at –80 °C until analysis.

Insulin and glucose levels

The serum levels of insulin and glucose were determined using commercial ELISA kits (Linco Research Inc., St. Louis, MO, USA and Labtest, MG, Brazil, respectively). Levels were expressed as ng/mL [29].

Transaminase activities and kidney function analyses

The levels of hepatic transaminases were assessed by measuring alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in U/L using specific kits (LABTEST, Lagoa Santa, MG, Brazil). Kidney function was assessed by measuring the creatinine and urea in mg/dL with specific kits (LABTEST, Lagoa Santa, MG, Brazil) [30].

Lipid contents and antioxidant enzyme activities in the liver

To quantify the hepatic lipid contents, the livers were entirely removed and weighed (±100 mg). Frozen liver samples were thawed on ice and homogenized in deionized water. Extraction and isolation of lipids were performed to yield dried lipid extracts using the technique described by Folch et al. [31]. The hepatic cholesterol and triglyceride contents of the lipid extracts were enzymatically assayed by colorimetry (Labtest Diagnostica S.A.) [32].

The catalase (EC 1.11.1.6) (CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance at 240 nm, and the results are expressed as CAT/mg protein [33]. The superoxide dismutase (EC 1.15.1.1) (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation at 480 nm, as previously described, and the results were expressed as SOD/mg protein [34]. The glutathione peroxidase (EC 1.11.1.9) (GPx) activity was measured according to Wendel [35]. One unit of GPx activity was defined as 1 µmol of NADPH consumed/min, and the specific activity was expressed as units/mg protein. The ratio of the SOD/CAT/GPx activities was evaluated to determine the effects of the protocols on these three coordinated oxidant-detoxifying enzyme activities, which act in sequence to convert superoxide anions into water [36].

Serum lipid profiles

The total serum levels of cholesterol and triglycerides were measured by standard enzymatic methods using Ortho-Clinical Diagnostics® reagents on a fully automated analyzer (Ortho Vitros Fusion® dry chemistry system; Johnson & Johnson, Rochester, NY, USA). Serum levels were expressed as mg/dL. HDL was measured in the serum supernatant after precipitation of apolipoprotein B-containing lipoproteins with dextran sulfate and magnesium chloride, as previously described [37]. OxLDL was derived from total cholesterol. The HDL in the animals was calculated using the Friedewald algorithm [27]. Serum levels were expressed as mg/dL.

Cytokine measurements

The serum concentrations of various cytokines were measured using ELISA for IL1, IL6, IL10 and TNF-α, which were purchased from R&D Systems. Levels were expressed as pg/mL [38].

Statistical analysis

Data are expressed as the mean ± standard error of the mean (M ± SEM.). All parameters were analyzed by a two-way ANOVA followed by the Bonferroni post hoc test [the factors were (1) concentration and (2) diet]. $p < 0.05$ was considered significant. *$p < 0.05$ versus control (LL); #$p < 0.05$, difference between hyperlipidic diet; $^{\$}p < 0.05$, difference between LPHSO and HL only.

Results

Effects of treatments on body weight gain

After 90 days of free access to different diets, no significant differences in body weight (Fig. 1a) or weekly cumulative weight gain (Fig. 1b) were observed among the four diet groups.

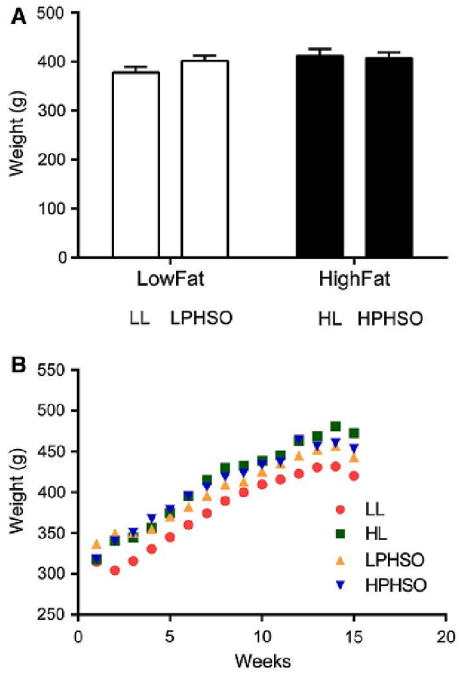

Fig. 1 Weight analysis (**a**, **b**) of the groups. *$p < 0.05$ versus control (LL); #$p < 0.05$, difference between hyperlipidic diet; $^{\$}p < 0.05$, difference between LPHSO and HL only. All groups were analyzed using a two-way ANOVA and Bonferroni post hoc test ($n = 15$ animals/group). Data are expressed as the mean ± SEM and $p < 0.05$

Effects of the PHSO diet and the lard diet on CSF parameters

OxLDL and LDL antibody analyses

For the OxLDL in CSF (Fig. 2a), our results showed that the PHSO diet had the highest values at both the normolipidic and hyperlipidic concentrations, and there were no significant differences between LPHSO and HPHSO. It is interesting to note that there were no significant differences between the LPHSO and HL diets. The diets were significantly different among the groups [$F(1.40) = 29.13$, $p < 0.0001$]. The LDL antibody (Fig. 2b) showed significant increases in all of the parameters analyzed [concentration and diet, $F(1.45) = 31.68$, $p < 0.0001$; $F(1.45) = 86.03$, $p < 0.0001$, respectively].

Cytokine measurements

The levels of IL1, IL6, IL10 and TNF-α in CSF were analyzed. Both IL1 (Fig. 3a) and IL6 (Fig. 3b) showed no significant differences between the LPHSO and HPHSO diets and exhibited the highest values in both the normolipidic and hyperlipidic diets in comparison with the lard diets for IL1 and IL6 ($p < 0.0001$ and $p = 0.0015$, respectively). The diet parameters were significantly different for both IL1 and IL6 [$F(1.44) = 124.0$, $p < 0.0001$; $F(1.45) = 59.74$, $p < 0.0001$, respectively]. IL10 (Fig. 3c) had the lowest values for both the LPHSO and HPHSO diets and showed significant differences in all of the parameters analyzed [concentration and diet, $F(1.45) = 45.20$, $p < 0.0001$; $F(1.45) = 106.6$, $p < 0.0001$, respectively].

The PHSO diets were associated with higher TNF-α values than the lard diets ($p < 0.0001$ at both low and high concentrations) and were significantly different in all of the parameters analyzed [concentration and diet, $F(1.45) = 41.79$, $p < 0.0001$; $F(1.45) = 128.5$, $p < 0.0001$, respectively, Fig. 3d].

Effects of the PHSO diet and lard on serum parameters

Serum concentrations of glucose and insulin

Regarding the serum parameters, Wistar rats on PHSO diets had increased glucose levels in both the LPHSO and HPHSO diets compared with the lard diet. The glucose level of the LPHSO diet was higher than the HL diet ($p < 0.05$) (Fig. 4a).

The insulin level analyses showed significant differences. The PHSO diet had the highest values in both the LPHSO and HPHSO diets ($p < 0.0001$). The LPHSO diet

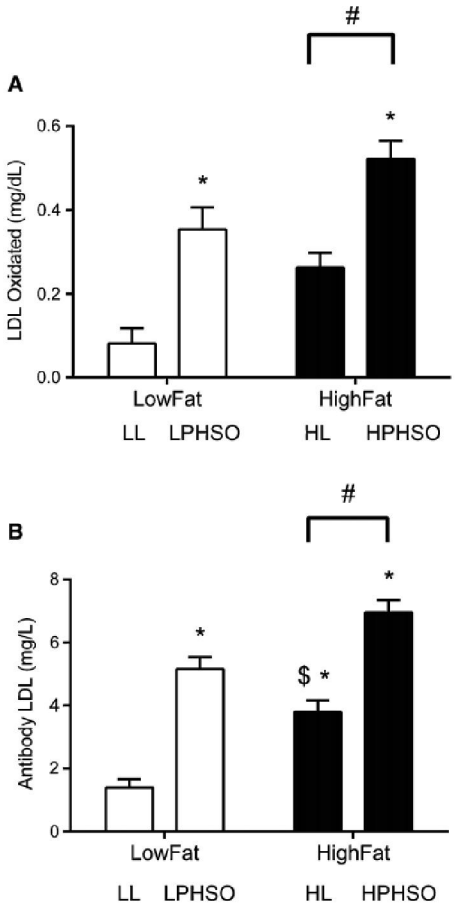

Fig. 2 Oxidized low-density lipoprotein **a** and low-density lipoprotein antibody **b** in CSF samples. $^{*}p < 0.05$ versus control (LL); $^{\#}p < 0.05$, difference between hyperlipidic diet; $^{\$}p < 0.05$, difference between LPHSO and HL only. All groups were analyzed using a two-way ANOVA and Bonferroni post hoc test ($n = 15$ animals/group). Data are expressed as the mean ± SEM and $p < 0.05$

had a higher level of insulin ($p < 0.0001$) than the HL diet (Fig. 4b). The concentrations and diet parameters showed significant differences [$F(1.43) = 23.25$, $p < 0.0001$; $F(1.43) = 161.1$, $p < 0.0001$, respectively].

Transaminase activities, lipid contents and antioxidant enzyme activities in the liver and serum parameters of the kidney

We evaluated the effects of our treatments on the serum parameters of the kidney and the liver after 90 days of intervention. The intervention did not show significant differences in the serum parameters among the experimental groups for urea (Fig. 5a), creatinine (Fig. 5b), ALT (Fig. 5c) and AST (Fig. 5d).

We found significant differences among LL compared with HL and LPHSO and observed significant differences between HL and the other groups in triglyceride analyses; however, the hepatic cholesterol measurement did not differ by experimental group (Table 3). Hepatic antioxidant enzymes had significantly different SOD levels in comparison with LL, and there were significant differences between LPHSO and both LL and HL in the ratio of SOD/CAT + GPx (Table 3).

Serum lipid profiles

After 90 days on the diets, the lipid profiles of the animals were assessed. Total cholesterol presented with altered values. The PHSO group had the highest cholesterol values in both the low and high PHSO diets in comparison with the lard diets ($p < 0.0001$) and was significantly different in all of the parameters analyzed [concentration and diet, $F(1.43) = 27.68$, $p < 0.0001$; $F(1.43) = 367.9$, $p < 0.0001$, respectively, Fig. 6a]. It is important to highlight that the LPHSO diet was associated with higher lipid profile values than the HL diet ($p < 0.0001$) and that no significant difference was observed with the HPHSO (Fig. 6a).

The LDL antibody (Fig. 6b) followed the same trend as total cholesterol: the PHSO diet had the highest concentration ($p < 0.0001$). In this case, there was no significant difference between the LPHSO and HPHSO diets. The PHSO diet had the highest OxLDL (Fig. 6c) concentrations, and similarly to the LDL antibody, there was no significant difference between the LPHSO and the HL diets.

However, HDL had the lowest values (presenting no significant difference) in the PHSO diet in both the LPHSO and HPHSO diets ($p < 0.0001$). The LPHSO had lower HDL values than either of the groups treated with lard (Fig. 6d).

Finally, we observed the effect of different diets on triglyceride levels. The PHSO diet significantly increased the triglyceride content in the blood in both the LPHSO and HPHSO groups ($p < 0.0001$, Fig. 6e).

Cytokine measurements

Rats fed with the PHSO diet showed the highest values of IL1 ($p < 0.0001$ in both concentrations, Fig. 7a) and IL6 ($p < 0.0001$; $p = 0.0004$, Fig. 7b) at both the low and high concentrations compared with the rats fed with the lard diet. It is important to highlight that there were no significant differences between the LPHSO and HPHSO diets for IL1 and IL6, and the values of the LPHSO diet were higher than the HL diet for both IL1 and IL6. However, IL10 (Fig. 7c) had the lowest values for the PHSO diet (low and

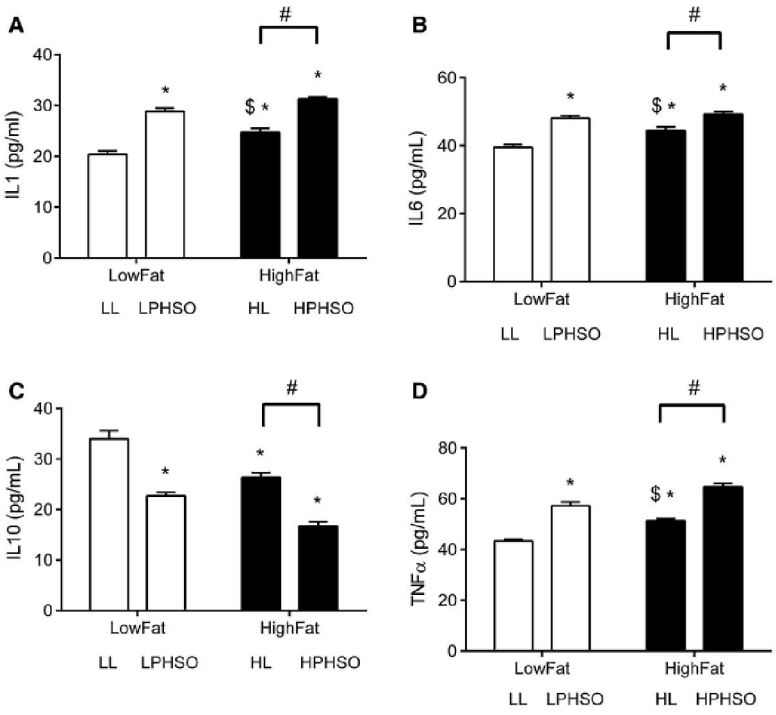

Fig. 3 IL1 (**a**), IL6 (**b**), IL10 (**c**) and TNF-α (**d**) in CSF samples. $^{*}p < 0.05$ versus control (LL); $^{\#}p < 0.05$, difference between hyperlipidic diet; $^{\$}p < 0.05$, difference between LPHSO and HL only. All groups were analyzed using a two-way ANOVA and Bonferroni post hoc test ($n = 15$ animals/group). Data are expressed as the mean ± SEM and $p < 0.05$

high) with $p < 0.0001$. In this case, the LPHSO diet was not significantly different than the HL diet. All treatments showed significant differences compared with the control diet ($p < 0.0001$). Finally, both LPHSO and HPHSO diets had the highest values for TNF-α ($p < 0.0001$, Fig. 7d).

Discussion

Our research focused on characterizing the physiological changes resulting from different diets in an animal model. In this study, body weight gain, CSF parameters, biochemical serum parameters and oxidative stress in hepatic tissue were evaluated in adult Wistar rats across four diets, two normolipidic and two hyperlipidic. Therefore, the metabolic alterations found in this study are likely explained by the different diet compositions, lard and PHSO. This discussion is important because changes in the fatty acid composition of the diet can have major effects on several critical physiological processes [39]. Our idea was to test diets that were similar to those of humans, i.e., small doses of harmful fats as part of a "Western diet." Therefore, we used lard and PHSO in our diet compositions. Some authors suggest that consumption of ruminant fats may impart health benefits, while others claim that the health effects of TFA from ruminant animals are less clear. However, there is considerable concern regarding the health risks associated with consumption of industrial TFA [2, 40].

Contrary to the belief that small doses of harmful fats do not affect health, we consistently showed weight gain with low-fat diets that exhibited the same characteristics as the HFD, which led to changes in most of the parameters evaluated.

CSF parameters

PHSO induced impairment of OxLDL and LDL antibodies

There is growing evidence that lipid metabolism, oxidative stress and inflammatory mechanisms may also participate in the pathogenesis of AD [41]. Kankaanpää et al. [42] commented that the role of antibodies in CSF is unknown and further investigations are needed. Results have shown that CSF IgG antibodies to OxLDL are significantly increased in AD patients and in patients with frontotemporal lobar degeneration compared with controls.

Our OxLDL results (Fig. 2a) demonstrated that in both treatments (PHSO and lard), the highest values were generated by the PHSO diets. Specifically for the LPHSO diet, this concentration of lipids resulted in the same harmful effects as both of the hyperlipidic diets (no significant difference), suggesting that the type of lipid

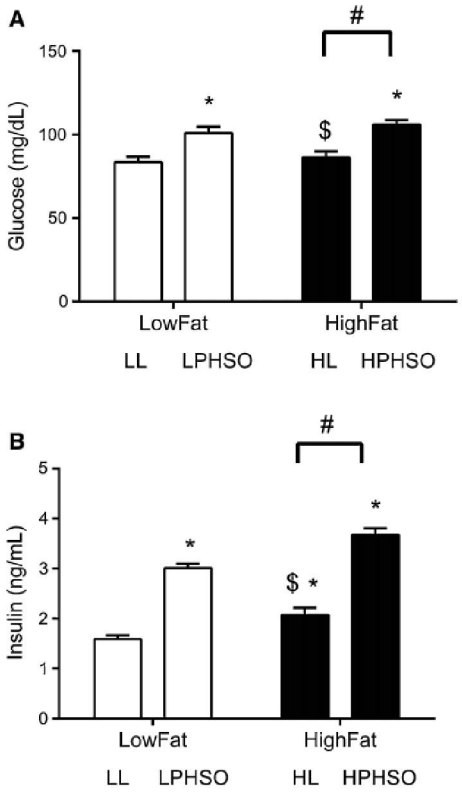

Fig. 4 Glycemic (**a**) and insulin (**b**) responses in blood samples. $^{*}p < 0.05$ versus control (LL); $^{\#}p < 0.05$, difference between hyperlipidic diet; $^{\$}p < 0.05$, difference between LPHSO and HL only. All groups were analyzed using a two-way ANOVA and Bonferroni post hoc test ($n = 15$ animals/group). Data are expressed as the mean ± SEM and $p < 0.05$

is more important than its concentration. Surprisingly, our animal models fed with PHSO in both the low and high lipid concentrations showed the highest LDL antibody values (Fig. 2b). In this analysis, the LPHSO diet elevated the CSF concentration of these parameters more than the HL diet, again supporting a possible inflammatory component to the PHSO diet that is independent of concentration.

Here, we demonstrate a possible relationship between the PHSO diet and increased lipoprotein in the brain. This increase has been linked to neurodegenerative diseases, such as AD. TFA intake may increase the risk of AD and may be associated with accelerated cognitive decline [18, 43, 44]. Grimm et al. [45] reported in vitro mechanisms, suggesting that TFA intake potentially increases AD risk or causes earlier disease onset.

PHSO effects on cytokine levels

Interleukin 1 is believed to mediate neuroinflammation in neurodegenerative conditions, including Alzheimer's disease [46]. IL6 is a potential marker of CNS diseases, and high IL6 levels have recently been reported in the CSF of patients with inflammatory CNS diseases, indicating the possible role of IL6 as a biomarker of inflammatory CNS conditions [47]. As an extension to our findings, we consistently showed elevated IL1 and IL6 in our PHSO diets at both concentrations (Fig. 3a, b).

Recent evidence suggests that IL10 has a notable therapeutic (anti-inflammatory) effect on rat neuropathic pain induced by spinal cord injury, chronic sciatic nerve constriction injury and intrathecal administration of the HIV-1 envelope protein. Following the same trend, animals treated with the PHSO diet had low levels of IL10 in CSF (Fig. 3c), supporting that this inflammatory characteristic is due to the diet to which the animals were exposed [48].

TNF-α is a potent proinflammatory and multifunctional cytokine. Its upregulation was shown to precede leukocyte infiltration to the site of injury [49]. Our animal models treated with PHSO at both concentrations demonstrated elevated TNF-α after 90 days on the diet (Fig. 3d), supporting the hypothesis that the PHSO diet leads to inflammation. To test this idea, Yan et al. [50] proposed that TNF-α could be increased in rats subjected to a combined diffuse brain injury and hypoxia. Our rats only received a dietetic treatment and still showed elevated TNF-α.

Serum parameters

The PHSO diet decreased glucose utilization and insulin sensitivity

Our data demonstrated that treating rats with the PHSO diet led to worse fasting glycemia than the lard diet at both the low and high concentrations, in accordance with the results of Lessa et al. [51] and Duque-Guimaraes et al. [52], in which the fasting glucose of the TFA group was significantly higher than the level of the control group. Our results also showed a strong relationship between TFA and glucose impairment because the LPHSO diet led to significantly higher blood glucose in comparison with HL. Therefore, the quality rather than the quantity of fatty acids consumed should be considered (Fig. 4a). This assay followed the insulin analysis results.

Serum Insulin in the PHSO diet was significantly higher at all concentrations, suggesting a possible inflammatory response to this lipid. Similarly to glucose, the insulin level was significantly higher in the LPHSO group than in the HL group (Fig. 4b). Estadella et al. [53] commented

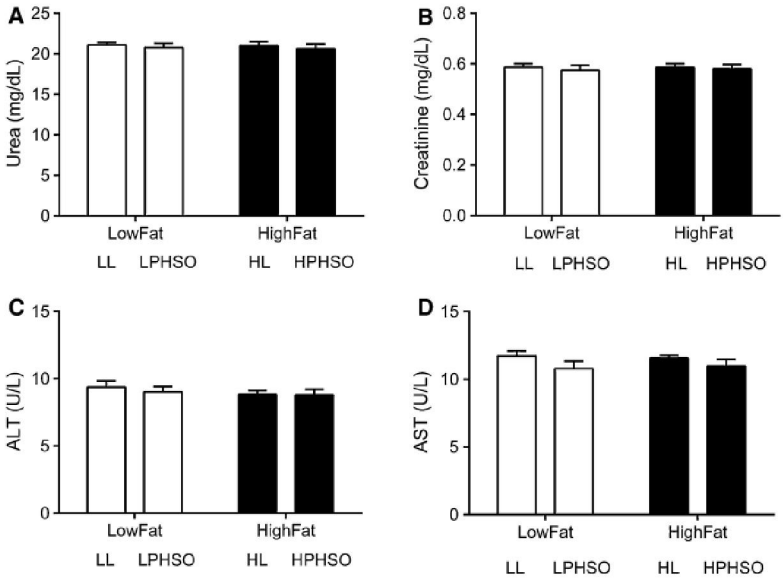

Fig. 5 Urea (**a**), creatinine (**b**), ALT (**c**) and AST (**d**) responses in blood samples. $^{*}p < 0.05$ versus control (LL); $^{\#}p < 0.05$, difference between hyperlipidic diet; $^{\$}p < 0.05$, difference between LPHSO and HL only. All groups were analyzed using a two-way ANOVA and Bonferroni post hoc test ($n = 15$ animals/group). Data are expressed as the mean ± SEM and $p < 0.05$

Table 3 Hepatic lipids and antioxidant enzyme activities

	LL	HL	LPHSO	HPHSO
Hepatic lipids				
Triglycerides (mg of Trig/100 mg tissue)	2.37 ± 0.35^{b}	4.33 ± 0.53^{a}	$1.37 \pm 0.16^{a,b}$	2.53 ± 0.28^{b}
Cholesterol (mg of Chol/100 mg tissue)	0.18 ± 0.02	0.19 ± 0.003	0.15 ± 0.01	0.15 ± 0.003
Hepatic antioxidant enzymes				
Superoxide dismutase (U/mg protein)	34.4 ± 1.69	55.8 ± 2.28^{a}	61.9 ± 1.7^{a}	56.21 ± 2.49^{a}
Glutathione peroxidase (U/mg protein)	28.03 ± 2.85	40.84 ± 2.31	40.01 ± 6.03	44.2 ± 3.51
Catalase (U/mg protein)	128.8 ± 7.75	139.5 ± 9.55	127.07 ± 10.3	123.7 ± 16.55
Ratio SOD/CAT + GPx	0.23 ± 0.02	0.29 ± 0.015	$0.43 \pm 0.03^{a,b}$	0.35 ± 0.05

[a] Different to LL

[b] Different to HL

in their review that TFA ingestion has no effect on fasting plasma glucose, insulin, or oral glucose tolerance in animal models, which contradicted the results of our research and that of Tardy et al. [54]; the Tardy study showed that dietary fatty acids are potential inductors of insulin resistance.

Trans fatty intervention did not alter kidney and liver function (evaluated by serum parameters) but altered lipid contents and antioxidant enzyme activities in hepatic tissue

Nonalcoholic fatty liver disease (NAFLD) is a common condition throughout the world. The pathogenesis of NAFLD is not fully clarified yet. Insulin resistance, oxidative stress and inflammatory signaling have synergistic effects on the pathogenesis of NAFLD [55]. Because our results showed changes in these markers, we evaluated the liver and kidney serum parameters (Fig. 5a–d). Our results did not show significant differences among the different groups for hepatic and kidney serum parameters. However, our results indicated effects of the PHSO diet on hepatic tissue: alterations in triglyceride levels, SOD activities and the ratio of SOD/CAT + GPx in the LPHSO diet (Table 3).

According to Kawano and Cohen [56], a hallmark of NAFLD is accumulation of triglycerides in the cytoplasm of hepatocytes, which is caused by an imbalance between the acquisition and removal of lipids. Cho et al. [57] examined the effects of fat from corn oil. Compared with high *trans* fat in mice, triglyceride accumulation in liver was lower in the corn oil group compared with the *trans* fat group. However, mice were administered either a *trans* fat, standard high-fat or control diet (lard) by Koppe et al. [58] (high-fat and *trans* fat diets were isocaloric). After 8 weeks, the high-fat and *trans* fat groups had similar increases in hepatic triglyceride contents. The authors

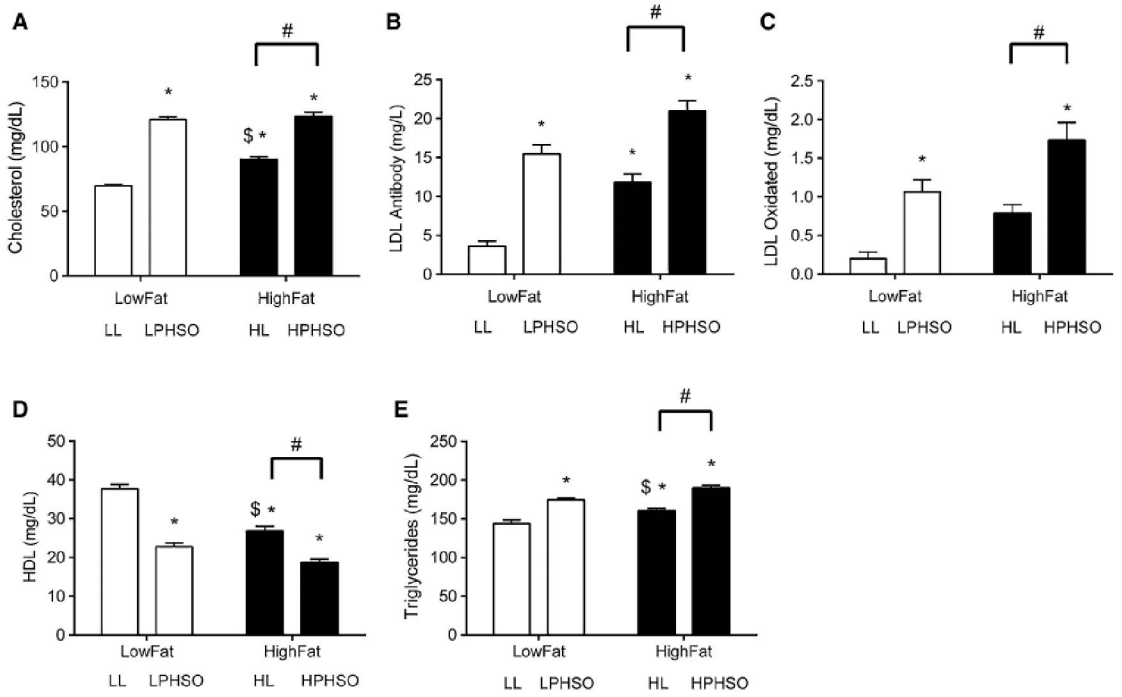

Fig. 6 Total cholesterol (**a**), low-density lipoprotein antibody (**b**), oxidized low-density lipoprotein (**c**), high-density lipoprotein cholesterol (**d**) and triglyceride (**e**) responses in blood samples. $^{*}p < 0.05$ versus control (LL); $^{\#}p < 0.05$, difference between hyperlipidic diet; $^{\$}p < 0.05$, difference between LPHSO and HL only. All groups were analyzed using a two-way ANOVA and Bonferroni post hoc test ($n = 15$ animals/group). Data are expressed as the mean ± SEM and $p < 0.05$

emphasized that the specific impact of *trans* fats on the liver has not been intensely reported in the literature. These results showed different effects of TFA. Accordingly, our results indicated that *trans* diets (low and high) decreased hepatic triglyceride levels compared with lard diets (Table 3).

In relation to hepatic antioxidant enzymes, when SOD activity is high, the conversion of superoxide anion to hydrogen peroxide is facilitated. High SOD activity in conjunction with low GPX activity leads to increased levels of H_2O_2 and H_2O_2-derived reactive species, such as hydroxyl radicals. These activities may play a role in initiating and propagating oxidative damage [59].

Altered lipid profiles and triglycerides with the PHSO diet compared with the lard diet

Our diets produced variations in serum OxLDL levels with the same trend. Treating rats with PHSO led to higher serum OxLDL levels compared with the lard treatment at both the low and high concentrations (Fig. 6c). It is important to note that the LPHSO diet was not significantly different than the HL diet, but was significantly different than the HPHSO diet.

According to, Kankaanpää et al. [27], antibodies to OxLDL have been extensively studied during the past few years in different age-related diseases, especially in atherosclerosis, but their origin, antigenic stimuli, as well as their possible association with and underlying mechanisms behind AD remain to be further discussed.

Total cholesterol and HDL levels were not significantly different between the LPHSO and HPHSO diets, which once again suggests that quality rather than quantity in fatty acid consumption is the determining factor. This is very interesting because the difference between the LL and HL diets was $p < 0.0001$ for both total cholesterol and HDL levels. The total cholesterol and HDL levels showed worse results in the PHSO diet compared with the lard diet for both the low and high concentrations ($p < 0.0001$ for Fig. 6a, d).

Lastly, the triglyceride levels (Fig. 6e) followed the same trend, in which the PHSO diet had the highest levels compared with the lard diet at both the low and high concentrations ($p < 0.0001$ for all). Shah et al [60] showed that

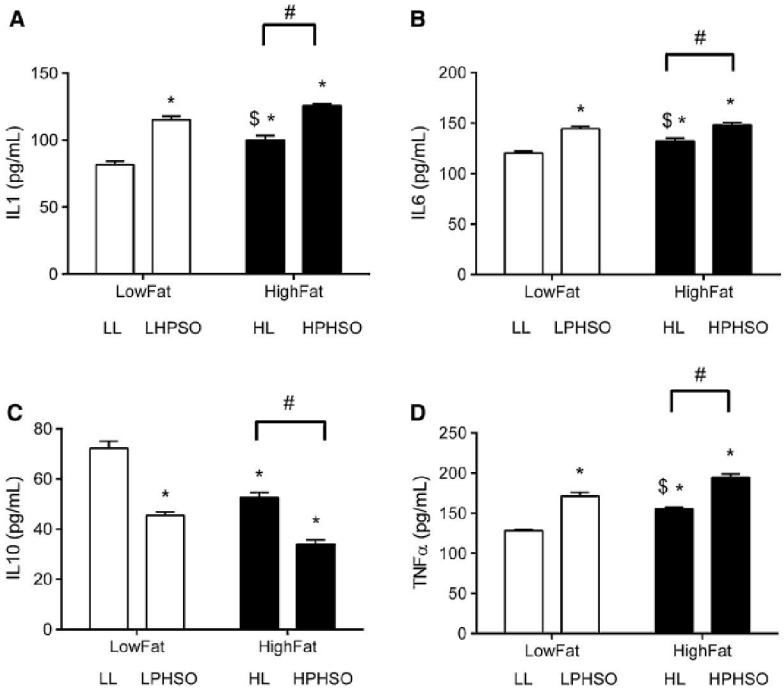

Fig. 7 IL1 (**a**), IL6 (**b**), IL10 (**c**) and TNF-α (**d**) responses in blood samples. $^{*}p < 0.05$ versus control (LL); $^{\#}p < 0.05$, difference between hyperlipidic diet; $^{\$}p < 0.05$, difference between LPHSO and HL only. All groups were analyzed using a two-way ANOVA and Bonferroni post hoc test ($n = 15$ animals/group). Data are expressed as the mean ± SEM and $p < 0.05$

serum triglycerides, total cholesterol and LDL levels were significantly increased with the HFD after eight weeks ($p < 0.05$), which was similar to our results. Likewise, Jia et al. [61] reported that the LDL levels in the HFD group were increased compared with those in the control group after 4 weeks, whereas the levels of HDL in the HFD group at week 4 were significantly decreased compared with those in the control group ($p < 0.05$ in both analyses).

Changes in serum cytokines with the PHSO diet

The PHSO treatment showed the highest values for IL1 with $p < 0.0001$ for both the LPHSO and HPHSO concentrations; there was no significant difference between the LPHSO and the HPHSO diets (Fig. 7a). Due to the link between PHSO and inflammatory processes, our group measured IL1, IL6, IL10 and TNF-α in serum for both the *cis* and *trans* treatments.

According to Maric et al. [62], increases in circulating basal IL6 levels are most commonly associated with obesity. In this study, levels of this cytokine were compared (Fig. 7b), and our results showed a significant increase in basal IL6 levels in the serum of animals treated with the PHSO diet at the low and high concentrations ($p < 0.0001$ and $p < 0.0004$, respectively). Okada et al. [10] reported that a high-TFA diet induces production of proinflammatory cytokines and other markers of inflammation (IL6 and C-reactive protein) without overt inflammation, even in healthy subjects. This information makes our results more interesting because we showed an increase in IL6 in the LPHSO diet and no significant difference with the HPHSO diet. Sarvas et al. [63] and Matthews et al. [64] commented that chronically elevated IL6 mediates inhibitory effects on insulin signaling and glucose metabolism; therefore, IL6 has been linked to insulin resistance in peripheral tissues.

Glucose and insulin levels were altered in our study as well (Fig. 4a, b). However, the role of IL6 in insulin resistance is still unclear and a subject of debate [65]. Cho et al. [66] commented that induction of the proinflammatory cytokines IL6 and TNF-α by a HFD has been shown to induce hepatic inflammation and subsequent cancer development.

In our study, the level of IL10 in the PHSO diet was significantly lower ($p < 0.0001$) at all concentrations in serum (Fig. 7c), and there was no significant difference between the LPHSO and HPHSO diets. According to Hong et al. [67], IL10 affects peripheral glucose metabolism, and preliminary observations suggest it may be a positive regulator of insulin sensitivity. Charles et al. [68] reported that IL10 is negatively correlated with body mass index (BMI), percent fat mass and fasting glucose levels, all of which were modified in our study.

According to Hotamisligil [69] and Ding et al. [70], obesity results in the recruitment of immune cells into adipose

tissue beds, which then secrete inflammatory cytokines, such as TNF-α. Our results showed that the TNF-α level in a PHSO diet was significantly higher ($p < 0.0001$) at all concentrations (Fig. 7d). Again, TNF-α was significantly higher ($p = 0.0078$) in the LPHSO group than in the HL group.

Conclusions

In this study, we observed worsened metabolic responses with diets enriched in *trans* fatty acids (PHSO) compared with diets enriched in *cis* fatty acids (lard). The PHSO diet was associated with impaired serum parameters, cerebrospinal fluid parameters and hepatic antioxidant enzyme activities at both low and high concentrations. Overall, these findings suggest that industrially produced *trans* fatty acids can induce an increased risk of metabolic diseases and a possible risk for brain diseases. This evidence corroborates our primary hypotheses that (1) industrial TFA is worse than lard, and (2) the composition of fat is more important than the quantity of fat consumed in the context of *cis* and *trans* fatty acid diets.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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4. *Artigo II*

Effect of a *Trans* Fatty Acid-Enriched Diet on Mitochondrial, Inflammatory and Oxidative Stress Parameters in the Cortex and Hippocampus of Wistar Rats

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EFFECT OF A *TRANS* FATTY ACID-ENRICHED DIET ON MITOCHONDRIAL, INFLAMMATORY AND OXIDATIVE STRESS PARAMETERS IN THE CORTEX AND HIPPOCAMPUS OF WISTAR RATS.

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Abstract

Purpose

Recent data from our group indicates that dietary *trans* fatty acids (TFAs) may cause systemic inflammation and affect the central nervous system (CNS) in an animal model. We showed a possible relationship among dietary TFA levels and increased levels of cytokines in the cerebrospinal fluid (CSF) and serum[1].

Here, we aimed to clarifying the impact of diets with different TFA concentrations on cerebral tissue in the hippocampus and cortex of adult Wistar rats, focusing on mitochondrial parameters (mass and membrane potential), oxidative stress (based on a DCFH analysis), TNF-α levels and behavioral performance.

Methods

Sixty adult male Wistar rats (n = 15/group) weighting 250–350 g were fed either a normolipidic diet or a hyperlipidic diet for 90 days. Both diets were similar except for fat composition and concentration and total calories. We used lard in the *cis* fatty acid group and partially hydrogenated soybean oil (PHSO) in the *trans* fatty acid group. The treatment groups were [1] Low Lard (LL), [2] High Lard (HL), [3] Low Partially Hydrogenated Soybean Oil (LPHSO), and [4] High Partially Hydrogenated Soybean Oil (HPHSO).

Results

Dietary supplementation with PHSO caused a reduction in mitochondrial mass and membrane potential in the cortex, impaired inflammatory and oxidative parameters in the cortex and hippocampus and resulted in alterations in the open field task performance of Wistar rats.

Conclusions

Overall, these findings suggest that fat composition is more important than the quantity of fat consumed in terms of dietary *cis* and *trans* fatty acids.

Keywords: diet, lipids, brain damage, neurodegeneration.

1.Introduction

There is increasing evidence suggesting that diet, an important modifiable lifestyle factor, may play a role in cognitive decline, such as that seen in Alzheimer's disease (AD), which is a relevant public health problem[2]. Several studies have identified an association between *trans* fat intake and a risk of dementia, including AD [3]. Saturated fatty acids (SFAs) have been reported to induce the production of proinflammatory cytokines, such as interleukin 1 (IL-1), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α), which are major effectors of the neuroinflammatory cascade[4]. Our group has demonstrated that similar

to the effects of SFAs, *trans* fatty acids (TFAs) also induce the production of proinflammatory cytokines in animal models fed normo- and hyperlipidic diets[1]. TFAs are unsaturated fatty acids that contain at least one non conjugated double bond in the *trans* configuration, resulting in a more linear shape. As a result of the reduced fluidity of TFAs, they also have a higher melting point than the corresponding *cis* fatty acids[5]. The presence of a *trans* double bond in a fatty acid chain results in a smaller bond angle, or kink, than in a *cis* double bond, resulting in a fatty acid chain conformation that is more similar to a saturated fatty acid than to an unsaturated fatty acid[6].

Fatty acid (FA) composition is closely related to the biophysical properties of membranes, and has an influence on neurotransmission. Considering the growing consumption of processed foods in Western countries, recent reports have associated their chronic intake with neuropsychiatric diseases[7], cognitive dysfunction[8] and the possible promotion of proinflammatory responses[9].

According to Spielman et al. (2014), an excess in peripheral pro-inflammatory mediators, some of which can cross the blood brain barrier (BBB), may trigger neuroinflammation, subsequently exacerbating neurodegeneration[10]. One of these pro-inflammatory mediators, TNF-α is involved in the regulation of the morphological development of the hippocampus[11] and may also be involved in the decrease of neurogenesis[12].

A plethora of studies have been performed examining the mechanisms connecting neurodegenerative diseases with mitochondrial dysfunction, the overproduction of reactive oxygen species (ROS) by mitochondria, the impairment of mitochondrial fission/fusion inducing alterations in mitochondrial number and mitochondrial morphology, and oxidative damage to DNA, suggesting an important role of mitochondrial dysfunction in the ageing process and neurodegeneration (β-amyloid fragments can target mitochondria and cause

mitochondrial dysfunction in AD)[13-16]. Corona and Duchen (2015) suggest that the sustained integrity of the mitochondrial population is critical for maintaining cellular health, and that a disruption of that integrity is strongly linked to human diseases, such as neurodegenerative disorders[17]. However, little is known about the effects of long-term high fatty diets on brain mitochondria[18].

Due to the observed effects of dietary TFAs on mitochondria and the brain, the aims of this study were to (1) analyze the differences in mitochondrial mass and membrane potential in the brain between distinct dietary treatments, (2) investigate the inflammatory response of TFA levels in brain tissue, (3) investigate the oxidative response of TFA levels in brain tissue, and (4) evaluate how dietary *cis* and *trans* fatty acids affect performance in an open field task in rats.

2. Materials and Methods

2.1. Animal Treatment

Sixty adult male Wistar rats (60 days old, weighing 250–350 g) were divided into 4 groups and were exposed to a 12-hour light/dark cycle (lights on at 7:00 AM) at a constant temperature of 22±1 °C. The rats were housed in plastic cages (5 per cage) with water and food available *ad libitum*. The different groups of rats were fed specific diets as explained below. The behavioral testing procedure was conducted between 9:00 AM and 5:00 PM. Animals were maintained in accordance with the Brazilian Society for Neuroscience and Behavior's recommendations for animal care. All protocols were approved by the Ethical Committee of the Federal University of Rio Grande do Sul (UFRGS).

2.2. Diets

During the 90 days of the treatment, 2 types of fat were used in the diets: i) lard (*cis*

fatty acid group) and ii) partially hydrogenated soybean oil (PHSO) (*trans* fatty acid group - TFA). The TFA levels in the original soybean oil have been shown to range between 0.8% and 2.6%[19]. For this study, after undergoing an industrial hydrogenation processes, the TFA content was 57.3% according to the manufacturer (Table A.2). The normolipidic and hyperlipidic treatment groups were [1] Low Lard (LL), [2] High Lard (HL), [3] Low Partially Hydrogenated Soybean Oil (LPHSO), and [4] High Partially Hydrogenated Soybean Oil (HPHSO). Diets were prepared according to AIN-93 (a diet for rodents recommended by the American Institute of Nutrition) by adding vitamins and mineral salts to ensure that the daily needs of the animals were fulfilled (Tables A.1 and A.2). The fatty acid compositions of the lard were based on dos Santos et al. (2013)[20]. The TFA content in the lard and PHSO diets were (values in g/Kg): LL (0.28), HL (1.44), LPHSO (34.38), and HPHSO (223.4). The calories (calculated based on diet composition, see Table A.1) of each diet were 4230 Kcal (low fat diet) and 5880 Kcal (high fat diet). Pellets were produced every 48 h and stored in a refrigerator.

2.3. Mitochondrial Mass and Membrane Potential Measurements

2.3.1. Tissue Preparation and Measurements of Mitochondrial Mass and Membrane Potential

The brain was rapidly removed from the skull and the cerebral cortex and hippocampus were isolated. Cell suspensions were obtained by mechanical dissociation with PBS containing collagenase. The dissociated contents were then filtered into sterile 50 mL Falcon tubes (BD Biosciences) through a 40-μm nylon cell strainer (Cell Filter Strainer—BD Biosciences) and kept on ice until mitochondrial staining[21].

MitoTracker Red (MTR or Chloromethyl-X-rosamine) and MitoTracker Green (MTG) dyes were employed to assess mitochondrial function. MTR is a lipophilic cationic fluorescent dye that is concentrated inside mitochondria due the negative mitochondrial membrane potential [22]. The loss of membrane potential results in release of MTR from the

mitochondria and a subsequent decrease in fluorescence[23]. MTG is a green-fluorescing fluorophore which is electrophoretically taken up into mitochondria and has been used as a measure of mitochondrial mass independent of mitochondrial membrane potential. MTR and MTG were dissolved in dimethyl sulfoxide (DMSO) to a 1 mM concentration. . Dissociated, filtered cells were stained with 100 nm MTR and 100 nm MTG for 45 min. at 37 °C in a water bath in a dark room according to previously described methods[22, 24], with some modifications. Immediately after staining, cell suspensions were removed from the water bath and analyzed via flow cytometry.

2.3.2. Flow Cytometry Analysis

Samples stained with MTR and MTG dyes were analyzed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). MitoTracker dyes were excited at 488 nm using an air-cooled argon laser. Negative controls (samples without stain) were included for setting up the machine voltages. Control cells stained with a single dye were used to set the compensation values. The emission of fluorochromes was recorded through specific band-pass fluorescence filters: red (FL-3; 670 nm long pass) and green (FL-1; 530 nm/30). Fluorescence emission data were collected using logarithmic amplification. In brief, data from 10.000 events were acquired and the mean relative fluorescence intensity was determined after the exclusion of debris events from the data set. All flow cytometric acquisitions and analyses were performed using CELLQuest Pro data acquisition (BD Biosciences) and FlowJo analysis software. Flow cytometry data were analyzed and plotted by density as a single-parameter histogram, which shows the relative fluorescence on the x-axis and the number of events (cell count) on the y-axis. The samples produced a single distinct peak that can be interpreted as a positive dataset. The histogram was divided into 2 halves (indicated as cells with low and high mass or with low and high potential) based on the controls peak for MTR and MTG, and this evaluation was applied to all data in each parameter for both

analyzed parameters. Analyses resulted in 2 cell populations with different mitochondrial potentials (Δψ) and masses. The first population presented low Δψ and mass and the second, high Δψ and mass[25, 26].

2.4. Cortical and Hippocampal Inflammatory and Oxidative Levels

2.4.1. Cortical and Hippocampal Tumor Necrosis Factor Alpha Levels (TNF-α)

The levels of TNF-α were measured in hippocampal and cortical homogenates, which had been prepared with an extraction kit following the manufacturer's instructions, using a specific rat ELISA kit for TNF-α (PeproTech, Rocky Hill, NJ, EUA). The results are expressed as a percentage of the control.

2.4.2. Cortical and Hippocampal 2′7′-dichlorofluorescein Levels (DCFH)

According to Wang and Joseph (1999), 2′7′-dichlorofluorescein (DCFH) is oxidized to the highly fluorescent dichlorofluorescein (DCF) in the presence of reactive oxygen species (ROS), and the intracellular DCF fluorescence can be used as an index to quantify the overall oxidative stress in cells[27]. This method has been used previously to measure intracellular ROS production[28]. DCFH-DA (2′7′- dichlorofluorescein diacetate) is hydrolyzed by intracellular esterases to dichlorofluorescein (DCFH), which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent DCF by actions the of cellular oxidants. The fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. ROS production was calculated as units of fluorescence (UF/mg protein) and is expressed as a percentage of the control. Slices were analyzes (300μm in 10μM) for 30min.

2.5. Open Field Task

The exploratory activity, locomotor activity and anxiety-like behavior of rats were

evaluated during a 10-min open field (OF) session. The task was performed by placing each individual animal without previous habituation in the center of a square arena (50×50×50 cm, length×width×height) with a black floor and walls. All sessions were recorded by a video camera (positioned above and at 90° to the square arena) connected to a monitor. Videos were blind-scored by 2 trained observers using dedicated software (ANY-maze®). The videos were subsequently placed in randomized order in a separate ANY-maze protocol for the observers, who were blinded to the treatment group, to score using a keyboard-based behavioral tracking system. The total distance travelled (exploratory activity and locomotor activity) and the distance travelled in the center area of the apparatus (anxiety features) were analyzed. After each session, the apparatus was cleaned with 70° alcohol and dried before the next animal was tested[29].

2.6. Statistical Analysis

Data are expressed as the mean ± standard error of the mean (M ± S.E.M.). All brain parameters were analyzed using a two-way ANOVA, followed by *post-hoc* tests. The independent variables were: (1) FA concentration and (2) diet. In all analyses, $p<0.05$ was considered to indicate a significant difference. Open field task results were first analyzed using ANY-maze software and then using a two-way ANOVA, followed by *post-hoc* tests. Ten to fifteen rats in each in group were analyzed, and "$" indicates a significant difference ($p<0.05$) among the behaviors between the individual minutes analyzed in the same treatment.

3. Results

3.1. Cortical and Hippocampal Mitochondrial Mass and Membrane Potential Measurement.

In the cortex, the mean mitochondrial mass (Figure A.1) was not affected by diet. However, the percentage of cells with a high mitochondrial mass in the cortex (Figure A.2)

was affected by the TFA content in the diets: there was a significant decrease in this value in rats fed the LPHSO (p=0.003) and HPHSO (p=0.0105) diets in comparison to those fed the LL diet, reflecting the significant effect of diet on this parameter [diet, F (1.17) = 10.84, p=0.0043].

Following a similar pattern as shown by the cortex analyses, the mean mitochondrial membrane potential (Figure A.3) was lower in rats fed the LPHSO (p=0.0108) and HPHSO (p=0.029) diets than in those fed the LL diet, reflecting the significant effect of diet on this parameter [diet, F (1.17) = 6.54, p=0.0204]. The percentage of cells with high mitochondrial membrane potentials in the cortex followed the same trend: rats fed the LPHSO and HPHSO (p=0.0079 and p=0.0241, respectively) diets had the lowest percentages (Figure A.4) in comparison with the percentage in LL-fed rats, reflecting the significant effect of diet on this parameter [diet, F (1.17) = 8.923, p=0.0083].

The hippocampal parameters were not affected by diet (Figure A.5 and A.6). Therefore, the percentage of cells with high mitochondrial mass and membrane potential were not evaluated in the hippocampus.

3.2. Levels of the Pro-Inflammatory Mediator Tumor Necrosis Factor-α in Hippocampus and Cortex

The PHSO (TFA) diets (both concentrations, $p<0.05$) were associated with higher TNF-α values than the LL diet in the hippocampus (Figure B.1). Concentration and diet were significantly different in this analysis [concentration and diet, F (1.14) = 5.058, p=0.0411; F (1.14) = 76.23, $p<0.0001$, respectively]. Specifically, in the cortex (Figure B.2), both diet and the concentration of lipids in the diets increased the levels of TNF-α [F (1.14) = 9.861, $p<0.0072$; F (1.14) = 6.37, $p<0.0243$, respectively]. In comparison to the values in the LL group, the *p* values in the cortex were as follows: LPHSO=0.0074, HPHSO=0.0078, HL=0.0252.

It is important to highlight that the LPHSO group had the same pro inflammatory profile as the hyperlipidic treatments, specifically in the hippocampus, and that the values were higher than in the HL treatment group ($p=0.0026$), indicating that quality is as important as quantity when the effects of CFAs and TFAs are compared.

3.3. Reactive Oxygen Species (ROS) in Hippocampus and Cortex

After 90 days on the specific diets, ROS content increased in the hippocampus and cortex in relation to TFA content and/or in relation to the overall lipid content (Fig. B.3 and B.4). As seen in the TNF-α results in both the hippocampus and cortex, the LPHSO group had the same oxidative profile as those of the hyperlipidic treatments. Specifically, in the hippocampus (Figure B.3), there was no significant difference between rats fed the LPHSO, HPHSO and HL diets, and there was a significant difference in all of the parameters analyzed [concentration and diet, $F(1.14) = 44.63$, $p<0.0001$; $F(1.14) = 92.45$, $p<0.0001$, respectively]. In the cortex (Figure B.4), the LPHSO group showed the highest ROS content in comparison to that of the LL group ($p<0.0001$), reflecting the significant effect of diet on this parameter [diet, $F(1.14) = 113.7$, $p<0.0001$]. In both analyses, our results suggest that quality as more important as quantity when the effects CFAs and TFAs on ROS levels are compared.

3.4. Open Field Task

For the total distance traveled in the OF tests (Figures C.1), all groups had the same results for the habituation period (measured in the first 3 min) and for locomotion (measured in the last 7 min) in this 10-minute session. When comparing the 1st and the 3rd minutes of the OF task, (Figure C.2) there was apparent habituation to the new environment observed in all groups ($p<0.05$ for all groups). The total distance traveled was also evaluated in relation to locomotion in the central zone (Figure C.3), showing that hyperlipidic diets reduced the tendency for rats to enter into the center of the box. Specifically, the total distance traveled in

the center between the 1st and the 3rd minutes were compared (Figure C.4). The analysis showed no significant difference between these time points in the hyperlipidic groups, pointing to an anxiogenic effect. This effect was not observed in normolipidic groups, which did show a significant difference between the 1st and 3rd minutes (*p* values, LL=0.0301, LPHSO=0.049), indicating habituation to the box.

4. Discussion

In the present study, we evaluated mitochondrial, inflammatory and oxidative brain parameters, as well as performance in an open field task, in Wistar rats fed diets enriched with *cis* and *trans* fats for 90 days. The experimental diets differed only in the FA composition and/or amount (Table A.1. and A.2). Corroborating our previous study[1], herein we show that hyperlipidic diets (*cis* and/or *trans*) promoted increases in inflammatory mediators and oxidative stress markers and resulted in changes in cortical mitochondrial parameters and behavioral alterations.

4.1. Changed Mitochondrial Mass and Membrane Potential with PHSO in the Cortex

Mitochondrial dysfunction is associated with the development of metabolic alterations and is thought to contribute to the pathogenesis of a variety of disorders in humans, including neurodegenerative diseases[30]. The maintenance of energy-dependent processes (ATP) depends on adequate mitochondrial activity in neural cells. The brain is extremely sensitive to hypoxia, particularly in specific brain regions such as the cortex and hippocampus[31]. Increased ROS production in neurodegenerative processes might affect mitochondrial parameters, intercellular ATP levels and membrane potential[32]. Our results demonstrate alterations in the mitochondrial membrane potential in cortex cells (Fig. A.3 and A.4) and in the levels of pro-inflammatory and oxidative stress mediators in response to TFA-enriched diets (Figure B.1 to B.4), suggesting that TFAs can be a risk factor for neurodegenerative processes. For the results from the hippocampus, our findings of no significance differences

are completely in agreement with the OF results. In this case, our results showed no significance difference in the total distance travelled, and only found a difference in the distance traveled in the central zone, suggesting an increase in anxiety without any loss of spatial learning. This makes sense as the relationship between spatial learning and the hippocampus is well documented[33, 34].

In the present work we investigated the effects of dietary lipids on the mitochondrial membrane potential and mass in the cerebral cortex and hippocampus of rats. Changes in both of these parameters have been shown to be risk factors for a diminished bioenergetic activity[35]. Dietary TFAs provoked a reduction in mitochondrial content (deceases in mass and membrane potential) in the cerebral cortex of rats (Figures A.1 to A.4). These data represent an important decrease in the capacity to generate ATP via oxidative phosphorylation in the cortex[36], which is a risk factor for neurodegeneration[37]. Likewise, Chen et al. (2005) have suggested that cells deficient in mitochondrial fusion show a loss of mitochondrial membrane potential and reduced rates of mitochondrial respiration, an critical event in cellular dysfunction[38]. Because our results were demonstrated in response to a chronic treatment, this effect could be due to diminished mitochondrial biogenesis. Cells having less mitochondria are more sensitive to an insult. It is important to remember that mitochondrial dysfunction is a hallmark of neurodegenerative diseases and that mitochondrial biogenesis is associated with neuroprotection[39, 40]. As TFAs are currently a part of our daily meals, dietary habits could be contributing to the neuronal loss that is characteristic of neurodegenerative diseases or brain insult. An elegant review by Lezi and Swerdlow (2012) explains that neurons are relatively intolerant to mitochondrial dysfunction and that an alteration in mitochondrial function is associated with Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS)[37].

4.3. Alterations in Pro-Inflammatory and Oxidative Mediators in Cortex and Hippocampus in

Response to TFA Diets

TNF-α, a potent pro-inflammatory and multifunctional cytokine[41], can be synthesized in the CNS by microglia, astrocytes, and some populations of neurons after brain injury[42]. Our results show that the TNF-α levels in the cortex and hippocampus were significantly higher in rats fed TFA and/or hyperlipidic diets (Figure B.1. and B.2.) It has been shown that elevated levels of TNF-α are associated with the pathological effects observed in neurological, neurodegenerative, and neurotoxic conditions[43].

In their review, Fischer and Maier (2015) commented on two important aspects of TNF. Their results indicated a dual and region-specific role for TNF in the brain: a promoter of neurodegeneration in the striatum and a protector against neurodegeneration in the hippocampus. Specifically, it can contribute to chronic neurodegeneration by promoting the generation and release of ROS[44]. In this study, we showed increases in the levels of TNF-α and ROS in the cortex and hippocampus (Figure B.1 to B.4), and oxidative damage in the brain can cause nervous system impairment, which is a risk factor for depression, anxiety and neurodegeneration[45].

These results could be related to our previous study where we demonstrated an increase in cytokines in the CSF and serum that was caused by dietary TFAs, suggesting that TFAs in the diet may have whole-body effects[1]. Woodcock and Morganti-Kossmann (2013) suggest that TNF-α is produced early the in response to neuronal injury by resident brain cells[41], and Yan et al. (2011) showed that TNF-α is increased in rats subjected to a combined diffuse brain injury and hypoxia but not in response to a diffuse brain injury alone [46]. Gage and Mu (2011) showed that the hippocampus is a brain region critical for learning and memory and is especially vulnerable to damage in the early stages of AD[47], with seems to be in accordance with the results of this study, which indicate dysfunctional mitochondrial activity caused by dietary TFAs (Figures A.1 to A.6).

4.4. Effects of TFAs on Open Field Test Performance

The open field (OF) task is a widely used task for studying locomotor and exploratory behaviors in rats, as well as anxiety-like behaviors (see the review[48]). We analyzed the details the OF task behaviors during the first minutes of the test and over the 10-min session with special attention to possible effects on locomotor habituation and anxiety features. When we analyzed the total distance travelled (Figure C.1), our results showed that all treatments had the same locomotor activity and that habituation to the new environment was not different among the conditions in the first minutes of the task (Figure C.2), showing that all groups were able to learn the task. In relation to the distance travelled in the central zone (exploratory activity), the rats from the hyperlipidic treatment groups did not show significant differences over the first 3 minutes. Both the HL and HPHSO groups exhibited a behavioral aversion to the central zone, with less crosses in the central zone than the other groups, showing a possible aversive response (Figure C.3. and C.4). This indicates that these groups had a fear of exploring new environments, suggesting a possible cognitive impairment as a result of the dietary treatment. According to Koob et al., (2006), the OF score measures an animal's mobility and exploratory behavior, and animals that are impaired may not be able to explore either due to motor or cognitive defects[49]. Our results suggest that dietary TFAs may cause deleterious effects, putatively linking cognitive impairment and/or anxiety-like behavior with altered brain parameters such as i) a decrease in mitochondrial mass and mitochondrial membrane potential in the cortex and ii) an increase in oxidative and inflammatory parameters in the cortex and hippocampus. Interesting, a previous study by our group showed no significant effects of dietary manipulation (as used in this study) on weight gain[1].

5. Conclusion

In this study, we observed worsened metabolic responses in rats fed diets enriched in *trans* fatty acids (TFAs) compared with the parameters in those fed diets enriched in *cis* fatty

acids (lard). TFA-enriched diets affected mitochondrial number and activity, were associated with increased inflammatory and oxidative parameters in the cortex and hippocampus and resulted in altered behavioral parameters. Taken together, these qualitative results provide evidence linking dietary fat composition to the risk of developing neurodegenerative conditions.

Overall, these findings suggest that industrially produced *trans* fatty acids in food can induce an increased risk of brain damage and exacerbate oxidative stress and inflammatory processes in the brain. This evidence supports our primary hypotheses that (1) industrial TFAs are worse than lard in relation to their effects on neurological function and (2) that the composition of fat is more important than the quantity of fat consumed when considering *cis* and *trans* fatty acid diets.

Conflict of interest

The authors declare no conflict of interest.

6. Acknowledgments/Grant Support

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Figure Legends:

Figure A.1. The mean mitochondrial mass in the cortex. Evaluation of the following groups: LL, LPHSO, HL and HPHSO. Two-way ANOVA followed by uncorrected Fisher's LSD *post-hoc* tests were used. No significant differences were observed.

Figure A.2. The percentage of cells with high mitochondrial mass in the cortex. TFA treatments caused a decrease in the percentage of cells with high mitochondrial mass. Evaluation of the following groups: LL, LPHSO, HL and HPHSO. * Represents a significant difference ($p<0.05$) in the TFA treatments in comparison to the LL treatment after a two-way ANOVA followed by uncorrected Fisher's LSD *post-hoc* tests.

Figure A.3 The mean mitochondrial membrane potential in the cortex. TFA treatments caused a decrease in the mean mitochondrial membrane potential. Evaluation of cells in the

following groups: LL, LPHSO, HL and HPHSO. * Represents a significant difference ($p<0.05$) in the TFA treatments in comparison to the LL treatment after a two-way ANOVA followed by uncorrected Fisher's LSD *post-hoc* tests.

Figure A.4. The percentage of cells with high membrane mitochondrial potential in the cortex. TFA treatments caused a decrease in the percentage of cells with high membrane mitochondrial potential. Evaluation of cells in the following groups: LL, LPHSO, HL and HPHSO. * Represents a significant difference ($p<0.05$) in the TFA treatments in comparison to the LL treatment after a two-way ANOVA followed by uncorrected Fisher's LSD *post-hoc* tests.

Figure A.5. Mean mitochondrial mass in the hippocampus. No significant difference was observed after a two-way ANOVA followed by uncorrected Fisher's LSD *post-hoc* tests. Evaluation of cells in following groups: LL, LPHSO, HL and HPHSO.

Figure A.6. Mean membrane potential in the hippocampus. No significant difference was observed after a two-way ANOVA followed by uncorrected Fisher's LSD *post-hoc* tests. Evaluation of cells in following groups: LL, LPHSO, HL and HPHSO.

Figure B.1. Tumor necrosis factor-α (TNF-α) levels in the hippocampus in the following groups: LL, LPHSO, HL and HPHSO. TFAs and/or hyperlipidic diets increased the TNF-α levels in comparison to those of the LL diet. * Represents a significant difference in comparison to the LL treatment. ** Represents a significant difference between the LPHSO and HL treatments. $^{\#}$ Represents a significant difference between both hyperlipidic treatments after a two-way ANOVA followed by Tukey's *post-hoc* tests. All analyses significant at $p<0.05$.

Figure B.2. Tumor necrosis factor-α (TNF-α) levels in the cortex in the following groups: LL,

LPHSO, HL and HPHSO. * Represents a significant difference in comparison to the LL treatment after a two-way ANOVA followed by Tukey's *post-hoc* tests. All analyses significant at $p<0.05$.

Figure B.3. Oxidative stress parameters in the hippocampus in the following groups: LL, LPHSO, HL and HPHSO. TFAs and/or hyperlipidic diets increased the DCFH level (a measure of intracellular ROS production) in comparison to the LL treatment after a two-way ANOVA followed by Tukey's *post-hoc* tests. All analyses significant at $p<0.0001$. * Represents a significant difference.

Figure B.4. Oxidative stress parameters in the cortex in following groups: LL, LPHSO, HL and HPHSO. TFAs and/or hyperlipidic diets increased the DCFH levels (a measure of intracellular ROS production) in comparison to the LL treatment after a two-way ANOVA followed by Tukey's *post-hoc* tests. All analyses significant at $p<0.0001$. * Represents a significant difference.

Figure C.1. Locomotor activity in open field task. All groups presented the same activities during a 10-min session. This figure represents the following groups: LL, LPHSO, HL and HPHSO. No significant difference among the groups after a two-way ANOVA followed by Tukey's *post-hoc* tests. All analyses significant at $p<0.05$.

Figure C.2. Locomotor activity in the open field task. All groups presented habituation to the environment. $ Represents a significant difference between the 1st and the 3rd min in the same group ($p<0.05$) after a two-way ANOVA followed by Tukey's *post-hoc* tests. All analyses significant at $p<0.05$. This figure represents the following groups: LL, LPHSO, HL and HPHSO.

Figure C.3. Exploratory activity in the center of the box in the open field task (a measure of

anxiety-like behavior). This figure represents the distance travelled in the center zone in the OF task in the following groups: LL, LPHSO, HL and HPHSO. Data represent the activity in a 10-minute session. A two-way ANOVA followed by Tukey's *post-hoc* tests ($p<0.05$) was used.

Figure C.4. Exploratory activity in center of the box in open field task (a measure of anxiety-like behavior). There was no significant difference between the 1st and the 3rd min in the hyperlipidic groups after a two-way ANOVA followed by Tukey's *post-hoc* tests ($p<0.05$), indicating a possible anxiogenic behavior. This figure represents the distance travelled in the center zone in the open field task in the 1st and the 3rd min of session in following groups: LL, LPHSO, HL and HPHSO. * Represents a significant difference between the 1st and the 3rd min of the same treatment ($p<0.05$).

Figure A.1.

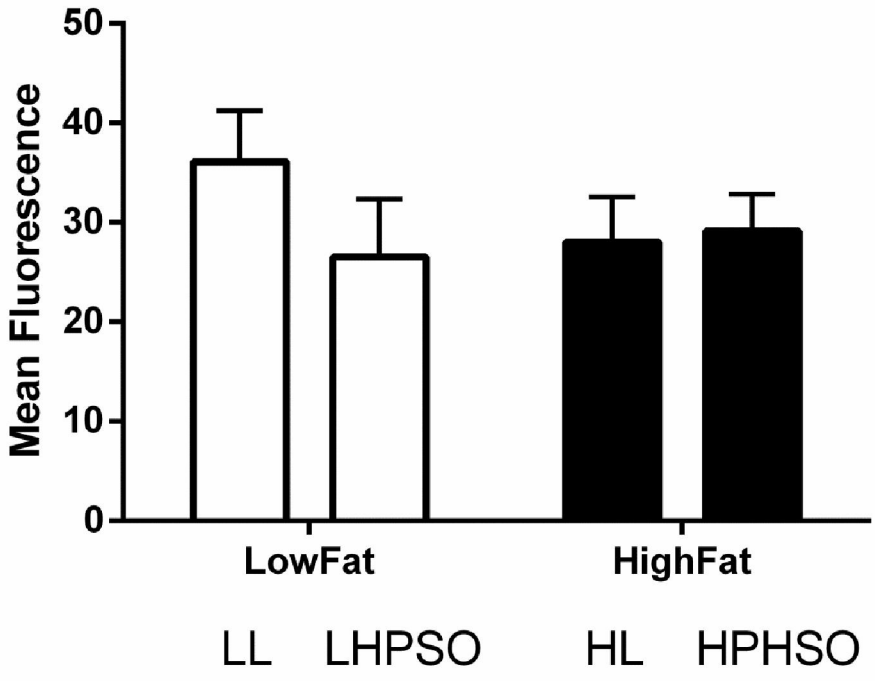

Figure A.1. Mean Mitochondrial Mass

Figure A.2.

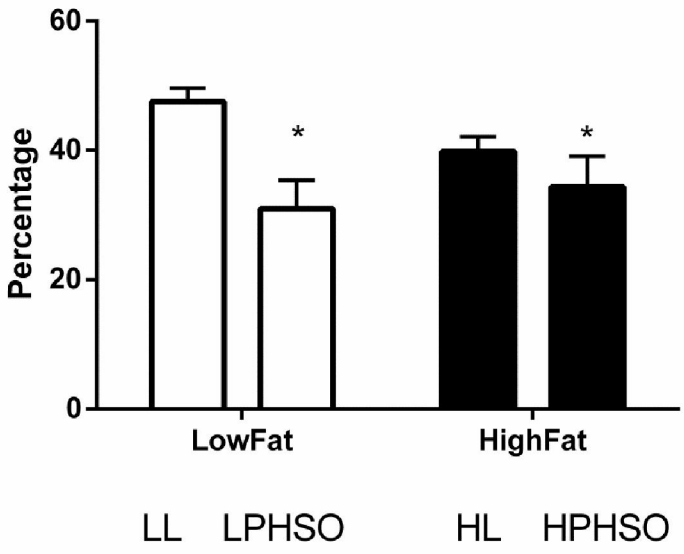

Figure A.2. Percentage of cells with high mitochondrial mass

Figure A.3.

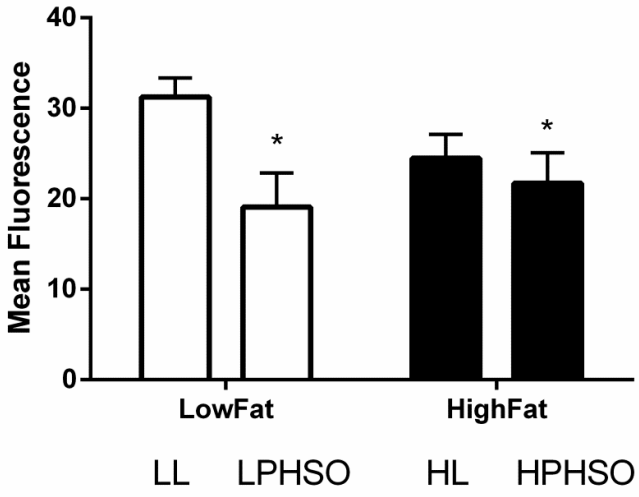

Figure A.3. Mean mitochondrial membrane potential in Cortex

Figure A.4.

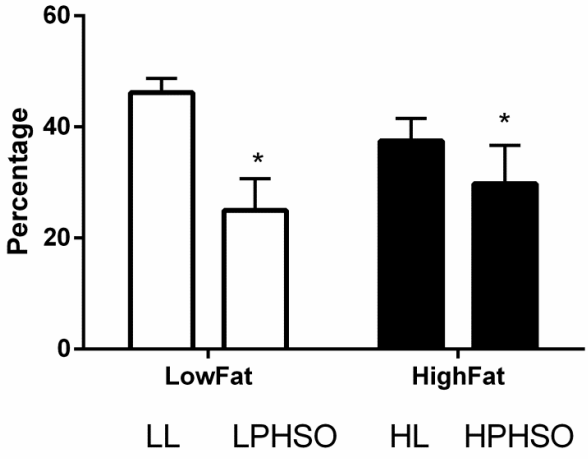

Figure A.4. Percentage of cells with high mitochondrial potential in Cortex

Figures A.5.

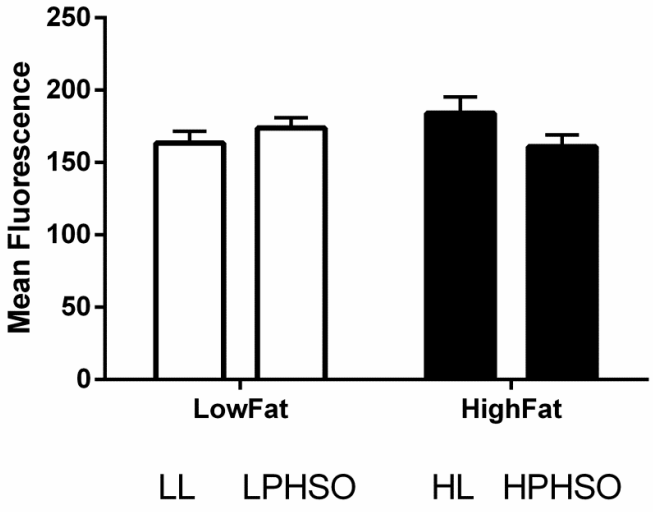

Figure A.5. Mean mitochondrial mass in Hippocampus

Figure A.6.

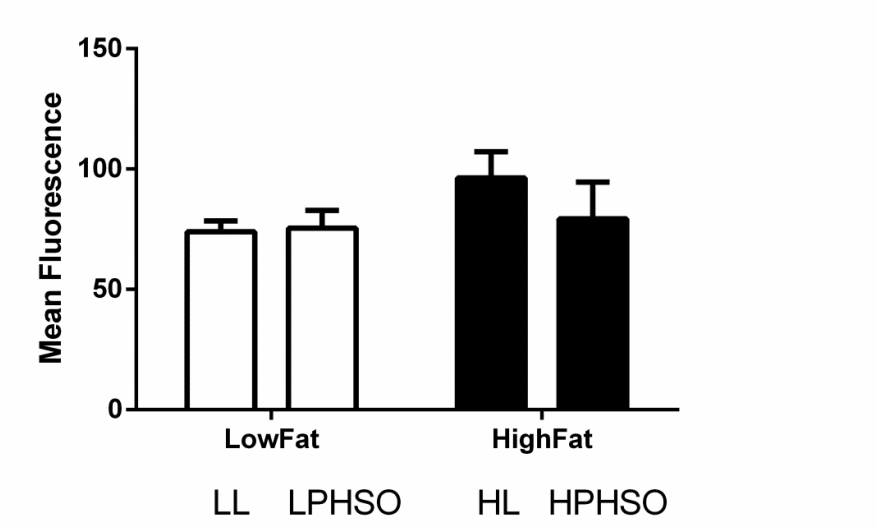

Figure A.6. Mean membrane potential in Hippocampus

Figure B.1.

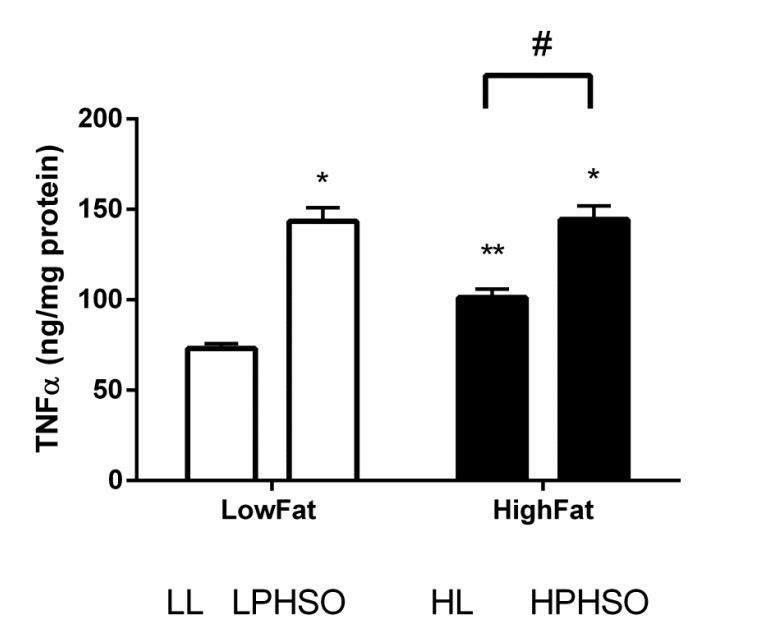

Figure B.1.TNFα in Hippocampus

Figure B.2.

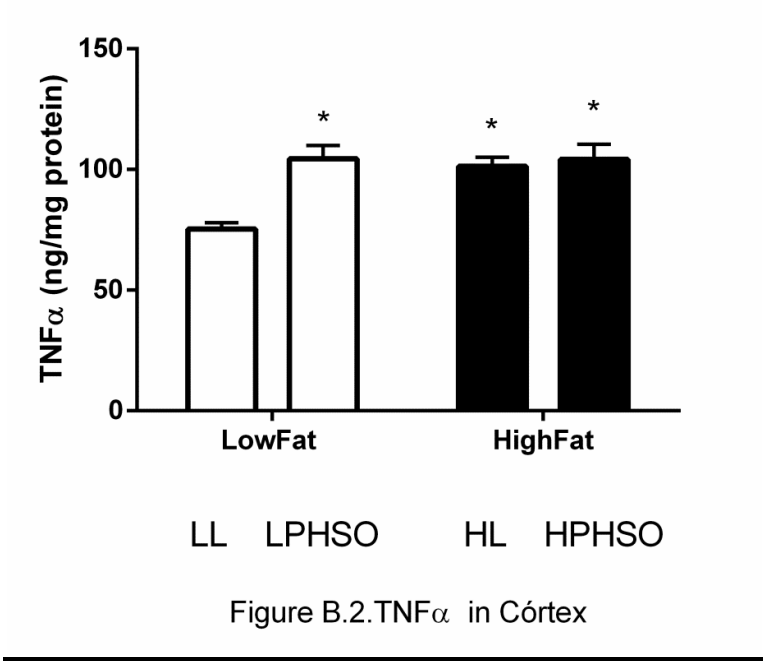

Figure B.2.TNFα in Córtex

Figure B.3.

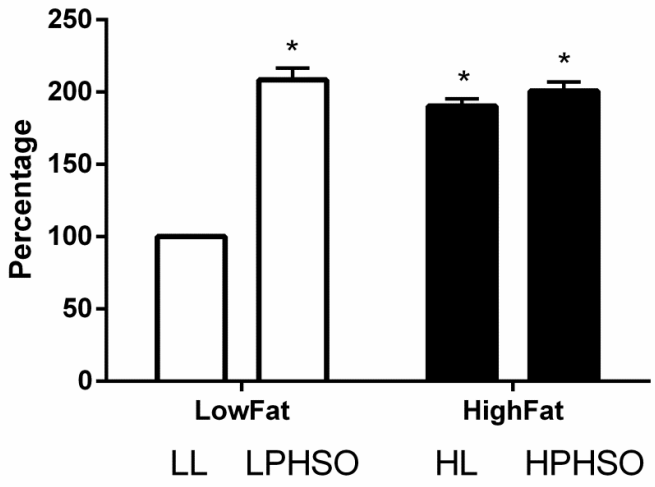

Figure B.3. DCFH in Hippocampus

Figure B.4.

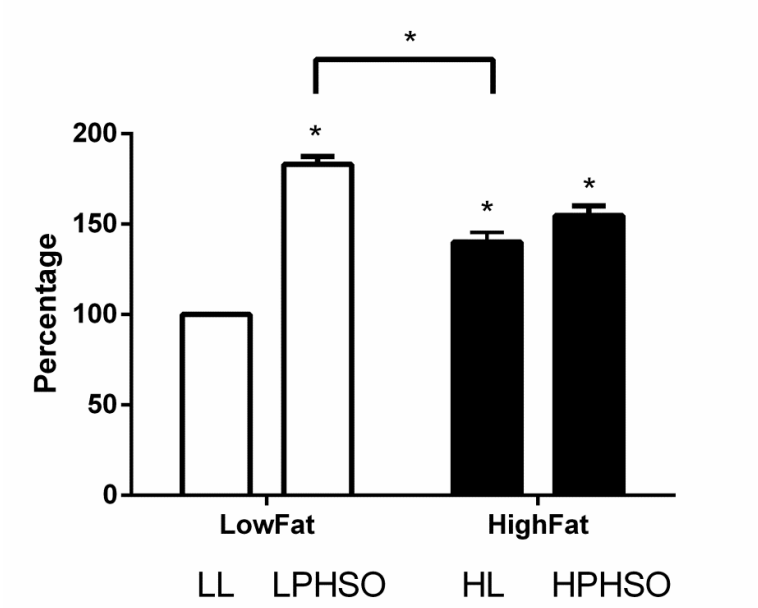

Figure B.4. DCFH in Córtex

Figure C.1.

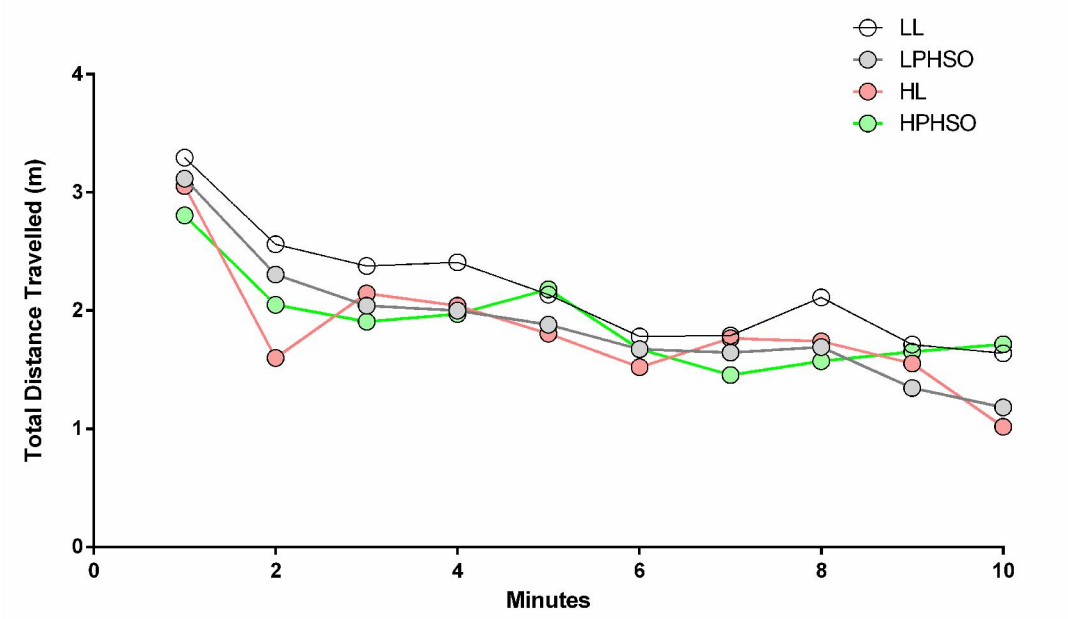

Figure C.1.Total Distance Travelled (m)

Figure C.2.

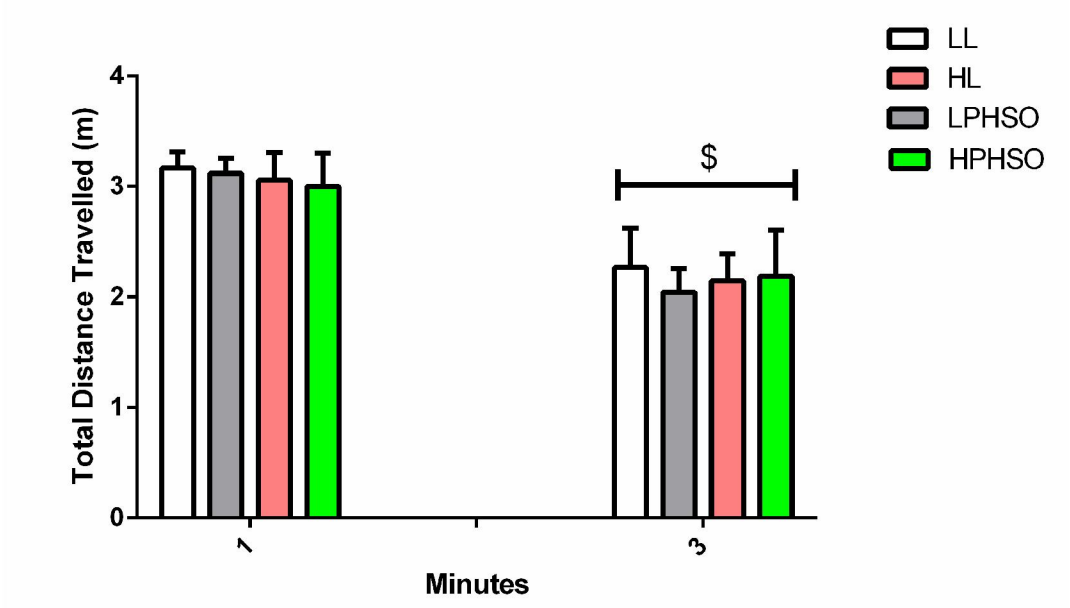

Figure C.2. Total Distance Travelled in the 1st and 3rd minutes of the task

Figure C.3.

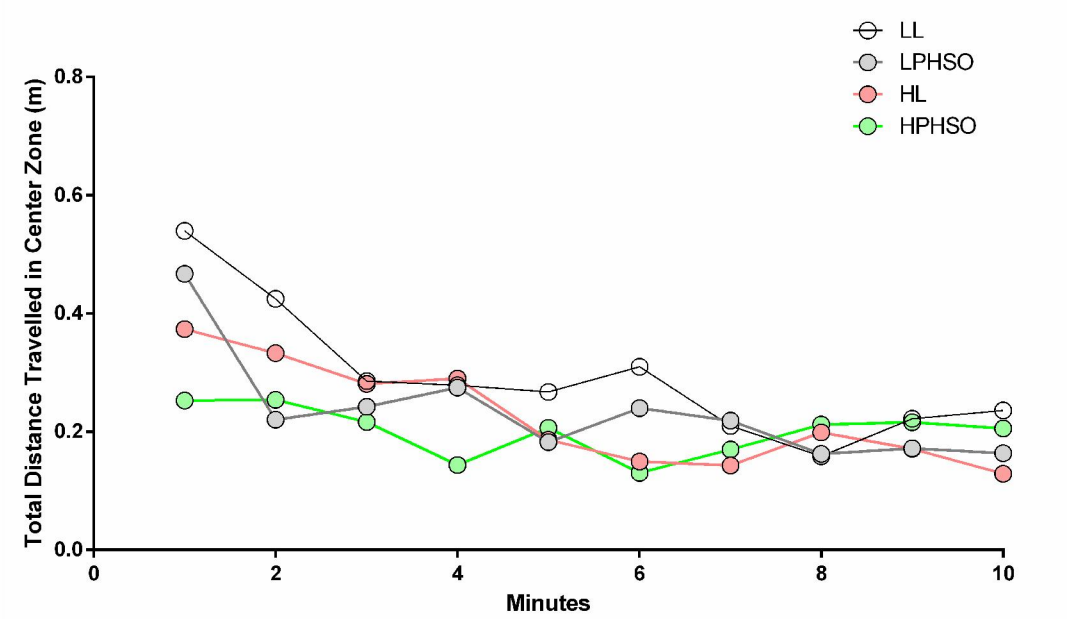

Figure C.3. Total Distance Travelled in Center Zone (m)

Figure C.4.

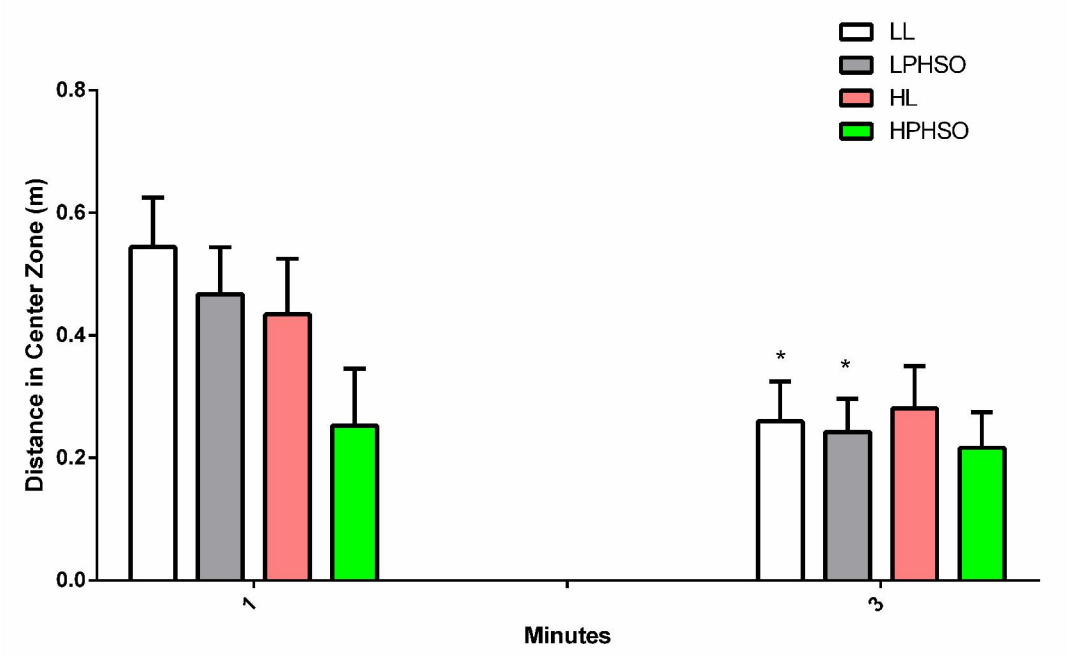

Figure C.4. Distance in Center Zone travelled in the 1st and the 3rd minutes of the task

Tables:

Table A.1: The composition in grams/kg of LL, HL, LPHSO and HPHSO diets.

Composition	LL	HL	LPHSO	HPHSO
Starch	575	250	57	250
Soy Protein	269	264	26	264
Sucrose	50	50	50	50
Vitamin mix[b]	10	10	10	10
Mineral salt mix[c]	20	20	20	20
DL-Methionin[d]	3	3	3	3
DL-Lysin[e]	3	3	3	3
Soy Oil	10	10	10	10
Lard[f] (TFA in grams)	60 (0.28[g])	390 (1.44[g])	-	-
Partially Hydrogenated Soybean Oil content in grams)	-	-	60 (34.38[h])	390 (223.4[h])

Salt and vitamin composition are according to Horwitz, 1980.

[a]Soy protein isolate, purity 97% (from Solae, Esteio, Brazil)

[b]Vitamin mixture: mg/100 g of diet (from Roche, São Paulo, Brazil): vitamin A (retinyl acetate), 4; vitamin D (cholecalciferol), 0.5; vitamin E (DL-a-tocopheryl acetate), 10; menadione, 0.5; choline, 200;
PABA, 10; inositol, 10; niacine (nicotinic acid), 4; pantothenicacid (calcium D-pantothenate), 4; riboflavin, 0.8; thiamin (thiamine hydrochloride), 0.5; pyridoxine (pyridoxine hydrochloride), 0.5; folic acid, 0.2; biotin [D-(+)-biotin], 0.04; vitamin B12, 0.003

[c] Mineral salt mixture: mg/100 g of diet (from Roche, SãoPaulo, Brazil): NaCl, 557; KI, 3.2; KH2PO4, 1556; MgSO4, 229; CaCO3, 1526; FeSO4–7H2O, 108; MnSO4–H2O, 16; ZnSO4–7H2O, 2.2; CuSO4–
5H2O, 1.9; CoCl–6H2O, 0.09

[d] DL-Methionin (from Merk, Rio de Janeiro, Brazil)

[e] DL-Lysine (from Merk, Rio de Janeiro, Brazil)

[f] Lard (from Sadia, São Paulo, Brazil)

[g] (C16:1 9t + C18:1 9t + C18:1 11t) X g /100. Values in the Table 2

[h] C18:1 9t X g/100. Values in the Table 2

Table A.2: Fatty Acid composition of the Partially Hydrogenated Soybean Oil and Lard diet*

Fatty Acids Composition PHSO diet	% Total Fatty Acids	Fatty acids composition in Lard	% Total Fatty Acids
C4	0,0	C14	1,14
C6	0,0	C16	22,7
C8	0,1	C16:1 9t	0,29
C10	0,1	C16:1 9c	1,66
C12	1,6	C17	0,37
C14	0,7	C17:1 10c	0,25
C16	10,8	C18	14,33
C16:1	0,1	C18:1 9t	0,09
C18	17,6	C18:1 11t	0,10
C18:1c	9,0	C18:1 9c	36,38
C18:2c	0,4	C18:1 11c	2,36
C18:3c	0,0	C18:2 9c, 12c (w6)	16,87
C20	0,5	C18:3 6c, 9c,12c	0,05
C20:1	0,0	C18:3 9c,12c,15c	1,67
C22	0,5	C19	0,09
C22:1	0,0	C19:1 7c	0,08
C24	0,0	C20	0,28
C18:1t	57,3	C20:2 11c,14c (w6)	0,61
C18:2t	1,4	C22:4	0,26
C18:3t	0,0		
Total TFA	57,3	Total TFA	0.48

* dos Santos et al., (2013)

Legend

Total TFA: Lard Diet: 0.48 (C16:19t = 0.29; C18:1t = 0.09; C18:1 11t = 0.10)
PHSO Diet: 57.3 (C18:1t)

Parte III

5. Discussão

Atualmente há um consenso mundial na necessidade em se diminuir o consumo de ácidos graxos *trans* (AGT), produzidos pela hidrogenação parcial dos óleos vegetais. Essas recomendações são baseadas em numerosos estudos demonstrando efeitos adversos à saúde a partir do seu consumo, independente da quantidade consumida (Kris-Etherton, Lefevre, et al. 2012).

Tendo em vista que AGT são um problema de grande relevância clínica devido à sua possível relação a doenças como o Diabetes, as cardiovasculares e mais recentemente ao Alzheimer, (Thompson, Minihane, et al. 2011; Morris, Evans, et al. 2003; Nestel, 2014), nossa proposta foi estudar os mecanismos envolvidos na ação desta gordura em modelos animais, investigando marcadores bioquímicos e inflamatórios no líquido cerebroespinhal e em marcadores séricos, ação enzimática e lipídica em tecido hepático, além de marcadores comportamentais e no tecido cerebral. Os resultados obtidos neste trabalho contribuem para avaliar os efeitos dos AGT em modelos animais que receberam dietas com diferentes concentrações do lipídio supracitado, visando o melhor entendimento do seu mecanismo bioquímico.

Os dois capítulos desta tese aprofundam o conhecimento acerca das consequências ao consumo de AGT em condições normolipídicas e hiperlipídicas. As concentrações foram confeccionadas para mimetizar, ao máximo, as características de uma dieta ocidental (ricas em AGT), a qual está relacionada à obesidade e a síndrome metabólica (Manzel, Muller, et al. 2014; Heinonen, Rinne, et al. 2014).

O primeiro capítulo desta tese buscou mostrar a relação dos AGT em marcadores séricos bioquímicos e inflamatórios e de líquido cerebroespinhal, além das enzimas antioxidantes e perfil lipídico em tecido hepático de modelos animais que receberam dietas em diferentes concentrações durante 90 dias. Para tanto, usamos 60 ratos adultos Wistar

pesando entre 250g e 350g (15 ratos/grupo) os quais receberam tratamento dietético a partir da conformação química do lipídio dietético. Dois grupos receberam dieta em concentrações normolipídica e hiperlipídica com gordura de conformação *trans* (gordura vegetal parcialmente hidrogenada) e outros dois grupos receberam dieta em concentrações normolipídica e hiperlipídica na conformação *cis* (banha de porco). A composição das dietas seguiu estudos prévios do nosso grupo, com modificações para garantir as características desta pesquisa (de Assis, Rech, et al. 2012). O segundo capítulo mostrou as ações dos AGT no tecido cerebral. As análises em córtex e em hipocampo foram feitas com o objetivo de avaliar atividade mitocondrial, ação inflamatória e oxidativa da dieta preconizada, além de teste comportamental (Campo Aberto) para avaliar possíveis efeitos ansiogênicos, locomotores, de habituação e de aprendizado após 90 dias de tratamento.

Nos experimentos relacionados ao capítulo 1, após os 90 dias de tratamento, ratos foram anestesiados e levados ao aparato estereotáxico para coleta do líquido cerebroespinhal seguindo protocolo publicado pelo nosso grupo (Almeida, Cereser, et al. 2010), feita a coleta, ratos foram decapitados após as 12h de jejum para a coleta do sangue e soro o qual foi armazenado após centrifugação, além da retirada de todo o tecido hepático (±100mg) para análise da atividade antioxidante e conteúdo lipídico.

Nos experimentos relacionados ao capítulo 2, mostramos os resultados das análises comportamentais feitas pelo Campo Aberto, técnica importante em modelos *in vivo* pois avalia atividade exploratória, locomotora e comportamental em uma sessão de 10 minutos (Prut, Belzung, 2003). Além do teste comportamental, marcadores mitocondriais, inflamatórios e oxidativos em córtex e hipocampo foram analisados a partir da total remoção e isolamento do tecido cerebral. Mitocôndrias foram coradas com MitoTracker Red (MTR) e MitoTracker Green (MTG) (Keij, Bell-Prince, et al. 2000), técnica utilizada para preparar o tecido para posterior análise na citometria de fluxo (Kalbacova, Vrbacky, et

al. 2003; Pendergrass, Wolf, et al. 2004). Nestes testes avaliamos a massa e o potencial de membrana mitocondrial, importantes marcadores de atividade bioenergética (Lukyanova, Kirova, 2015) e de produção energética aeróbia, respectivamente (Hroudova, Singh, 2014). Para análise do estresse oxidativo, avaliamos a presença de diclorofluorescína (DCF), subproduto da 2,7, diclorofluoresceína (DCFH) em córtex e hipocampo, parâmetro usado como um índice de quantificação do estresse oxidativo celular (Wang, Joseph, 1999) e para análises da possível ação pró-inflamatória dos AGT em córtex e hipocampo, medimos os níveis do Fator de Necrose Tumoral Alfa (TNF-α) a partir de kit específico.

Os resultados apresentados no capítulo 1 demonstram que os AGT alteraram o perfil oxidativo e inflamatório no líquido cerebroespinhal e no plasma, nos ratos tratados com AGT em ambas as intervenções, normo e hiperlipídicas, além de desequilíbrios no perfil lipídico. Quando avaliou-se as lipoproteínas de baixa densidade oxidada (LDLOx) e os anticorpos deste LDL (LDL-a), notou-se uma elevação nestes marcadores oxidativos tanto no líquido cerebroespinhal quanto no plasma, importante destacar que a intervenção normolipídica *trans* teve o mesmo comportamento que as intervenções hiperlipídicas em ambas análises.

LDLOx é considerado fator de risco não-tradicional para doenças cardiovasculares, por ser biomarcador para o estresse oxidativo (Gradinaru, Borsa, et al. 2015), um fator de risco para aterogênese (Gradinaru, Borsa, et al. 2015; Trpkovic, Resanovic, et al. 2016; Zelzer, Mangge, et al. 2015 ; Zhong, Zhao, et al. 2015). Outra relação importante ao aumento do LDLOx é a obesidade. Nascimento et al., (2015) e Park et al., (2015) relacionam a obesidade como fator independente ao aumento do LDLOx, onde é importante citar que em nossos resultados não houve diferença significativa entre o ganho de peso nos 4 tratamentos, mas houve uma significante elevação deste biomarcador nas intervenções com a gordura parcialmente hidrogenada (*trans*), ao contrário das intervenções

com gordura *cis*, no nosso caso a banha de porco. Com relação ao líquido cerebroespinhal, os resultados são mais escassos. Kankaanpää et al., (2009), cita o LDLOx como importante regra para a patogênese da DA e mais recentemente, Zhornitsky, et al., (2016) comenta sobre uma possível relação entre esses marcadores lipídicos no líquido cerebroespinhal, como potenciais candidatos a biomarcadores clínicos na Esclerose Múltipla (EM).

Colesterol total, lipoproteínas de alta densidade (HDL-c) e triglicerídeos também tiveram seus valores alterados nas condições hiperlipídicas e, especificamente, na dieta normolipídica enriquecida com AGT. Sabidamente, a dislipidemia é amplamente estudada e caracterizada como fator de risco para doenças cardiovasculares, esteatose hepática não-alcoólica (NAFLD), dentre outras doenças crônicas não transmissíveis (DCNT) (Cohen, Fisher, 2013; Chang, Wu, 2013; Sabatine, 2016). Destacando o caráter inovador desta tese, nossos resultados mostraram distúrbios dislipidêmicos em ratos Wistar alimentados com dietas normolipídicas enriquecidas com AGT, ao contrário da maioria dos artigos, até agora publicados, onde mostram esta relação apenas em dietas hiperlipídicas (Ebrahimi, Behdad, et al. 2015; Uetake, Ikeda, et al. 2015; Cai, Xi, et al. 2015; Zhang, Liu, et al. 2015). Outro caráter inovador refere-se ao artigo publicado por Jørgensen et al., (2015), no qual o autor oferece ao longo de 12 meses uma dieta hiperlipídica a ratos machos Wistar (os mesmos usados em nossos experimentos) rica em óleo de soja e banha de porco, ou seja, os mesmos ingredientes que foram utilizados em nossos grupos *cis*. Sua conclusão comenta significantes alterações na respiração celular mitocondrial no músculo esquelético, mas sem alteração em mitocôndrias do tecido cerebral analisado (cérebro com remoção apenas do cerebelo). No capítulo 2 desta tese avaliamos atividade mitocondrial em córtex e hipocampo e encontramos alterações significativas em córtex tanto em ratos alimentados com dietas hiperlipídicas quanto ratos que receberam dietas normolipídicas enriquecidas com AGT, dessa forma, sugere-se um caráter tóxico dos AGT independente da quantidade

consumida.

Quando avaliamos os marcadores de insulina e glicose, nossos resultados mostraram um aumento na glicemia e na insulina em ratos alimentados com dietas enriquecidas com AGT tanto em condições normo quanto em condições hiperlipídicas. Seguindo a mesma linha do caráter tóxico da intervenção *trans*, Manco et al., (2004) cita uma relação entre o aumento de ácidos graxos circulantes a desequilíbrios no transporte da glicose e sensibilidade à insulina, essa teoria baseia-se no acúmulo de triglicerídeos em tecidos não-adiposos gerando transtornos ao bom funcionamento pancreático. Cabe salientar que a referida teoria tem como elemento chave o consumo excessivo de lipídios, contrapondo nossos resultados, onde a dieta normolipídica *trans* mostrou desequilíbrios mais agressivos do que a dieta hiperlipídica *cis* e sem diferença significativa a sua análoga hiperlipídica.

Haag e Dippenaar (2005), ratificam a ideia de que o ácidos graxos são um importante fator de risco para o desenvolvimento de resistência à insulina, em sua revisão citam gorduras saturadas como os principais constituintes a esse transtorno metabólico. Especificamente se tratando de AGT, revisões citam que não há evidências de que o consumo de AGT possa gerar sensibilidade à insulina em pessoas saudáveis, tanto em estudos com animais quanto em estudos com humanos, mas reforçam a ideia de que a dieta ocidental pode ser um possível fator de risco (Risérus, 2006). Arnold et al.,(2014) relacionou resistência à insulina com declínio cognitivo a partir de seus resultados com camundongos alimentados com dieta hiperlipídica por 17 dias e esses dados corroboram com nossos resultados, pois encontramos desequilíbrios na insulina e possíveis transtornos cognitivos a partir dos resultados comportamentais no campo aberto (artigo 2).

Seguindo a mesma tendência, nossos marcadores inflamatórios (IL1, IL6, IL10 e TNF-α) também mostraram alterações mais significativas em ratos com a dieta enriquecida com AGT tanto no líquido cerebroespinhal quanto no plasma.

Importante marcador pró-inflamatório, a Interleucina-1 (IL1) é uma família de 11 membros dos quais incluem a IL1α, IL1β, IL18, IL33 dentre outras, mas todas com ação pró-inflamatórias (Dinarello, 2007). Seu mecanismo de ação está relacionado principalmente à proliferação e diferenciação celular, onde sinaliza diversas doenças inflamatórias pela iniciação e potencialização de respostas inflamatórias e imunes (Akdis, Burgler, et al. 2011). Especificamente em relação ao Sistema Nervoso Central (SNC), Gosselin e Rivest (2007) citam a IL1 como uma molécula extremamente importante, primeiro por ser efetiva em funções neuronais, comportamentais, neuroendocrinológicas e metabólicas e segundo, por ser capaz de modular funções cerebrais durante insultos inflamatórios localizados ou sistêmicos, como DA, EM e doença de Parkinson (DP) (Andre, Lerouet, et al. 2005).

Um dos mais importantes fatores neuroimunes, a IL6 também mostrou elevações em nossos resultados com dietas enriquecidas com AGT, esta interleucina tem sido envolvida ao desenvolvimento fisiológico do cérebro e a insultos neurológicos como esquizofrenia, DA e depressão, onde altos níveis aparecem sob condições inflamatórias (Wei, Alberts, et al. 2013; Lebherz, Kahles, et al. 2016). Hao-Ho, et al., (2015) avaliou a relação entre a inflamação periférica de alguns marcadores inflamatórios como a IL6 e o TNFα, induzida via lipopolissacarídeo de *E. coli* (LPS), e a neuroinflamação e o estresse oxidativo em hipocampo, seus resultados corroboram com os nossos (salientando que usamos uma ferramenta dietética), nos quais também obtivemos elevações desses marcadores tanto em líquido cerebroespinhal quanto no plasma e também exarcebação nos processos inflamatórios e oxidativos em hipocampo (discutidos no Artigo 2). Em modelos animais, TNFα tem seu aumento relacionado tanto a eventos agudos quanto a crônicos sugerindo forte relação a transtornos neurodegenerativos (Gosselin, Rivest, 2007). Baek, et al., (2013) mostra em seus resultados uma relação ao aumento do TNFα sérico com o uso de dietas

hiperlipídicas (60% das calorias provenientes de lipídios, não especificado pelo autor) em camundongos durante 12 semanas, nossos modelos animais tratados com dietas normolipídicas de conformação *trans* tiveram o mesmo comportamento inflamatório (elevação de TNFα sérico) com apenas 90 dias de tratamento. A ação pró-inflamatória dos AGT em condições normolipídicas apareceram em todos nossos marcadores inflamatórios, inclusive na IL10, onde houve significativa queda deste marcador, que sabidamente é conhecido como um fator anti-inflamatório, regulador de diversos aspectos da resposta imune (Baek, Hwang, et al.2013).

Com a finalidade de ampliar a investigação sobre os mecanismos de ação envolvidos na toxicidade do AGT, no artigo 2 propusemos investigar marcadores inflamatórios e oxidativos no SNC e uma possível relação a transtornos comportamentais. O excesso de mediadores pró-inflamatórios periféricos podem atravessar a barreira hematoencefálica (BHE), podendo ser um gatilho a neuroinflamação, subsequentemente, potencializando a neurodegeneração (Spielman, Little, et al. 2014).

De acordo com Kann e Kovács (2007), a saúde dos neurônios está intimamente dependente da função mitocondrial e suprimento de oxigênio, desta forma, a disfunção mitocondrial é fator de risco para a neurodegeneração. Nossos resultados apontam para uma queda na massa e no potencial de membrana mitocondrial em córtex de ratos alimentados com AGT nas duas concentrações. Diversos artigos apontam uma relação entre saúde mitocondrial e neurodegeneração, Stevens et al., (2015), avaliou a relação da massa mitocondrial a patogenia da DP, usando camundongos adultos *knockout-parkin*, seus resultados condicionam uma redução no número de mitocôndrias, e claro, da massa mitocondrial como fator de risco para DP, pelo fato de o gene causador da DP, o *Parkin,* regular a biogênese mitocondrial. Outros autores creditam a disfunção mitocondrial e o estresse oxidativo como importantes fatores para perda neuronal na patogênese das doenças

neurodegenerativas, como a DP e a DA, ou até mesmo uma diminuição na capacidade energética da célula (Stevens, Lee, et al. 2015; Tyurina, Polimova, et al. 2015; Lin, Liou, et al. 2009;).

Assim como a massa mitocondrial, o potencial de membrana também gera disfunção mitocondrial no tecido lesado. Wang et al., (2009), sugere o potencial de membrana como indicativo dessa disfunção mitocondrial. Onyango et al., (2010), cita que o envelhecimento mitocondrial cerebral está relacionado com a diminuição das atividades dos complexos I e IV da respiração celular gerando diminuição do potencial de membrana e o aumento na produção de Espécies Reativas de Oxigênio (ERO). Esta conclusão corrobora com nossos resultados, mostramos uma elevação das ERO em ratos tratados com AGT em ambas concentrações, além da disfunção mitocondrial de córtex, conforme supracitado. Estresse oxidativo e cérebro possuem uma estreita ligação, em clássico artigo de Halliwell (2006), o autor comenta que o dano oxidativo, o processo inflamatório e a disfunção mitocondrial são o foco principal para a morte neuronal. Assim como a elevação dos marcadores de estresse oxidativo em dietas enriquecidas com AGT, nossos resultados também atestaram um aumento de TNFα em córtex e hipocampo desses ratos, que segundo Fisher e Maier (2015), é um importante marcador para doenças neurodegenerativas e também está associado a memória e depressão, ou seja, ratificando o caráter inflamatório desta intervenção.

Nas questões comportamentais, avaliamos o caráter locomotor, exploratório e de habituação no campo aberto, durante uma sessão de 10 minutos e os resultados não mostraram diferença significativa nas características locomotoras dos modelos animais nos quatro tratamentos, bem como, a habituação ao novo ambiente. Houve diferenças quando avaliamos o perfil desses ratos em questões exploratórias na periferia e no centro do *box*, ratos tratados com dietas hiperlipídicas tiveram mais aversão ao centro do *box*, que segundo Koob et al., (2006) pode estar relacionado a déficit motor ou cognitivo, como nossos ratos

tiveram o mesmo perfil locomotor, sugere-se um possível dano cognitivo.

Para concluir, esta tese representou um importante avanço na contribuição para o entendimento dos mecanismos bioquímicos dos Ácidos Graxos *Trans* em modelos animais. Embora ainda existam dúvidas quanto à toxicidade desta gordura no organismo, esta tese deu um passo muito importante demonstrando que os AGT desequilibraram marcadores inflamatórios, oxidativos no líquido cerebroespinhal e no plasma, além da ação no tecido hepático e no córtex e no hipocampo através de avaliações de atividade mitocondrial, de estresse oxidativo, perfil inflamatório e comportamental, independente da quantidade consumida.

6. Conclusões

Os dados obtidos ao longo desta tese permite concluir que:

1. O tratamento com dietas enriquecidas com ácidos graxos *trans* alteraram marcadores oxidativos, inflamatórios, lipídicos e de tecido hepático, tais efeitos foram mediados pelos seguintes marcadores (Artigo 1):

- Aumento na atividade da LDLOx e da LDL-anticorpo tanto em líquido cerebroespinhal quanto em marcadores séricos nos modelos animais tratados com dietas enriquecidas com AGT em ambas concentrações;

- Alteração nos marcadores inflamatórios IL1, IL6, IL10 e TNFα, onde a dieta normolipídica enriquecida com AGT teve o mesmo comportamento que as dietas hiperlipídicas, especificamente nas análises de IL1, IL6 e TNFα, e o comportamento da dieta normolipídica *trans* foi mais agressivo do que a dieta hiperlipídica *cis*, em ambos líquidos cerebroespinhal e marcadores séricos;

- Alteração em glicose e insulina nos tratamentos enriquecidos com AGT, mostrando que a qualidade da dieta é mais importante que a quantidade, pois a dieta normolipídica *trans* gerou aumentos de glicose e de insulina em maiores teores do que a dieta hiperlipídica *cis*;

- Modulação dos marcadores lipídicos com dietas enriquecidas com AGT, redução do HDL-c e aumento de colesterol total, LDL-c e triglicerídeos em ambas as intervenções enriquecidas com AGT.

2. O tratamento com dietas enriquecidas com ácidos graxos *trans* alteraram marcadores oxidativos e inflamatórios no sistema nervoso central, além de alterar

atividade mitocondrial e marcadores comportamentais (Artigo 2):

- Alteração na atividade mitocondrial em córtex pela diminuição da massa e do potencial de membrana mitocondriais em ambas as dietas enriquecidas com AGT;

- Alteração no perfil inflamatório em córtex e hipocampo pelo aumento do marcador TNFα em ambas as dietas enriquecidas com AGT;

- Alteração na exposição ao estresse oxidativo em dietas enriquecidas com AGT, tanto em córtex quanto hipocampo pela elevação do marcador DCF

- Dietas hiperlipídicas tiveram uma alteração no perfil comportamental no campo aberto pela diminuição às passagens no centro do *box* desde o primeiro minuto do teste, sugerindo um possível declínio cognitivo.

7. Perspectivas

- Avaliar os possíveis efeitos do ômega 3 como estratégia neuroprotetora preventiva e/ou de diminuição dos marcadores avaliados;

- Avaliar os possíveis efeitos do resveratrol como estratégia neuroprotetora preventiva e/ou de diminuição dos marcadores avaliados;

- Avaliar os possíveis efeitos do exercício como estratégia neuroprotetora preventiva e/ou de diminuição dos marcadores avaliados;

- Monitorar o efeito sinérgico do exercício juntamente com o ômega 3, ou junto resveratrol ou o exercício e os dois suplementos alimentares na resposta aos insultos gerados neste trabalho.

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