UNIVERSIDADE DE SÃO PAULO
FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Programa de Pós-graduação em Ciência dos Alimentos
Área de Nutrição Experimental

Efeito do uso combinado de ácidos graxos ômega 3 e
fitosteróis sobre o perfil lipídico, estresse oxidativo e
biomarcadores de inflamação

Patrícia Borges Botelho

Orientadora:
Prof^a. Dr^a. Inar Alves de Castro

**São Paulo
2012**

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Tese apresentada à Faculdade
de Ciências Farmacêuticas da Universidade de
São Paulo para a obtenção do grau de DOUTOR

Orientadora:
Prof^a. Dr^a. Inar Alves de Castro

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AA	Ácido araquidônico
ABCA1	<i>ATP binding cassette 1</i>
ABCG5/ABCG8	<i>ATP binding cassette (ABC) transporters G5 and G8</i>
ACAT	Acetil-Coenzima A acetiltransferase
ACO	Acyl- CoA oxidase
AHA	<i>American Heart Association</i>
ALA	Ácido α -linolênico
AVC	Acidente vascular cerebral
COX-2	Ciclooxigenase 2
CRP	Proteína C reativa
CVD	Doenças cardiovasculares
EPA	Ácido eicosapentaenoico
FDA	<i>Food and Drugs Administration</i>
HDL	<i>High density lipoprotein</i>
IL-1	Interleucina – 1
IL-6	Interleucina 6
IL-18	Interleucina – 18
LDL	<i>Low Density Lipoprotein</i>
LNA	Ácido linoleico
LOX	Lipooxigenases
LXR	Receptor X do fígado
N-3 FA	Ácidos graxos poliinsaturados ômega 3
N-6 FA	Ácidos graxos poliinsaturados ômega 6
NF κ B- $\text{I}\kappa$ B	Complexo fator nuclear κ B – $\text{I}\kappa$ B kinase
NPC1L1	Niemann- Pick C1-like protein
PGE2	Prostaglandinas E2
PPAR α , γ	<i>Peroxisome Proliferator Activated Receptor α e γ</i>
SDA	Ácido estearidônico
SREBP-1c	Proteína ligante do elemento regulado por esteróis 1c
TG	Triacilglicerol
TNF- α	Fator de necrose tumoral – alfa
VLDL	<i>Very low density lipoprotein</i>

RESUMO

A aterosclerose é um processo inflamatório que se inicia na infância e progride com a idade, sendo o principal processo patológico que culmina nas doenças cardiovasculares. Ácidos graxos ômega 3 (N-3 FA) reduzem triacilgliceróis plasmáticos e inflamação, enquanto fitosteróis possuem ação hipocolesterolêmica. Portanto, o objetivo deste estudo foi de aplicar a combinação dessas duas classes de lipídios bioativos na infância, visando reduzir a aterosclerose na fase adulta. O estudo foi dividido em duas etapas. Inicialmente, o efeito de três ingredientes contendo N-3 FA foi avaliado em termos de perfil lipídico e biomarcadores inflamatórios. Camundongos adultos LDLr Knockout receberam uma dieta hiperlipídica e foram simultaneamente suplementados com água (CON), óleo de algas (ALG), óleo de peixe (FIS) e óleo de *Echium* (ECH) através de gavagem durante 4 semanas. Os animais suplementados com óleo de *Echium* apresentaram os maiores índices de redução de triacilgliceróis e VLDL, além de inibição da esteatose causada pela dieta hiperlipídica. Entretanto, diferente dos óleos marinhos, tais efeitos não envolveram ativação de fatores de transcrição envolvidos no metabolismo lipídico, como PPAR α e LXR α hepáticos. Na segunda etapa deste estudo, camundongos Knockout LDLr recém-desmamados foram suplementados durante 2 meses com emulsões preparadas com óleo de soja (CON), óleo de *Echium* (ECH), óleo de alga (ALG), fitosteróis isolados (PHY), óleo de alga + fitosteróis (ALG+PHY) e óleo de *Echium* + fitosteróis (ECH+PHY). A seguir, dislipidemia e estresse oxidativo foram induzidos através de uma dieta hiperlipídica por mais 2 meses. Todos os animais apresentaram estrias gordurosas na aorta, sendo que a área de lesão foi maior naqueles suplementados com fitosteróis isoladamente (PHY). Entretanto, esse efeito negativo foi totalmente revertido pela co-suplementação com N-3 FA. Observou-se que os fitosteróis isolados agiram como agonistas de LXR α , e que a reversão proporcionada pelos N-3 FA envolveu aumento da expressão do fator de transcrição PPAR α e redução do aumento de LXR α . Além disso, o óleo de *Echium* reduziu o estresse oxidativo no fígado através de mecanismos associados à modulação da atividade e expressão de enzimas antioxidantes. Nossos resultados sugerem que a melhor alternativa no desenvolvimento de alimentos funcionais para crianças, visando prevenção de aterosclerose na fase adulta, foi conferida pelos N-3 FA provenientes do óleo de *Echium*.

Palavras- chave: ácidos graxos ômega 3, fitosteróis, óleo de echium, aterosclerose, esteatose hepática

ABSTRACT

Atherosclerosis is an inflammatory process that begins in childhood and progresses with age, being the main pathological process that culminates in cardiovascular disease. Omega 3 fatty acids (n-3 FA) reduces triacylglycerol (TG) and inflammation, while phytosterols present hypocholesterolemic action. Therefore, the aim of this study was to apply the combination of these two classes of bioactive lipids in childhood to reduce atherosclerosis in adulthood. The study was divided into two steps. Initially, the effect of three ingredients containing n-3 FA was evaluated in terms of lipid and inflammatory biomarkers. LDLr knockout adult mice received a high-fat diet and were simultaneously supplemented with water (CON), algae oil (ALG), fish oil (FIS) and *Echium* oil (ECH) by gavage for 4 weeks. The animals supplemented with *Echium* oil presented higher reduction of TG and VLDL. Besides, *Echium* oil inhibited hepatic steatosis caused by high-fat diet. However, unlike of marine oils, this hypotriglyceridemic effect did not involve activation of transcription factors associated to lipid metabolism, such as PPAR α and LXR α . At the second step of this study, weaning LDLr knockout mice were supplemented with emulsions prepared with soybean oil (CON), *Echium* oil (ECH), algae oil (ALG), isolated phytosterols (PHY), algae oil + phytosterols (ALG + PHY) and *Echium* oil + phytosterols (PHY + ECH) for 2 months. Thereafter, dyslipidemia and oxidative stress were induced by a high fat diet for 2 months more. All animals showed fatty streaks in the aorta artery. The lesion area was greatest in mice supplemented with isolated phytosterol (PHY). However, this negative effect was completely reversed by co-supplementation with n-3 FA. It was observed that isolated phytosterols acted as LXR α agonists, and that the reversal provided by the N-3 FA involved the increase of PPAR α and reduction of LXR α expression. Furthermore, the *Echium* oil reduced oxidative stress in liver through mechanisms associated with modulation of antioxidant enzymes activity and expression. Our results suggest that the best alternative to develop functional foods for children, aimed at preventing atherosclerosis in adulthood, was conferred by the N-3 FA from *Echium* oil.

Key words: omega 3 fatty acids, phytosterol, *Echium* oil, atherosclerosis, hepatic steatosis.

1 INTRODUÇÃO

A aterosclerose, principal processo patológico que culmina na doença coronariana e vascular cerebral, inicia-se na primeira década de vida e sofre alterações substanciais entre 5 e 34 anos (DANIELS; GREER; COMMITTEE OF NUTRITION, 2008). Logo, a investigação de fatores envolvidos no desenvolvimento da aterosclerose em crianças e jovens é essencial para a elaboração de estratégias que visem à redução do risco de progressão da mesma na fase adulta. Mendis *et al.*(2005), investigaram sinais de aterosclerose na autópsia de 1277 indivíduos de diferentes padrões econômicos, socioculturais e nutricionais e observaram que 80% das crianças entre 5 e 14 anos já apresentavam estrias gordurosas na aorta, sendo que 29% apresentavam estrias na artéria coronária direita.

Embora haja uma ampla disponibilidade de fármacos capazes de exercer um efetivo controle das respostas inflamatórias ou mesmo da redução dos substratos que levam à gênese da aterosclerose, tais medicamentos não são comumente prescritos ao público infantil, tendo seu uso frequentemente descontinuado. Além disso, ainda não é reconhecida a segurança e eficácia desses tratamentos em crianças (DANIELS; GREER; COMMITTEE OF NUTRITION, 2008; RAGHUVeer, 2008). Dessa forma, apesar dos compostos bioativos presentes nos alimentos funcionais ou em suplementos nutracêuticos apresentarem potencial protetor inferior ao dos fármacos, eles não apresentam efeitos adversos e podem ser facilmente incorporados à dieta de crianças e adolescentes. Entre os compostos bioativos com alegação de saúde já aprovados pelos órgãos de fiscalização e controle, como o *Food and Drugs Administration* - FDA e a Agência Nacional de Vigilância Sanitária – ANVISA, para redução do risco de doenças cardiovasculares (CVD) destacam-se os ácidos graxos poliinsaturados ômega 3(N-3 FA) e os fitosteróis.

Os N-3 FA podem modular a sinalização celular, função protéica e a expressão de genes envolvidos no metabolismo lipídico e no processo inflamatório (DE CATERINA; MASSARO, 2005). Já os fitosteróis reduzem os níveis de colesterol total e da *Low Density Lipoprotein* (LDL), uma vez que diminuem a absorção intestinal de colesterol por meio da competição com o colesterol biliar e dietético na solubilização nas micelas

(MARINANGELI; VARADY; JONES, 2006). Portanto, a combinação desses dois compostos bioativos poderia contribuir para reduzir a concentração de LDL e atenuar processos inflamatórios responsáveis pela maior geração de espécies reativas, reduzindo, assim, a oxidação da LDL considerada um dos agentes causais do desenvolvimento da placa aterosclerótica.

Desta forma, o objetivo deste estudo foi de combinar a ação hipocolesterolêmica dos fitosteróis com a ação hipotriglicidêmica e antiinflamatória dos N-3 FA, visando reduzir o processo aterosclerótico na infância, contribuindo, assim, para a menor formação de placas de ateroma na fase adulta.

2 REVISÃO DE LITERATURA

2.1 Doenças cardiovasculares

As CVD são as principais causas de morte nos países ocidentais e representam cerca de 41% de todas as causas de mortes na Europa. As principais formas de CVD são: angina, infarto do miocárdio e acidente vascular cerebral (AVC) (WORLD HEALTH REPORT, 2004). As lesões vasculares que acompanham essa doença estão associadas à aterosclerose. Considerando sua multicausalidade, alguns dos fatores de risco para essa afecção são obesidade, sedentarismo, hipertensão arterial e tabagismo (TAPIERO; TOWNSEND; TEW, 2004).

A aterosclerose consiste em lesões focais da íntima arterial, caracterizadas pela deposição de colesterol, fibrose e inflamação. As lesões ateroscleróticas precoces, denominadas estrias gordurosas, consistem em depósitos de macrófagos contendo lipídios e linfócitos T na região subendotelial (CHOY *et al.*, 2004). As lesões avançadas são caracterizadas pela presença de uma “capa” fibrosa de tecido conectivo e células musculares lisas, estando frequentemente associadas à calcificação (COSTOPOULOS; LIEW; BENNETT, 2008).

A artéria possui três camadas distintas. A “íntima” formada por células endoteliais em contato com sangue. A túnica “média” que contém células musculares lisas embebidas de matriz extracelular e a “adventícia” que apresenta mastócitos, nervos e microvasos (**Figura 1A**). A aterosclerose se inicia quando leucócitos presentes na monocamada endotelial ativada penetram a íntima arterial e se diferenciam em macrófagos que, por sua vez, são capazes de fagocitar a LDL que foi oxidada por espécies reativas presentes no local, formando as células espumosas. A presença das células espumosas na íntima contribui para o aumento da síntese de citocinas inflamatórias, que levam a quimiotaxia de mais leucócitos para o local, contribuindo para exacerbar a resposta inflamatória (**Figura 1B**). Células musculares lisas migram da média para a íntima arterial, ocasionando um aumento da produção de matriz extracelular e, conseqüentemente, formação de uma capa fibrosa (**Figura 1C**) (LIBBY; RIDKER; HANSSON, 2011).

Macrófagos e células musculares lisas podem sofrer apoptose ao longo do desenvolvimento da placa. Os lipídeos derivados dessas células acumulam-se na região central da placa formando uma região necrótica (**Figura 1D**). Fatores teciduais presentes nessa região ao entrarem em contato com o sangue ativam a cascata de coagulação, levando a formação de um trombo que se estende ao longo da luz do vaso, podendo obstruir a passagem de sangue, culminando no infarto ou AVC (**Figura 1D**) (LIBBY; RIDKER; HANSSON, 2011).

Figura 1 – Formação da placa de ateroma (LIBBY; RIDKER; HANSSON, 2011).

O desenvolvimento das estrias gordurosas, fase inicial da aterosclerose apresentada nas **Figuras 1 B e C**, inicia-se na infância e progride gradualmente na adolescência e juventude. Entretanto, poucos estudos, até o momento, avaliaram a relação entre o desenvolvimento da aterosclerose e fatores de risco presentes em crianças e jovens (MENDIS *et al.*, 2005).

Alterações na íntima arterial são evidentes em estudos post –morte realizados em jovens (FREEDMAN *et al.*, 1988, MENDIS *et al.*, 2005), sendo que a extensão da lesão está relacionada ao número e a intensidade dos fatores de risco cardiovascular,

como dislipidemia, estresse oxidativo e inflamação crônica presentes desde a infância (HARRINGTON *et al.*, 2010, MORRISON *et al.*, 2010).

Estudos têm demonstrado a associação entre moléculas pró-inflamatórias e futuros eventos cardiovasculares (**Figura 2**). Entre elas destacam-se interleucina 6 (IL-6), Fator de necrose tumoral – alfa (TNF- α), proteína C reativa (CRP) e moléculas de adesão (HADDY *et al.*, 2003).

Figura 2- Placa aterosclerótica destacando um sítio de inflamação (HANSSON; HERMANSSON, 2011). APC, célula apresentadora de antígeno; T_H1, células que produzem citocinas que ativam macrófagos; oxLDL, lipoproteína de baixa densidade oxidada.

A IL-6 é uma citocina inflamatória produzida e secretada principalmente pelo tecido adiposo (EDER *et al.*, 2009). Aproximadamente 30% da interleucina circulante é derivada do tecido adiposo (GALIC; OAKHILL; STEINBERG, 2010), sendo 50% produzida pelos macrófagos infiltrados nesse tecido. Outras fontes incluem leucócitos ativados e células endoteliais (HARKINS *et al.*, 2004). A IL-6 está envolvida na regulação da inflamação, hematopoiese, resposta imune e mecanismos de defesa do hospedeiro, além de ser capaz de regular o peso corporal e o metabolismo lipídico (STRVJECKI ; MUTCH, 2011). É um dos determinantes primários da produção de CRP (HADDY *et al.*, 2003). TNF- α é uma citocina pró-inflamatória produzida principalmente por macrófagos. A secreção de TNF- α promove a síntese de outras citocinas pró-inflamatórias, como a IL-6, e redução das antiinflamatórias, resultando em um ambiente pró-inflamatório (STRVJECKI ; MUTCH, 2011).

A CRP é uma proteína de fase aguda que prediz eventos cardiovasculares em indivíduos com ou sem doença cardiovascular estabelecida (HADDY *et al.*, 2003). É sintetizada primariamente nos hepatócitos e regulada pela IL-6, interleucina 1 (IL-1), TNF- α e outras citocinas. Altos níveis de CRP contribuem para o aumento do risco das CVD, uma vez que a CRP pode se ligar à LDL e à *very low density lipoprotein* (VLDL) ativando o sistema complemento. Esse sistema, constituído por proteínas envolvidas na resposta imune inata e adquirida, estimula a produção de fatores teciduais pelos macrófagos, iniciando assim o processo de coagulação e formação de trombos. Além disso, a CRP induz o aumento da expressão de moléculas de adesão como as *Inter-Cellular Adhesion Molecule 1*, *vascular cell adhesion molecule 1* e e-selectina, contribuindo para o aumento da infiltração local de monócitos e linfócitos (**Figura 2**) (FERRANTI; RIFAI, 2002).

Além das citocinas inflamatórias, eicosanóides (**Figura 2**), como as prostaglandinas E2 (PGE2) e D2 produzidas a partir do ácido araquidônico, contribuem para aumentar a inflamação local. Entretanto, a mesma via sinalizadora envolvida na produção dessas prostaglandinas, também ativam a transcrição de enzimas envolvidas na geração de outras classes de eicosanóides, gerados a partir do ácido araquidônico, tais como as lipoxinas, bem como mediadores lipídicos gerados a partir dos N-3 FA conhecidos como resolvinas e protectinas, que podem dominar a fase de resolução da inflamação (SERHAN; SAVILL, 2005).

Adiponectina é uma citocina antiinflamatória secretada exclusivamente pelo tecido adiposo, sendo inversamente associada ao acúmulo de gordura. Possui propriedades antiinflamatórias e anti-aterogênicas agindo no metabolismo de lipídeos, na adesão de monócitos nas células endoteliais, no acúmulo de lipídeos nos macrófagos e na produção de óxido nítrico. Em adultos, a adiponectina tem sido inversamente correlacionada com a gordura corporal e os seus níveis reduzidos podem predispor ao desenvolvimento de diabetes (ARNAIZ *et al.*, 2010). Entre crianças, evidências são escassas sobre a relação entre baixos níveis de adiponectina e disfunção endotelial e /ou aumento da espessura da íntima arterial da carótida (WEISS *et al.*, 2003, PILZ *et al.*, 2005, CHU *et al.*, 2005, PUNTHAKEE *et al.*, 2006, BEAULOYE *et al.*, 2007).

Portanto, observa-se que o processo inflamatório, a dislipidemia e a produção excessiva de espécies reativas são fatores intimamente envolvidos na aterosclerose e consequentemente no desenvolvimento das CVD. Considerando que tais fatores já se manifestam na infância, torna-se evidente a importância de se desenvolver intervenções que visem à redução do processo inflamatório e oxidativo nas fases iniciais de crescimento, proporcionando uma melhor qualidade de vida aos indivíduos na fase adulta.

Intervenções medicamentosas não são comuns no tratamento de crianças que apresentam fatores de riscos para CVD, devido a baixa aderência e aos poucos estudos na área que comprovem a eficácia, garantindo assim a segurança do medicamento. Segundo o *ESC/EAS Guidelines for the management of dyslipidaemias- The Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS)* (2011), a dieta é a principal forma de tratamento para a dislipidemia em crianças. Somente em casos de hipercolesterolemia familiar, é que se deve considerar a utilização de drogas redutoras de lipídeos. Nos demais casos, o foco deve ser a dieta e o tratamento das desordens metabólicas que ocasionam a dislipidemia (CATAPANO et al, 2011).

Nesse sentido, cresce o mercado de alimentos funcionais, que apesar de apresentarem eficácia inferior a dos medicamentos, sofrem menor descontinuidade na ingestão, uma vez que os compostos bioativos podem ser incorporados a alimentos que fazem parte da dieta das crianças. Entre os compostos bioativos relacionados à redução de risco de doenças cardiovasculares destacam-se os N-3 FA e fitosteróis.

Os N-3FA desfrutam de considerável popularidade comercial como resultado das consistentes descobertas em relação aos benefícios à saúde associados ao seu consumo. Globalmente, o mercado de suplementos contendo N-3FA é estimado em torno de US\$ 700 milhões, com uma taxa de crescimento de 8% ao ano (CLOUGH, 2008). Já os alimentos contendo fitosteróis apresentaram um crescimento de 91% em 2005, com mercado gerando em torno de 1,4 bilhões de euros em 2009 (BASHIN, 2010).

2.2 Relação entre ácidos graxos ômega 3, inflamação e aterosclerose

Os N-3 FA são ácidos monocarboxílicos alifáticos nomeados de acordo com o número de carbonos que constitui sua cadeia, bem como o número e a localização das duplas ligações (MC MANUS; MERGA; NEWTON, 2011, CALDER, 2012).

O mais simples N-3 FA é o α -linolênico (C18:3 n-3) (ALA). Este pode ser sintetizado a partir do ácido linoleico (C18:2 n-6) (LNA) por meio de reações de dessaturação catalizada pela Δ -15 dessaturase. Entretanto, roedores e humanos não possuem essa enzima o que torna o ALA um ácido graxo essencial. Embora animais não sejam capazes de sintetizá-lo, eles podem metabolizá-lo a ácidos graxos de cadeia mais longa e insaturada por meio de reações de dessaturação e enlogação. Desta forma, o ALA pode ser convertido a ácido estearidônico (SDA – C18:4 n-3) pela Δ -6 dessaturase e este pode ser enlogado e dessaturado a ácido eicosatetraenóico (C20:4 n-3) e ácido eicosapentaenoico (EPA) (C20:5 n-3), respectivamente (CALDER, 2012).

Existe considerável interesse no aumento da ingestão desse ácido graxo comumente presente em óleos vegetais, sendo frequentemente consumidos pela população por meio da dieta. Entretanto, estudos que comprovem a eficácia do ALA ainda são escassos e inconclusivos. O que se sabe é que a resposta fisiológica do ALA difere do EPA e DHA na manutenção da saúde cardiovascular (POUDYAL *et al.*, 2011). Segundo Wang *et al.* (2004) os efeitos e mecanismos atribuídos aos N-3 FA são restritos ao EPA e DHA, uma vez que ALA não reduz mortalidade relacionada a problemas cardíacos.

Além disso, a conversão do ALA a EPA e DHA é ineficiente em humanos (ARTEBUM; HALL; OKEN, 2006, BURDGE & CALDER, 2006). Estima-se que somente 1-5% de ALA seja convertido em EPA e menos que 0,1% em DHA (HARRIS *et al.*, 2008), de forma que o aumento da ingestão de ALA não reproduz os efeitos benéficos decorrentes do consumo direto dos ácidos graxos EPA e DHA. A baixa conversão pode estar relacionada com a competição entre os ácidos graxos ômega 6 (N-6 FA) e N-3 FA pela enzima Δ -6 dessaturase (**Figura 3**) que converte o ALA em SDA e LNA em ácido araquidônico (AA) (JAMES; URSIN; CLELAND, 2003, CALDER, 2012). Apesar da enzima Δ -6 dessaturase apresentar maior afinidade pelos N-3 FA, a alta

concentração de N-6 FA na dieta ocidental (5 a 10 vezes maior que a ingestão de ALA) (BLASBALG *et al*, 2011), desloca a enzima para a via de síntese do AA, limitando assim a conversão do ALA em EPA e DHA (GUIL-GUERRERO, 2000). Dessa forma, o SDA, poderia ser melhor convertido em EPA que o ALA (MILES; BARNEJIE; CALDER, 2006, WHELAN, 2009), conforme se observa na **Figura 3**, pela vantagem competitiva na primeira etapa de dessaturação em relação ao LNA, apresentando uma eficácia de conversão a EPA em fosfolipídeos plasmático que varia de 17-30% (WHELAN, 2009).

Figura 3 - Esquema da formação do ácido araquidônico (AA), ácido eicosapentaenóico (EPA) e ácido docosahexaenóico (DHA) a partir do ácido linoleico (LNA) e e linolênico (ALA) por meio de reações de alongamento e dessaturação (Adaptado de RUSSO, 2009). GLA, ácido gamma linolênico; DAS, ácido estearidônico; DPA, ácido docosapentaenóico; COX, ciclooxigenase; LOX, lipooxigenase; PGE, prostaglandinas E; PGF, prostaglandinas F; PGI, prostaglandinas I; TXA, tromboxano A; LTB, leucotrieno B; LTC, leucotrieno C; LTE, leucotrieno E.

As principais fontes de ALA encontradas na natureza são sementes, nozes, castanhas e seus respectivos óleos. O óleo de linhaça é constituído de 45-65% de ALA, ao passo que óleo de soja e girassol contém apenas ~ 10 % (CALDER, 2012). Estima-se que na dieta ocidental a ingestão de ALA corresponda somente a 0,5-2 g/dia (BRITISH NUTRITION FOUNDATION, 1999, BURDGE & CALDER, 2006).

Já o SDA é encontrado no óleo de peixe e em algumas plantas da família *Boraginaceae*, sendo as principais o *Echium* (nome popular: soagem-viperina) e o *Borage*. O óleo de *Echium* é a principal fonte de SDA disponível em plantas (3,5%-9%), (GUIL-GUERRERO, *et al.*, 2000, SURETTE; EDENS; CHILTON, 2004) seguida pelas espécies da família *Grossulariaceae* (2-6%) (DEL CASTILLO *et al.*, 2004). Outras fontes de SDA é o *Black currant seed* e o óleo de soja geneticamente modificado formado a partir da adição dos genes relativos a $\Delta 6$ e $\Delta 5$ dessaturase que convertem o ALA presente no óleo de soja a SDA (15 a 30%) (HAMMOND *et al.*, 2008, WHELAN; GOUFFON; ZHAO, 2012).

Enquanto os efeitos do EPA e do ácido docosahexaenóico (DHA) dietéticos têm sido largamente estudados, poucos estudos têm investigado o efeito do SDA na redução de risco cardiovascular (JAMES; URSIN; CLELAND, 2003, HARRIS *et al.* 2008, LEMKE *et al.*, 2010).

James, Ursini & Cleland (2003) compararam o efeito de etil esters de SDA com etil esters de ALA e EPA (dose de 0,75-1,5 g/dia) na concentração de triacilglicerol (TG), LDL e *High density lipoprotein* (HDL) em 45 indivíduos, não observando nenhuma diferença significativa entre os grupos. Harris *et al.* (2008) avaliaram o efeito do SDA proveniente do óleo de soja enriquecido com SDA na redução do risco cardiovascular. A dose utilizada neste estudo foi de 4,2 g/dia de SDA e 1g/ dia de EPA (controle positivo). Nenhuma diferença significativa para LDL, TG e HDL foi observada entre os grupos. Em um estudo randomizado, duplo-cego, envolvendo participantes com sobrepeso, mas perfil lipídico aparentemente normal, foram administradas 3,7g de SDA e 1g de EPA como controle positivo. Assim como nos dois estudos acima apresentados, não foi observada nenhuma diferença significativa na concentração dos biomarcadores entre os dois grupos.

Apesar dos estudos descritos acima não terem apresentado diferenças significativas no perfil lipídico, James; Ursin; Cleland (2003) observaram uma maior

concentração de EPA nos lipídios plasmáticos e celulares de indivíduos suplementados com etil-ester de ácido estearidônico, quando comparados a aqueles suplementados com a mesma quantidade de etil-ester de ALA. Esses resultados suportam a hipótese de que a atividade da $\Delta 6$ -dessaturase é o fator limitante na conversão de ALA a EPA em humanos e roedores.

Miles, Banerjee & Calder (2006) reportaram que a suplementação de homens jovens saudáveis com óleo de *Echium* (nome popular: soagem-viperina) na dosagem de 1g/dia aumentou a concentração de EPA em células mononucleares, mas apresentou limitado impacto na resposta imune. Já Surette; Edens; Chilton (2004) observaram que a suplementação de indivíduos com 1,9 g SDA/dia proveniente do óleo de *Echium* aumentou o conteúdo de EPA nos fosfolipídios e neutrófilos plasmáticos, reduzindo a concentração de triglicerídeos plasmáticos. Horia & Watkins (2005) avaliaram o efeito do SDA e do ALA sobre os níveis de PGE2 e ciclooxigenase 2 (COX-2) e observaram que o SDA foi mais efetivo que o ALA em converter-se em EPA/DHA, e em reduzir a expressão protéica e gênica da COX-2.

Diante do exposto, acredita-se que o SDA possa ser uma fonte vegetal sustentável de N-3 FA, podendo contribuir para alcançar as recomendações de ingestão relacionadas ao mesmo. No entanto, são necessárias mais pesquisas que busquem avaliar o efeito deste ácido graxo nos fatores de risco e no desenvolvimento das CVD, bem como avaliar se os efeitos da sua suplementação são decorrentes da sua conversão em EPA e DHA, da ação direta do SDA ou ainda de outros metabólitos formados nesse processo (DECKELBAUM; TORREJON, 2012).

EPA e DHA são encontrados principalmente na gordura do peixe e em suplementos alimentares (LEE *et al.*, 2009). Entretanto, a concentração de N-3 FA e a proporção de EPA e DHA irão variar de acordo com a espécie, a concentração de gordura em partes comestíveis do peixe e as condições ambientais (temperatura da água, dieta e estação do ano). Por exemplo, uma porção de bacalhau pode prover 0,2 a 0,3 gramas de N-3 FA, enquanto que uma de salmão contém 1,5 a 3,5 g (CALDER, 2012).

A *American Heart Association* (AHA) e a *European Society of Cardiology* têm encontrado evidências suficientemente fortes e concisas quanto aos benefícios obtidos pelo consumo de N-3 FA, de forma que recomendam o aumento da ingestão desses

ácidos graxos tanto para a prevenção primária quanto secundária das doenças cardiovasculares. A AHA recomenda que pacientes com CVD consumam um total de 1g\dia de EPA e DHA, preferencialmente da gordura de peixe (KRIS-ETHERTON; HARRIS; APPEL, 2002). Já o FDA recomenda a ingestão de até 2 g\dia de EPA e DHA.

O EPA e DHA têm mostrado ação protetora contra as CVD. Esses benefícios parecem resultar primariamente do enriquecimento dos fosfolipídeos de membrana, levando a uma melhora da função endotelial e vascular, redução da agregação plaquetária, melhora do tônus muscular arterial e redução da pressão sanguínea (LEE *et al.*, 2009). Segundo Calder (2012), alguns efeitos dos N-3 FA parecem ser mediados por mudanças na composição de membranas celulares de forma a alterar a fluidez da mesma, a interferir na formação e composição dos *lipids rafts*, alterando dessa forma a sinalização celular e a produção de mediadores peptídicos e lipídicos.

Lipid rafts são subdomínios de membranas celulares que possuem uma distinta e característica composição, rica em esfingolipídios e colesterol. São potencialmente modificados pelo N-3 FA dietético uma vez que estes, ao serem incorporados próximos aos *lipids rafts*, influenciam a sua formação e funcionalidade (CALDER, 2012). Estudos *in vitro* e *in vivo* sugerem que ácidos graxos poliinsaturados modulam a estrutura e composição de *lipids rafts* em células imunes, modificando proteínas sinalizadoras chaves e alterando o transporte de proteínas intracelulares (KIM *et al.*, 2008, SHAIKH *et al.*, 2009, ROCKET *et al.* 2011)

Os N-3 FA são reconhecidos também pelo seu efeito antiinflamatório, uma vez que competem com os N-6 FA na produção de eicosanóides, aumentando a produção de prostaglandinas de série 3 (vasodilatador e inibidor da agregação plaquetária), tromboxanos A3 (fraco agregador plaquetário) e leucotrienos B5 (fraco indutor de inflamação). Eicosanóides das séries 3 e 5 possuem ação antiinflamatória e antiagregatória quando comparado aos eicosanóides de séries 2 e 4 produzidos a partir do ácido araquidônico (ROBINSON; STONE, 2006).

Além disso, a partir dos N-3 FA são produzidos metabólitos com efeitos antiinflamatórios, através de oxidação enzimática (COX e lipooxigenases- LOX), como as resolvinas, protectinas e ácidos graxos eletrofílicos ou de oxidação não enzimática como os neuroprostanos ciclopentanos eletrofílicos (**Figura 4**) (GROEGER *et al.*, 2010).

As resolvinas, as quais promovem sua ação pela ativação de receptores acoplados a proteína G, incluem as de série E formadas a partir do EPA e de séries D (D1 ou RvD1), neuroprotectinas/protectinas formadas a partir do DHA. Os efeitos antiinflamatórios das resolvinas incluem a redução da produção de $\text{TNF-}\alpha$ e interleucina-13 e a inibição da migração transendotelial de neutrófilos, prevenindo, desta forma, a infiltração de neutrófilos no local de inflamação (POUDYAL *et al.*, 2011, CALDER, 2012). Portanto, N-3 FA podem agir sobre a estabilidade da placa aterosclerótica, uma vez que por meio do aumento da concentração de resolvinas e redução da inflamação, são capazes de promover um decréscimo da infiltração de células inflamatórias e imunes, reduzindo, assim, os fatores de risco que contribuem para a formação da placa. Estudos *in vivo* têm mostrado que N-3 FA são incorporados em placas de ateroma e que aquelas com maior conteúdo de EPA são mais estáveis (THIES *et al.*, 2003, CAWOOD *et al.*, 2010)

Figura 4 – Ação antiinflamatória dos AGPI n-3. COX, ciclooxigenase; LOX, lipooxigenase; $\text{NF}\kappa\text{B}$ - $\text{I}\kappa\text{B}$, fator nuclear κB – $\text{I}\kappa\text{B}$ kinase; IL- interleucina; PCR, proteína C reativa, $\text{TNF-}\alpha$, fator de necrose tumoral alfa; ERO, espécies reativas de oxigênio.

A inibição da fosforilação do complexo fator nuclear κ B – I κ B kinase (NF κ B- κ B) constitui uma outra forma pelo qual os N-3 FA reduzem o processo inflamatório. Essa inibição ocorre via interação com receptores acoplados a proteína G- GPR 120 e pelo aumento da expressão de *Peroxisome Proliferator Activated Receptor* γ e α (PPAR- γ , PPAR- α), que pode interagir fisicamente com o NF κ B prevenindo sua translocação para o núcleo (CALDER, 2012) (**Figura 5**). Outro efeito que justifica a ação antiinflamatória dos N-3 FA é a redução da interleucina – 18 (IL-18), um fator de risco independente para as doenças arteriais coronarianas. IL-18, citocina da família da IL-1 descrita recentemente, tem ação pleiotrópica, participando da resposta imune inata e adquirida. Entre suas principais funções, está a estimulação da produção de interferon gama pelos linfócitos T, células *Killers* naturais e macrófagos (SOUZA *et al.*, 2008). Troseid *et al.* (2009), observaram que concentrações reduzidas de IL-18 foram positivamente correlacionadas com aumento das concentrações séricas de EPA e DHA em homens idosos com alto risco de aterosclerose tratados com 2,4 g/ dia de EPA/DHA. Em resumo, os efeitos antiinflamatórios dos N-3 FA são decorrentes dos metabólitos produzidos a partir do mesmo e/ou da supressão da via do N-6 FA (POUDYAL *et al.*, 2011).

Figura 5- Ação antiinflamatória do N-3 FA via PPAR- α (A) e PPAR γ (B) e sua interação com o sistema NF κ B-I κ B. Modificado de Calder et al (2012). (n-3) PUFA, ácidos graxos poliinsaturados ômega 3; COX-2, ciclooxigenase -2; TNF, fator de necrose tumoral; IL-1, interleucina 1; ACO, acil-CoA oxidase; FABP, fatty acid binding protein; ApoA, apolipoproteína A; αP2, proteína que estimula diferenciação de adipócitos; ADRP, adipocyte differentiation-related protein; LPL, lipase lipoprotéica; C/EBP, proteínas que aumentam a ativação de receptores de insulina.

Além dos efeitos antiinflamatórios, o EPA e DHA apresentam ação hipotrigliceridêmica (CASLAKE *et al.*, 2008). Os mecanismos moleculares pelos quais o EPA e o DHA reduzem a concentração sérica de TG ainda não estão completamente esclarecidos, mas várias hipóteses têm sido sugeridas a partir de estudos pré-clínicos. Esses estudos sugerem que o EPA e o DHA podem reduzir a síntese e a secreção hepática de VLDL-TG, aumentando o *clearance* de TG dos quilomícrons e das VLDL via fatores de transcrição que regulam a expressão de enzimas envolvidas com a síntese e a degradação de ácidos graxos. De forma resumida, os N-3 FA ativam os receptores retinóide X e farnesóide X. A ativação desses receptores inibe o receptor X do fígado (LXR), inibindo a ativação da proteína ligante do elemento regulado por esteróis 1c (SREBP-1c), reduzindo, assim, a síntese de ácidos graxos. Os N-3 FA também ativam PPARs que, além de modularem a expressão de enzimas envolvidas na oxidação dos ácidos graxos, também atuam inibindo a SREBP-1c. Somado à ação direta dos N-3 FA na inibição da SREBP-1c, essas formas indiretas de inibição contribuem para a redução da síntese de ácidos graxos, uma vez que a SREBP-1c modula enzimas envolvidas nesse processo, como ácido graxo sintase, acetil CoA carboxilase, dentre outras (FERNANDEZ; WEST, 2005; DAVIDSON, 2006; HARRIS *et al.*, 2008; SCHMITZ; ECKER, 2008). Logo, considerando que a modulação da ação do SREBP-1c é, um processo parcialmente dependente de PPAR- α , acredita-se que os PPARs sejam os alvos primários dos N-3 FA na manutenção da homeostase lipídica no organismo (POUDYAL *et al.*, 2011).

O aumento da oxidação dos ácidos graxos mediado pelo N-3 FA também pode ser atribuído a sua ação sobre a mitocondrial carnitina palmitoil transferase 1 que facilita o transporte de ácidos graxos do citoplasma para o interior da mitocôndria para sofrerem β -oxidação, sendo este processo regulado por PPARs (POUDYAL *et al.*, 2011).

Uma rota alternativa para a oxidação lipídica é aquela mediada pela *peroxisomal acyl-CoA oxidase (ACO)*, uma via relativamente ineficiente para a oxidação de lipídeos, uma vez que gera 30% mais calor e portanto menos ATP que a β -oxidação mitocondrial (POUDYAL *et al.*, 2011). Baillie *et al.* (1999) observaram um aumento da expressão da ACO no fígado, músculo esquelético e coração em ratos suplementados com óleo de peixe.

Em relação ao estresse oxidativo, apesar do maior número de insaturações aumentar a susceptibilidade dos ácidos graxos à oxidação *in vitro*, não está estabelecido que isso também ocorra *in vivo* (THOLSTRUP *et al.*, 2004). Pesquisas que se propuseram a avaliar o efeito da lipoperoxidação *in vivo* em modelos com humanos e animais, observaram aumento (FUHRMAN; VOLKOVA; AVIRAM, 2006, OARADA *et al.*, 2008, CARRAPEIRO *et al.*, 2011), nenhum efeito (CARRAPEIRO *et al.*, 2007) ou redução (NYBY *et al.*, 2005) da susceptibilidade à oxidação. A variabilidade desses resultados pode ser atribuída a vários fatores, incluindo o nível de suplementação anterior, concentração de antioxidantes no óleo de peixe e diferenças na metodologia utilizada para avaliar o estado oxidativo (LEIGH-FIRBANK *et al.*, 2002).

Tem sido reportado que produtos da oxidação do EPA poderiam inibir a adesão de neutrófilos e monócitos nas células endoteliais pela ativação do PPAR- α , sugerindo que EPA oxidado também apresente efeitos antiinflamatórios. Musiek *et al.* (2008) demonstraram que neuroprostanos ciclopentano formados a partir da oxidação do DHA são potentes inibidores da via de sinalização do NF-kB, contribuindo para os efeitos anti-inflamatórios do DHA.

Apesar de ainda não ser conclusivo a ação dos N-3 FA sobre o estresse oxidativo, alguns autores acreditam que este possa exercer alguns efeitos antioxidantes, como consequência da redução da síntese de ácido araquidônico que constitui o principal componente responsável pela ativação da Nicotinamida adenina dinucleotideo fosfato oxidase (NADPH oxidase) que catalisa a produção de espécies reativas de oxigênio. Outro mecanismo plausível seria a redução da produção de óxido nítrico a partir da redução da atividade da óxido nítrico sintase endotelial (VIGNAIS, 2002, POUDYAL *et al.*, 2011).

Além de todos os benefícios citados anteriormente, os N-3 FA são necessários para o crescimento e desenvolvimento das crianças. Em humanos, o ALA é convertido a DHA, o principal constituinte de fosfolipídios presentes nas membranas do cérebro e da retina, sendo de extrema importância para a manutenção das funções cerebrais (MUTHAYYA *et al.*, 2009). O DHA é capaz de regular fatores de transcrição envolvidos no desenvolvimento cognitivo, mental e visual da criança, uma vez que interage com

PPARs, modulando a expressão de genes alvos que influenciam a neurogênese e o metabolismo de neurotransmissores (HSIEH; BRENNAN, 2009).

Portanto é evidente a ação do N-3 FA sobre o perfil lipídico, desenvolvimento cerebral bem como sobre o processo inflamatório. Além disso, considerando que sinais subclínicos de aterosclerose já são evidentes em crianças obesas (GIANINI *et al.*, 2009), uma suplementação com N-3 FA já na infância poderia preparar melhor o organismo ao impacto do estresse oxidativo na fase adulta.

2.3 Fitoesteróis e doenças cardiovasculares

Esteróis vegetais ou fitosteróis são importantes componentes da dieta, sendo particularmente abundantes em cereais, nozes, sementes e óleos vegetais. Fitosteróis são estruturalmente semelhantes ao colesterol (**Figura 6**), diferindo somente pela presença de um grupo metil ou etil no carbono 24 ou ainda pela dupla ligação no carbono 22, o que confere ao fitosterol maior hidrofobicidade e, conseqüentemente, maior afinidade às micelas no intestino quando comparados ao colesterol (ESCURRIOL *et al.*, 2009).

Figura 6 – Estruturas dos fitosteróis e do colesterol (GARCÍA-LLATAS; RODRIGUEZ-ESTRADA, 2011).

Mais de 40 diferentes tipos de esteróis vegetais tem sido identificados, dos quais o β-sitosterol, campesterol e estigmasterol são os mais abundantes. Os estanois se

diferenciam dos fitosteróis pela ausência da dupla ligação no anel estrutural. Enquanto a absorção do colesterol varia de 30 a 60% (HEINEMANN; AXTMANN; VON BERGMANN, 1993, BOSNER *et al.*, 1999), a absorção dos fitosteróis é extremamente reduzida. Os níveis de absorção do campesterol (9,4-14,8%) são aproximadamente 3 vezes mais altas que do sitosterol (3,1-4,5%) e estigmasterol (~4%). A absorção das respectivas formas estanóis é ainda mais baixa (0,1-2%) HEINEMANN; AXTMANN; VON BERGMANN, 1993, MIETTINEN *et al.*, 2000, PIIRONEN *et al.*, 2000, SANDERS *et al.*, 2000). A diferença na absorção entre os fitosteróis se deve principalmente a estrutura dessas moléculas, sendo que as duplas ligações e o menor número de carbonos favorecem a absorção (GARCÍA- LLATAS; RODRIGUES – ESTRADA, 2011).

Os fitosteróis são absorvidos da mesma forma que o colesterol (**Figura 7**). Após sua solubilização nas micelas, os fitosteróis são transportados para o enterócito por meio da *Niemann- Pick C1-like protein* (NPC1L1). No enterócito, os fitosteróis são esterificados e incorporados aos quilomícrons. Entretanto, esses compostos possuem baixa afinidade pela *Acetil-Coenzima A acetiltransferase* (ACAT), enzima que cataliza essa esterificação. Os fitosteróis não esterificados tendem a retornar para o lúmen intestinal por meio dos transportadores *ATP binding cassette (ABC) transporters G5 e G8* (ABCG5/ABCG8), explicando a baixa taxa de absorção desses compostos (GARCÍA- LLATAS; RODRIGUES –ESTRADA, 2011).

colesterol. Entretanto, segundo Mel'nikov *et al.*(2004) esse mecanismo apresenta baixo impacto no efeito hipocolesterolêmico;

- 2- Competição pela atividade da esterase, enzima que catalisa a hidrólise dos ésteres de colesterol, etapa necessária para absorção do mesmo;
- 3- Competição por transportadores de colesterol na borda em escova, tais como NPC1L1;
- 4- Inibição da atividade da ACAT. A ACAT intestinal re-esterifica os fitosteróis e colesterol absorvidos. Acredita-se que os fitosteróis possam causar a supressão da atividade da ACAT, levando à redução da incorporação do colesterol aos quilomícrons e o aumento de colesterol livre intracelular, estimulando a expressão do *ATP binding cassette* (ABCA), que por sua vez leva ao efluxo de colesterol do enterócito para o lúmen intestinal (PLAT; MENSINK, 2002, JONG; PLAT; MENSINK, 2003, TRAUTWEIN, 2003, PLAT; MENSINK, 2005)

Segundo Calpe- Berdiel; Escola-Gil; Blanco-Vaca (2009) fitosteróis podem ainda influenciar a concentração sérica de colesterol via ativação de LXR. LXR α é altamente expresso no fígado e em menor nível no intestino. Estudos têm sugerido que este fator de transcrição possui influência no metabolismo intestinal de colesterol, uma vez que contribui para o aumento da expressão de outros membros transcricionais da família ABC, tais como ABCA1 e ABCG5 e 8 no intestino e em macrófagos. Entretanto, estudos conclusivos utilizando camundongos knockout para esses transportadores demonstraram que os fitosteróis podem mediar a inibição da absorção do colesterol de forma independente dos mesmos (PLOSCH *et al.*, 2006, CALPE-BERDIEL *et al.*, 2007)

O decréscimo da absorção do colesterol está associado ao aumento compensatório da síntese de colesterol e ao aumento da expressão dos receptores para LDL, de forma que o aumento dessa expressão pode levar não somente ao aumento do *clearance* da LDL, mas também da IDL, lipoproteína precursora da LDL. Portanto, como resultado da menor absorção de colesterol e maior expressão dos receptores da LDL, ocorre a redução da concentração sérica da LDL e do colesterol total (PLAT; MENSINK, 2005, JONG; PLAT; MENSINK, 2003).

Os fitosteróis tem distintas atividades biológicas que incluem modulação de vias de sinalização e ativação de respostas ao estresse celular, tendo implicações no

metabolismo lipídico, inflamação e no desenvolvimento de CVD. Sitosterol, campesterol e estigmasterol têm mostrado reduzir a síntese de colesterol em linhagens de células hepáticas e intestinais (HO & PAL, 2005). Estigmasterol reduziu a absorção de colesterol, decresceu colesterol hepático e suprimiu a expressão da 3-hidroxi-3-metilglutaril-CoA reductase e da enzima colesterol 7- alfa-hidroxilase (BATTA *et al.*, 2006) . Estigmasterol e campesterol, mas não sitosterol, interferiram na expressão do SREBP e reduziram a expressão de genes envolvidos na biosíntese do colesterol. Além disso, o estigmasterol tem mostrado ser capaz de se ligar ao LXR, promovendo a expressão dos transportadores ABCG1 (*ATP-binding cassette sub-family G member 1*) e ABCA1, envolvidos no transporte reverso de colesterol em células adrenais (YANG *et al.*, 2006), sendo que este efeito não foi observado em células hepáticas (CARTER *et al.*, 2007). Essas observações indicam que a atividade biológica dos fitosteróis depende do tipo de célula e do tipo de esterol (SABEVA *et al.*, 2011).

Desde 2001, o *National Cholesterol Education Program* recomenda uma ingestão de 2 g/dia de fitosteróis para adultos concomitantemente a mudanças no estilo de vida, o que proporciona uma redução em torno de 6 a 15% da LDL, sem influência sobre os níveis de HDL e TG. Alguns estudos têm demonstrado que fitosteróis em quantidades acima de 2,5 g/dia reduzem LDL tanto em indivíduos normocolesterolêmicos quanto hipercolesterolêmicos (POLI *et al.*, 2008, VAN HORN *et al.*, 2008). Uma meta-análise com 41 estudos reportou que a ingestão de 2g/d de fitosteróis reduziu o LDL em torno de 10% sendo que ingestões superiores conferiram poucos benefícios adicionais (KATAN *et al.*, 2003). De acordo com a Comissão Européia (EC, 2004) a ingestão acima de 3 g/d de fitosteróis não proporcionariam efeitos benéficos adicionais.

Fitosteróis são bem tolerados e nenhum efeito adverso tem sido reportado (VAN HORN *et al.*, 2008). Porém, um decréscimo dos níveis sanguíneos de carotenóides tem sido documentado seguido do consumo de esteróis. Entretanto, essa redução parece inexpressiva quando a concentração de carotenóides é expressa em relação a concentração de colesterol total (KATAN *et al.*, 2003).

2.4 Combinação de ácidos graxos ômega 3 e fitosteróis

Os N-3 FA são reconhecidos pelo seu efeito cardioprotetor em função da sua ação anti-arrítmica, hipotrigliciridêmica e antiinflamatória (CASTRO; BARROSO; SINNECKER, 2005). Já os fitosteróis reduzem o LDL colesterol por competir com o colesterol dietético e biliar na absorção intestinal (OSTLUND, 2002), reduzindo assim o substrato para oxidação, e consequentemente, a concentração de LDL oxidada, que é considerada um fator causal para o desenvolvimento das estrias gordurosas. Logo, a combinação de N-3 FA e fitosteróis poderia potencializar os efeitos cardioprotetores e antiinflamatórios exercidos por cada um isoladamente em indivíduos hiperlipidêmicos.

Micallef & Garg (2008) e Micallef & Garg (2009) suplementaram 60 indivíduos hiperlipidêmicos com 1.4 g/d N-3 FA e 2 g/ d de fitosteróis e observaram que a combinação desses dois compostos bioativos reduziu a concentração de TG (25,9%) após três semanas de tratamento. Mudanças também foram observadas na concentração de colesterol total, LDL (redução de 13,3% e 12,5%, respectivamente), HDL (aumento de 8,6%) e parâmetros inflamatórios (redução de CRP, TNF, IL-6 e aumento de adiponectina). Além disso, os autores reportaram uma redução de 22,6% do risco cardiovascular total, sugerindo que a redução da inflamação na hiperlipidemia representa um importante mecanismo pelo qual os N-3 FA associados aos fitosteróis conferem benefícios cardiovasculares.

Segundo Micallef & Garg (2008), a presença de N-3 FA e fitosteróis no trato gastrointestinal pode aumentar a substituição micelar do colesterol, resultando em uma maior inibição da sua absorção. Além disso, a suplementação com N-3 FA está associada com aumento do tamanho da partícula de LDL, enquanto que os fitosteróis são conhecidos por reduzir a concentração de LDL. Dessa forma, o efeito da combinação dos dois compostos poderia proporcionar a redução tanto do tamanho quanto da concentração de LDL, contribuindo, assim para a redução do risco aterogênico.

Portanto, considerando que o processo inflamatório, a dislipidemia e o estresse oxidativo são fatores de risco para o desenvolvimento da aterosclerose, e que sinais de aterosclerose já são evidentes em crianças, a associação na infância de N-3 FA com fitosteróis poderia contribuir para a redução do processo inflamatório e oxidativo, reduzindo assim o risco de desenvolvimento de CVD na fase adulta.

3 HIPÓTESES

H₁: O efeito da suplementação com N-3 FA provenientes do óleo de peixe, de algas e do óleo de *Echium* sobre parâmetros inflamatórios e sobre o perfil lipídico de camundongos *Knockout LDLr* é diferente.

H₂: A suplementação na infância de uma mistura contendo N-3 FA e fitosteróis apresenta potencial sinérgico na redução do risco cardiovascular na fase adulta.

4 OBJETIVOS

O objetivo deste estudo foi de suplementar camundongos *Knockout LDLr* com uma combinação de fitosteróis e N-3 FA na infância, e avaliar o efeito dessa suplementação nos biomarcadores associados à aterosclerose após a indução de dislipidemia, estresse oxidativo e inflamação na fase adulta.

5 ETAPAS

Na primeira fase, foi selecionada a forma de suplementação com N-3 FA, onde três ingredientes foram avaliados: óleo de peixe contendo EPA e DHA, óleo de alga contendo DHA e óleo de *Echium* contendo SDA. A seguir, foi avaliado o efeito da combinação de fitosteróis e dos N-3 FA selecionados na etapa anterior (óleo de *Echium* e óleo de alga) sobre os biomarcadores associados à aterosclerose, através da suplementação de camundongos recém-desmamados submetidos à dieta hiperlipídica na fase adulta.

CAPÍTULO 1

HIPÓTESE 1 O efeito da suplementação com N-3 FA provenientes do óleo de peixe, de algas e do óleo de *Echium* sobre parâmetros inflamatórios e sobre o perfil lipídico de camundongos *Knockout LDLr* é diferente.

RESEARCH

Open

Effect of Echium oil compared with marine oils on lipid profile and inhibition of hepatic steatosis in LDLr knockout mice

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Abstract

Background: In an effort to identify new alternatives for long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) supplementation, the effect of three sources of omega 3 fatty acids (algae, fish and Echium oils) on lipid profile and inflammation biomarkers was evaluated in LDL receptor knockout mice.

Methods: The animals received a high fat diet and were supplemented by gavage with an emulsion containing water (CON), docosahexaenoic acid (DHA, 42.89%) from algae oil (ALG), eicosapentaenoic acid (EPA, 19.97%) plus DHA (11.51%) from fish oil (FIS), and alpha-linolenic acid (ALA, 26.75%) plus stearidonic acid (SDA, 11.13%) from Echium oil (ECH) for 4 weeks.

Results: Animals supplemented with Echium oil presented lower cholesterol total and triacylglycerol concentrations than control group (CON) and lower VLDL than all of the other groups, constituting the best lipoprotein profile observed in our study. Moreover, the Echium oil attenuated the hepatic steatosis caused by the high fat diet. However, in contrast to the marine oils, Echium oil did not affect the levels of transcription factors involved in lipid metabolism, such as Peroxisome Proliferator Activated Receptor α (PPAR α) and Liver X Receptor α (LXR α), suggesting that it exerts its beneficial effects by a mechanism other than those observed to EPA and DHA. Echium oil also reduced N-6/N-3 FA ratio in hepatic tissue, which can have been responsible for the attenuation of steatosis hepatic observed in ECH group. None of the supplemented oils reduced the inflammation biomarkers.

Conclusion: Our results suggest that Echium oil represents an alternative as natural ingredient to be applied in functional foods to reduce cardiovascular disease risk factors.

Keywords: Atherosclerosis, Inflammation, Echium, Stearidonic, Omega 3, Steatosis

Background

The increased intake of omega-6 fatty acids during the 20th century as a result of an elevation in vegetal oil consumption (of more than 1,000-fold) contributed to a decline in the tissue concentration of long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) [1,2], which might be associated with the increased incidence of inflammatory disorders, such as atherosclerosis [3]. The development of atherosclerotic plaques is associated with several clinical cardiovascular events. Considering the health effects of LC n-3 PUFA toward the reduction

of cardiovascular disease (CVD) risk [4,5], many industries have added eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from marine oils to food formulations or supplements, aiming to explore this health claim. In 2004, the Food and Drug Administration (FDA) qualified the health claim of products containing EPA and DHA [6]. A similar recommendation was also provided by the American Heart Association (AHA), who suggested consumption of 1 g/day of EPA + DHA for patients with CVD and 2–4 g/day for patients with hypertriglyceridaemia [7].

The cardioprotective effects of LC N-3 PUFA appear to be due to a synergism between multiple mechanisms including triacylglycerol (TG) lowering, improving membrane fluidity, anti-inflammatory, antiarrhythmic and

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antithrombotic effects [5]. The scientific evidence concerning the beneficial effects of the LC N-3 PUFA on lipid profile and inflammation were obtained from several studies using animal and human models. However, these effects and the mechanisms by which they occur are restricted to the action of EPA and DHA [8]. Other non-marine sources of omega 3 fatty acids (N-3 FA), such as alpha-linolenic acid (ALA) or stearidonic acid (SDA), can be converted in vivo to EPA and DHA by the desaturase and elongase enzymes in a tissue-dependent manner, the liver being the major site of this conversion [9]. It has been reported that the conversion rate of ALA is low (5-10% for EPA and < 1% for DHA), which diminishes the efficacy of these alternative sources in the reduction of cardiovascular risk [10-12]. However, due to dietary preferences, safety, sustainability, cost and oxidative stability aspects, other non-marine oils alternatives must be evaluated [3,9-11,13,14]. It has been suggested that the low rate by which ALA is converted to EPA is a result of the limited activity of 6-desaturase when linoleic acid (LNA) is also present [15]. However SDA, a pre-cursor of EPA that is found in plants, such as Echium (*Echium plantagineum*), black currant seed and other genetically modified seeds, does not need 6-desaturase activity to be converted into EPA [3,10]. In an effort to identify new alternatives for LC N-3 PUFA supplementation, the objective of this study was to compare the effects of three sources of N-3 FA (algae, fish and Echium oil) on lipid composition and some inflammatory bio-markers using LDL receptor deficient mice (LDLR knockout mice) as model.

Methods

Oils and reagents

The N-3 FA used in this study were commercial products: the algae oil containing 40% DHA (DHASCO) was obtained from Martek Biosciences^W (Winchester, KY, USA), the fish oil (EPA1T1600 MEG-3TM) containing EPA (20%) + DHA (12%) was obtained from Ocean Nutrition^W (Dartmouth, NS, Canada) and the Echium oil containing 11.5% SDA (AW39144ECH) was obtained from Oil Seed Extraction^W (Ashburton, New Zealand). All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Merck (Darmstadt, Germany), Calbiochem Technology Inc. (Boston, MA, USA) and GE Healthcare (Little Chalfont, Bucks, UK). The aqueous solutions were prepared with ultra-pure Milli-Q water (Millipore Ind. Com. Ltd., SP, Brazil), and the organic solvents were of HPLC grade. Fatty acids profile of the oils used in this study was analyzed by gas chromatography and is shown in Table 1.

Animals and diets

Forty male homozygous LDL receptor-deficient mice (LDLR Knockout mice, C57BL/6) weighing 25–29 g

Table 1 Fatty acids composition of the edible oils applied in this study

Fatty acids (g/100 g total FA)	Fish oil	Algae oil	Echium oil
C10:0 – capric	-	1.12	-
C12:0 – lauric	-	4.92	-
C14:0 – miristic	7.93	10.19	-
C16:0 – palmitic	17.05	6.97	5.15
C18:0 – stearic	3.05	0.92	2.57
C16:1 – palmitoleic	9.76	2.82	-
C18:1 – oleic (n-9)	13.20	27.48	12.77
C18:2 – (LNA) linoleic (n-6)	1.19	1.17	27.52
C18:3 – (ALA) α-linolenic (n-3)	0.68	-	26.75
C18:3 – (GLA) γ-linolenic (n-6)	-	-	13.11
C18:4 – (SDA) stearidonic (n-3)	3.28	-	11.13
C20:4 – (ARA) arachidonic (n-6)	1.82	-	-
C20:5 – (EPA) eicosapentaenoic (n-3)	19.97	-	-
C22:5 – (DPA) docosapentaenoic (n-3)	2.20	0.38	-
C22:6 – (DHA) docosaenoic (n-3)	11.51	42.89	-
Σ saturated FA	28.03	24.12	7.72
Σ monounsaturated FA	22.96	30.30	12.77
Σ polyunsaturated FA	40.65	44.44	78.51
N-6 FA	3.01	1.17	40.63
N-3 FA	37.64	43.27	37.88
N-6/N-3 FA ratio	0.08	0.03	1.07

(4.0-4.5 months of age) were purchased from the Faculty of Pharmaceutical Sciences (São Paulo, Brazil). The mice were housed in plastic cages (5 animals/cage) at constant temperature ($22 \pm 2^\circ\text{C}$) and relative humidity ($55 \pm 10\%$), with a 12-h light–dark cycle. Food and water were available ad libitum, and animals were divided into four groups. All groups were fed with a high fat diet for 4 weeks (Table 2), and were supplemented with an oil-in-water emulsion ($190 - 240 \mu\text{L/d}$) per mouse containing fish oil (FIS), algae oil (ALG), Echium oil (ECH) or water (CON) by gavage. Amount of EPA, DHA, SDA and ALA ingested from added oil per day is presented in Table 3. We opted to compare the effect of the same amount of EPA+DHA in all groups. To achieve this objective the rate of conversion from SDA to EPA (4:1) proposed by Whelan [16] was adopted in our study. By this way, the EPA+DHA dosage applied was 0.7, 0.8 and 0.7 mg/day using Fish, Algae and Echium oil respectively (Table 3). The emulsions were prepared weekly by mixing the respective oil with water using a high-pressure homogenizer (Homolab mod A-10, Alitec, São Paulo, Brazil). The emulsions were prepared in less than 2 minutes, and the temperature was kept below 40°C , during this short time. After, the emulsions were transferred to 2 mL eppendorf tubes, immediately

Table 2 Composition and major fatty acids profile of the high fat diet

Composition ²	High fat diet ¹
Moisture (g/100 g)	7.22 ± 0.05
Ashes (g/100 g)	2.94 ± 0.10
Protein (g/100 g)	17.99 ± 0.49
Lipids (g/100 g)	30.72 ± 0.57
Carbohydrate (g/100 g)	41.13 ± 0.33
Energy (Kcal/100 g)	512.96 ± 3.32
Fatty acids (g/100 g total FA)	
C10:0 – capric	0.81 ± 0.00
C12:0 - lauric	1.03 ± 0.17
C14:0 - miristic	1.68 ± 0.01
C16:0 - palmitic	26.20 ± 0.62
C18:0 - stearic	17.30 ± 0.05
C16:1 – palmitoleic	0.96 ± 0.07
C18:1 n-9 - oleic	32.73 ± 0.12
C18:2 – (LNA) linoleic (n-6)	11.94 ± 0.97
C18:3 – (ALA) α-linolenic (n-3)	0.60 ± 0.06
C18:3 – (GLA) γ- linolenic (n-6)	0.25 ± 0.06
C18:4 – (SDA) stearidonic (n-3)	-
C20:0 - arachidic	0.27 ± 0.01
C20:1 - eicosaenoic (n-9)	0.59 ± 0.05
C20:2- eicosadienoic (n-6)	0.26 ± 0.02
C20:4- (ARA) arachidonic (n-6)	-
C20:5 – (EPA) eicosapentaenoic (n-3)	-
C22:5 – (DPA) docosapentaenoic (n-3)	-
C22:6 – (DHA) docosaenoic (n-3)	-
NI	5.15

¹ Based on Safwat et al. [30].² Diet was composed by 187.0 g casein, 133.5 g sucrose, 300.0 g lard, 193.5 g corn starch, 67.0 g cellulose, 53.5 g soybean oil, 46.5 g minerals, 2.4 g L-cystine, 3.3 g choline bitartrate and 13.35 g vitamin mix/ kg diet.

NI, not identified.

immersed in nitrogen and kept at -80°C until the time of gavage. All this procedure was repeated twice a week. Emulsion characteristics are presented in Table 3. Diet consumption was measured daily and animals were weighed individually twice a week. After 4 weeks, the mice were fasted for 12 h, and anaesthetised with a mixture containing xylazine 2% (Sespo Ind. e Com. Ltda., Paulínia, Brazil), ketamine (Syntec do Brasil Ltda., Cotia, Brazil) and acepromazine (Vetnil Ind. e Com. de Prod. Veterinários Ltda., Louveira, Brazil). Blood samples collected from the brachial plexus were immediately centrifuged ($1,600 \times g$ for 15 min at 4°C), frozen under liquid nitrogen and stored (-80°C) for further analysis. The liver was excised, dried with lint and weighed. Small pieces of the larger lobe were frozen for Western blotting and further analysis, and a piece of the smaller lobe was immersed in 10% buffered

Table 3 Characteristics of the emulsions prepared with three N-3 FA sources

Characteristics ¹	Emulsions		
	Fish	Algae	Echium
Emulsion volume (μL/d)	240	190	270
Oil in the emulsion (%)	1	1	10
Oil supplementation (μL/d)	2.4	1.9	27.0
Oil supplementation (mg/d)	2.2	1.8	25.1
C20:5 – (EPA) eicosapentaenoic (n-3) (mg/d)	0.44	-	-
C22:6 – (DHA) docosaenoic (n-3) (mg/d)	0.25	0.77	-
C18:4 – (SDA) stearidonic (n-3) (mg/d)	0.07	-	2.792
C18:3 – (ALA) linolenic (n-3) (mg/d)	0.01	-	6.71
C22:5 – (DPA) docosapentaenoic (n-3) (mg/d)	0.05	0.01	-
N-6 FA from gavage (mg/d)	0.07	0.02	10.20
N-3 FA from gavage (mg/d)	0.82	0.78	9.501
N-6 FA from diet (mg/d)	80.07	80.02	80.00
N-3 FA from diet (mg/d)	4.00	4.00	4.00
Total N-6 FA (mg/d)	80.07	80.02	90.20
Total N-3 FA (mg/d)	4.82	4.78	13.50
N-6/N-3 FA ratio	16.6	16.7	6.7

¹ Supplementation is expressed as mg/d/animal.² Assumed no conversion from ALA to EPA and equivalence from SDA to EPA of 25%, according to Whelan [16], resulting in about 0.70 mg/d.

formalin solution for histopathological examination. Subsequently, the animals were perfused with a cold NaCl solution (0.9%, 240.0 mL) via their left ventricle to remove the excess of blood. The animal protocol was approved by the Ethics Committee for Animal Studies of the Faculty of Pharmaceutical Sciences (São Paulo, Brazil).

Measurements

Fatty acids were isolated from the liver and diets using the extraction methodology proposed by the Association of Official Analytical Chemists (method 996.06) [17]. The fatty acid methyl esters (FAME) were suspended in hexane and analyzed by a gas chromatograph (GC17A Shimadzu Class CG, Kyoto, Japan) equipped with a 30 m × 0.25 mm (i.d.), 0.25 μm film thickness fused silica capillary column (Supelcowax, Bellefonte, PA, USA) and a flame ionization detector. Helium was used as a carrier gas, and the fatty acids were separated using a $10^{\circ}\text{C}/\text{min}$ gradient from 80 to 150°C and then a $6^{\circ}\text{C}/\text{min}$ gradient from 150°C to 230°C . Standard mixtures with 37 FAME and PUFA 3 methyl esters from Menhaden oil (Sigma Chemical, St. Louis, MO, USA) was used to identify the peaks. The results were expressed as percentage of the total fatty acids present.

The serum lipoprotein concentrations, including total cholesterol, high density lipoprotein (HDL) and TG, were quantified using an enzymatic colorimetric test from Labtest (Lagoa Santa, MG, Brazil). The low density

lipoprotein levels (LDL) and VLDL were estimated using the Friedewald formula [18]. The inflammation biomarkers (C-reactive protein (CRP), interleukin-6 (IL-6) vascular cell-adhesion molecule-1 (VCAM), Inter-Cellular Adhesion Molecule (ICAM) and adiponectin) were analysed in serum samples using Multiplex commercial Kits (Millipore, St. Charles, MO, USA).

Liver histology

The representative liver fragments were fixed in a 10% buffered formalin solution for approximately 48 hours. Then, the fragments were fixed in paraffin. The material was sub-mitted to microtomy with a cut of 5 μ m and stained with hematoxylin and eosin for histopathological evaluation [19].

Western blotting analysis of hepatic peroxisome proliferator activated receptor α (PPAR α) and liver X receptor α (LXR α)

The total nuclear protein was extracted from the frozen liver tissue samples using the specific commercial Kit NEPER (GE Healthcare, Little Chalfont, Bucks, UK). Subsequently, 15 μ g of the proteins present in the supernatant was separated on a 10% SDS-PAGE gel and transferred to AmershamTM HybondTM-ECLTM nitrocellulose membranes (GE Healthcare UK Ltd.-Amersham Place, Little Chalfont, Buckinghamshire, UK) using a humid system composed of buffer containing 25 mM Tris base, 192.0 mM glycine, 0.02% SDS, and 10% methanol (GE Healthcare, Little Chalfont, Bucks, UK). The membranes were then blocked with 5% ECL advance blocking agent in TBST (Tris-buffered saline and Tween 20) for 1.5 h to prevent the occurrence of nonspecific binding, and they were incubated with primary antibodies (rabbit

polyclonal to LXR α – ab 82774 and rabbit polyclonal to PPAR α – ab 8934, ABCAM plc., Cambridge, MA, USA) diluted in a ratio of 1:345 and 1:500, respectively. After this step, the membranes were incubated for 1 h at 4°C with a secondary antibody conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, Bucks, UK). Following three washes in TBST, the immunoreactive bands were visualised using the ECL advance detection system (Amersham Biosciences, Pittsburgh, PA, USA). To standardise the immunoblots, a digital detection system was applied (IMAGE QUANTTM 400 version 1.0.0, Amersham Biosciences, Pittsburgh, PA, USA) using proliferating cell nuclear antigen – PCNA (ABCAM plc., Cambridge, MA, USA) as standard. The densitometry was quantified using the Discovery seriesTM Quantity oneW Analysis Software version 4.6.3 (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Statistical analysis

The effect of each N-3 FA source on biomarkers was compared by one-way ANOVA and Tukey HSD test. Equivalent non-parametric ANOVA was applied when there was no homogeneity of variances (Hartley test). A probability value of 0.05 was adopted to reject the null hypothesis. All calculations and graphs were performed using the software Statistica v.9 (Statsoft Inc., Tulsa, USA).

Results

All groups showed the same weight gain and diet consumption (Table 4). Animals supplemented with Echium oil presented lower total cholesterol and triacylglycerol concentrations than Control, and lower

Table 4 Body weight, diet consumption, plasma lipid profile and inflammatory biomarkers observed in the supplemented groups

	CON	FIS	ALG	ECH	P [†]
Initial weight (g)	26.9 $\pm$ 1.3	26.8 $\pm$ 0.6	27.3 $\pm$ 0.1	27.8 $\pm$ 0.8	0.670
Final weight (g)	28.9 $\pm$ 2.0	28.3 $\pm$ 0.6	29.8 $\pm$ 1.4	28.8 $\pm$ 0.5	0.731
Weight gain (g)	2.0 $\pm$ 0.7	1.5 $\pm$ 0.0	2.5 $\pm$ 1.3	1.1 $\pm$ 1.3	0.542
Diet consumption (g/d/mouse)	2.3 $\pm$ 0.2	2.3 $\pm$ 0.1	2.3 $\pm$ 0.1	2.4 $\pm$ 0.3	0.886
Cholesterol (mg/100 mL)	371.7 $\pm$ 37.4a	349.8 $\pm$ 37.2ab	350.2 $\pm$ 56.3ab	312.9 $\pm$ 40.2b	0.040
LDL (mg/100 mL)	244.3 $\pm$ 32.2	240.4 $\pm$ 34.2	227.6 $\pm$ 50.4	199.3 $\pm$ 31.4	0.054
HDL (mg/100 mL)	95.9 $\pm$ 8.8	82.7 $\pm$ 13.7	95.0 $\pm$ 8.4	92.0 $\pm$ 13.7	0.050
VLDL (mg/100 mL)	29.7 $\pm$ 4.6a	26.7 $\pm$ 5.1a	27.6 $\pm$ 9.7 a	21.6 $\pm$ 3.5b	0.036
Triacylglycerol (mg/100 mL)	149.0 $\pm$ 23.6a	133.6 $\pm$ 25.4ab	115.8 $\pm$ 34.2b	109.1 $\pm$ 17.5 b	0.008
CRP (ng/mL)	121.6 $\pm$ 5.5	118.9 $\pm$ 6.7	118.0 $\pm$ 9.6	124.6 $\pm$ 8.6	0.232
IL-6 (pg/mL)	11.4 $\pm$ 2.3	10.6 $\pm$ 2.0	10.1 $\pm$ 1.9	11.2 $\pm$ 2.0	0.457
VCAM (pg/mL)	1.7 $\pm$ 0.4	1.7 $\pm$ 0.3	1.6 $\pm$ 0.5	1.3 $\pm$ 0.2	0.059
ICAM (ng/mL)	39.3 $\pm$ 6.7	36.9 $\pm$ 3.8	39.2 $\pm$ 4.4	35.4 $\pm$ 3.4	0.188
ADIPONECTIN (pg/mL)	13.9 $\pm$ 8.3	14.4 $\pm$ 6.0	11.5 $\pm$ 3.7	14.3 $\pm$ 9.3	0.805

[†] Probability value obtained by ANOVA. Values followed by the same letter are not significantly different at the 0.05; a,b: $p < 0.05$ (n = 10/group).

VLDL than all of the other groups, constituting the best lipoprotein profile observed in this study. None of the N-3 FA sources altered any of the inflammation bio-markers (Table 4). The effect of N-3 FA associated with high fat diet on the histological evaluation of the liver tissue is presented in Figure 1. Hepatic steatosis can be observed in the CON group with fatty infiltration around the portal space. Fish and Echium oils attenuated hepatic steatosis, whereas algae oil did not promote any protection against the hepatic steatosis induced by the high fat diet. The N-3 FA must be present in liver to exert their effects on lipid metabolism. Of the main N-3 FA applied in our study (ALA, SDA, EPA and DHA) (Figure 2), only EPA was observed in a higher concentration in the liver homogenate of animals supplemented with fish and Echium oils (Table 5). Moreover, a lower omega 6/omega 3 ratio (N-6/N-3 FA ratio) ($p < 0.001$) was observed in liver of animals supplemented with Echium oil (Table 5). In order to better investigate the action of N-3 FA on the hepatic steatosis, two transcription factors involved in lipid metabolism were evaluated. Figures 3 and 4 present the influence of the N-3 FA supplementation on the hepatic transcription factors $LXR\alpha$ and $PPAR\alpha$, which are associated with fatty acids synthesis and oxidation, respectively. Animals supplemented with algae oil

experienced a significant increase of $PPAR\alpha$ expression when compared to all other groups (Figure 3). A decrease in $LXR\alpha$ expression was observed in the groups supplemented with fish and algae oils (Figure 4), whereas Echium oil did not alter any of these two transcription factors.

Discussion

The three sources of N-3 FA fatty acids were effective in improving the plasma lipid profile of the LDLr knockout mice. Among them, Echium oil provided the best results in terms of VLDL and total cholesterol reduction and contributed to the attenuation of hepatic steatosis. None of these oils was able to reduce the inflammation caused by the high fat diet, according to the biomarkers evaluated in this study.

The supplementation with fish and Echium oils increased EPA concentrations in liver homogenate. The capacity of SDA to increase EPA content in different tissues is still controversial. Zhang et al. [20] reported that the supplementation of LDLr knockout mice with Echium oil (10% of total energy intake) resulted in a significant enrichment of EPA in plasma lipids. According Harris [21] the direct dietary intake of SDA has been proposed to be another strategy to increase tissue EPA levels, since SDA does not depend of 6-desaturase to be converted in EPA.

Figure 1 Representative photomicrographs of liver sections: (CON) - fatty infiltration around the portal space; (FIS) and (ECH) - antisteatogenic effect exhibiting well-defined cells and low-fat vacuoles in the cytoplasm, and (ALG): hepatocytes presenting fatty infiltration around the portal space. Original magnification 10X.

LXRs and PPARs are nuclear receptors that play crucial role in the regulation of fatty acid metabolism [22]. The hypolipidaemic effect of algae and fish oils has been partially attributed to the downregulation of LXR α , with a subsequent inhibition of fatty acid synthesis, associated with the upregulation of PPAR α , which promotes β -oxidation. Several studies have demonstrated that EPA and DHA reduce TG and VLDL acting as PPAR α agonists and LXR α antagonists [5,23,24]. According to Chilton et al. [3], SDA and EPA reduce the level of mRNA for Sterol Regulatory Element-Binding Protein 1C (SREBP1c), Fatty Acid Synthase (FAS) and stearoyl CoA desaturase 1 (SCD) in liver, suggesting that a possible

mechanism to explain TG reduction would be associated with a decrease in the LXR α and consequently in the genes that codify proteins involved in fatty acids hepatic synthesis. This mechanism could be clearly observed to the both marine oils applied in our study, but not to the Echium oil. Animals supplemented with Echium oil showed the most significant VLDL reduction and attenuated steatosis, although no differences had been in regards to LXR α and PPAR α expression. In fact, the mechanisms for the reduction of the plasma TG levels by Echium oil are unknown. Although the dose applied in our study was 5-fold lower, our results agree with those reported by Zhang et al. [20], who observed a reduction in TG and

Table 5 Major fatty acids composition in liver homogenate (g/100 g Total FA) observed in the supplemented groups

Fatty acids	CON	FIS	ALG	ECH	P [†]
C14:0 – myristic acid	0.47 ± 0.08	0.46 ± 0.06	0.50 ± 0.08	0.41 ± 0.08	0.079
C16:0 – palmitic acid	26.37 ± 1.96	28.73 ± 4.15	27.60 ± 3.20	29.99 ± 3.91	0.163
C18:0 – stearic acid	12.11 ± 3.85	13.43 ± 4.98	10.50 ± 2.74	15.61 ± 6.43	0.107
C18:1- oleic acid	27.09 ± 6.25	26.4 ± 6.33	29.5 ± 4.87	22.15 ± 7.34	0.074
C18:2 – (LNA) linoleic (n-6)	12.87 ± 3.22	11.40 ± 4.82	12.53 ± 5.81	10.07 ± 4.64	0.584
C18:3 – (ALA) α-linolenic (n-3)	0.15 ± 0.13	0.14 ± 0.14	0.19 ± 0.12	0.26 ± 0.22	0.744
C18:3 – (GLA) γ-linolenic (n-6)	0.30 ± 0.05	0.35 ± 0.15	0.31 ± 0.11	0.34 ± 0.09	0.396
C20:4- (ARA) arachidonic (n-6)	6.69 ± 1.85	5.31 ± 1.46	4.80 ± 1.26	4.55 ± 1.22	0.060
C20:5 – (EPA) eicosapentaenoic (n-3)	0.12 ± 0.04 ^a	0.17 ± 0.06 ^{ab}	0.11 ± 0.05 ^a	0.25 ± 0.04 ^b	0.004
C22:6 –(DHA) docosahexaenoic (n-3)	2.98 ± 0.75	2.92 ± 0.96	2.81 ± 0.88	2.76 ± 0.92	0.951
N-6 FA	19.86 ± 3.56 ^b	17.06 ± 5.78 ^{ab}	17.64 ± 6.61 ^{ab}	14.97 ± 5.81 ^a	0.017
N-3 FA	3.25 ± 0.70	3.23 ± 0.98	3.12 ± 0.95	3.27 ± 0.96	0.982
N-6/N-3 FA	6.11 ± 1.46 ^a	5.27 ± 1.08 ^a	5.65 ± 1.54 ^a	4.58 ± 1.41 ^b	<0.001

[†] Probability value obtained by ANOVA. Values followed by the same letter are not significantly different at the 0.05; a, b: $p < 0.05$ (n 6–10 animals/group).

VLDL levels after Echium oil supplementation without changes in PPARα and LXRα expression. These results suggest that Echium oil can exert its beneficial effect on lipid metabolism and hepatic steatosis via mechanisms other than those reported for marine oils. In addition, it

has been recommended [25] that studies involving SDA adopt a dose equivalent to EPA for supplementation. However, when this procedure was carried out in our study (Table 3), N-6/N-3 FA ratio of emulsion containing Echium oil (6.7) became lower than emulsions with algae

Figure 3 Changes in PPARα expression after 4 weeks of supplementation - water (CON), fish oil (FIS), algae oil (ALG) and Echium oil (ECH). * $p < 0.05$.

Figure 4 Changes in LXR α expression after 4 weeks of supplementation- water (CON), fish oil (FIS), algae oil (ALG) and Echium oil (ECH). **P<0.01

(16.7) and fish (16.6) oils. Thus, differences observed in biomarkers between ECH group and the other two supplemented groups (ALG and FIS) can have also been influenced by these difference in N-6/N-3 FA ratio.

Mice supplemented with Echium oil showed reduction of N-6/N-3 ratio in liver (Table 5). According to Parker et al. [26] the increase of N-6/N-3 FA ratio in liver is associated with higher steatosis, since this condition can favor lipogenesis and inflammation processes. These findings have also been confirmed in human and animal studies [27,28]. Thus, the lower N-6/N-3 FA ratio in liver homogenate can have contributed to the attenuation of steatosis observed in ECH group.

None of these three N-3 FA fatty acids sources was able to reduce serum inflammatory biomarkers. Ishihara et al. [29] observed that, in whole blood of Balb/c mice, the production of Tumor Necrosis Factor - α (TNF α) was suppressed by ALA, SDA and EPA supplementation. However, the dose applied by the authors was 53-fold higher than the dose used in our study. Our high-fat diet was formulated on basis of the diet applied by Safwat et al. [30] to promote hepatic steatosis in rats. The authors observed that after 10 weeks, the animals developed hepatic steatosis, insulin resistance, hypertriglyceridaemia, and increased VLDL levels, but they observed no evidence of hepatic inflammation or fibrosis, suggesting that the hepatic steatosis was in its

early stages. It is possible that the dose of EPA, DHA and SDA used in our study, although corresponding to an intake of 2 g/day for humans, was not sufficient to reduce the inflammation biomarkers when the process is at its initial steps. In spite of this, the high N6/N3 FA ratio present in the diet (16:1), typical of Western diet [5], may have annulled the potential LC N-3 PUFA anti-inflammatory action due to the higher availability of ARA than EPA as substrate to the oxidation mediated by cyclooxygenase and lipoxygenase enzymes.

Conclusions

In our study, the supplementation with three different sources of N-3 FA fatty acids was evaluated using LDLr knockout animals fed with a high fat diet. It was observed that the best combination of results, in terms of plasma lipid profile and steatosis, was achieved by the supplementation with Echium oil, and the mechanism involved in this favourable result seems to be different from those involved with EPA and DHA metabolism, maybe due to the lower N-6/N-3 FA ratio present in the liver of animals supplemented with Echium oil. Theoretically, it is possible to transfer a metabolic pathway for EPA and DHA synthesis from a marine organism to an oilseed crop plant [14]. However, while this option is not

available, our study confirms that Echium oil represents an alternative as natural ingredient to be applied in functional foods to reduce cardiovascular disease risk.

Abbreviations

ALA: α - Linolenic acid; ALG: Algae oil group; CON: Control group; CRP: C-reactive protein; CVD: Cardiovascular disease; DHA: Docosahexaenoic acid; ECH: Echium oil group; EPA: Eicosapentaenoic acid; FAME: Fatty acid methyl ester; FDA: Food and drug administration; FIS: Fish oil group; HDL: High density lipoprotein; ICAM: Inter-cellular adhesion molecule; IL- 6: Interleukin 6; LDL: Low density lipoprotein; LDL: Receptor deficient mice; LDL_r: Knockout mice; LNA: Linoleic acid; LXR- α : Liver X receptor α ; LC n-3 PUFA: Long-chain n-3 polyunsaturated fatty acids; N-3 FA: Omega 3 fatty acids; N-6/N-3 FA: Omega 6 fatty acids/omega 3 fatty acids; PPAR- α : Peroxisome proliferator activated receptor α ; SDA: Stearidonic acid; TG: Triacylglycerol; TNF- α : Tumor necrosis factor - α ; VCAM: Vascular cell adhesion molecule; VLDL: Very low density lipoprotein.

Competing interest

The authors have no conflict of interest to declare.

Authors' contributions

PBB conducted the research and wrote the manuscript. KRM performed part of the experiments. MMR participated in critical revision of the study. IAC contributed to the design of the study, analysis of data, drafting the manuscript and was responsible for the financial support. IAC had primary responsibility for the final content. All the authors read and approved the final manuscript

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CAPÍTULO 2

HIPÓTESE 2: A suplementação na infância de uma mistura de AGPI n-3 e fitosteróis apresenta potencial sinérgico na redução do risco cardiovascular na fase adulta.

Effect of supplementation with omega 3 fatty acids and phytosterols on atherosclerosis risk in LDLr knockout mice

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Abbreviations

ABCA1 (adenosine triphosphate binding cassette transporter A1), ABCG5 (adenosine triphosphate binding cassette transporter G5), ABCG8 (adenosine triphosphate binding cassette transporter G8), ALA (α -linolenic fatty acid) ALG (algae oil group), ALG + PHY (algae oil + phytosterol group), CETP (cholesterol Ester transfer protein), CON (control group), CVD (cardiovascular disease), DHA (docosahexaenoic fatty acid), DPA (docosapentaenoic fatty acid), ECH (echium oil group), ECH + PHY (echium oil + phytosterol group), EPA (eicosapentaenoic fatty acid), FAS (fatty acid synthase), FDA (Food and Drug Administration), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), GLA (gamma linolenic fatty acid), GPX (glutathione peroxidase), GR (glutathione reductase), HDL-c (High density lipoprotein), LDL-c (Low density lipoprotein cholesterol), LDL_r knockout mice (LDL receptor deficient mice), LNA (linoleic fatty acid), LXR- α (liver X receptor – α) MDA (malondialdehyde) n-3 FA (Omega 3 fatty acids), n-6-FA (Omega 6 fatty acids), NPC1L1 (Niemann Pick C1 like 1), PHY (phytosterol group), PPAR- α (peroxisome proliferator activated receptors – α), PUFA (polyunsaturated fatty acid), SDA (stearidonic fatty acid), SOD (superoxide dismutase), SREBP1C (sterol regulatory element-binding proteins 1C), TBA (thiobarbituric acid), TBST (Tris-buffered saline and Tween 20), VLDL (very low density lipoprotein)

ABSTRACT

Atherosclerotic process begins early in life and it is progressive throughout the life span. As drug therapy is not recommended for healthy children, supplementation with bioactive compounds may be an alternative approach by which atherosclerosis could be prevented. To evaluate this hypothesis, LDL_r knockout mice were supplemented by gavage with omega 3 fatty acids (n-3 FA) with or without phytosterols during the first 2 months of life. Subsequently, dyslipidemia and oxidative stress were induced by a high-fat diet intake for 2 months. Instead of being reduced, an increase was observed in fatty streaks lesion area following isolated phytosterol supplementation by gavage. This effect was reversed by co-supplementation with n-3 FA. The mechanisms by which n-3 FA reversed the phytosterol-induced increase in lesion area involved activation of fatty acid oxidation by Peroxisome Proliferator Activated Receptor α , reduction in fatty acid synthesis by Liver X Receptor α in the liver and reduction of oxidative stress by a mechanism that involved the modulation of GPx activity. In conclusion, the co-supplementation of weaning LDL_r knockout mice with phytosterols and n-3 FA did not reduce the cardiovascular risk in adulthood. However, the increase of fatty streak induced by isolated phytosterol was fully reserved by the presence of n-3 FA.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality in many countries, accounting for 15.6 million deaths annually (1). Atherosclerosis, a chronic inflammatory disorder, is the pathological process that underlies the CVD (2), being generally observed in middle-aged or elderly individuals. However, the process of atherosclerosis begins in childhood and is progressive throughout adulthood (3). Studies have demonstrated that the extension of atherosclerosis in children and young adults can be associated with the presence of the same risk factors that have been identified in adults, such as high concentration of low density lipoprotein cholesterol (LDL-c), obesity, hypertension, diabetes mellitus and cigarette smoking (4). Except for those with familial hypercholesterolemia, pharmacological interventions in children should be discouraged due to its adverse effects (5). Thus, functional foods could represent an alternative in terms of CVD prevention. Many bioactive compounds have potential to be added to food formulations with the aim of reducing CVD risk factors. Among these compounds, phytosterols and omega 3 fatty acids (n-3 FA) are approved and qualified by Food and Drug Administration, respectively (6). Although the effectiveness of functional foods is usually much lower when compared with medications, the former can easily be incorporated into the diet, are safe to be consumed by children and do not present any adverse effects.

Phytosterols are compounds with a molecular structure similar to that of cholesterol and are found in seeds, vegetable oils and cereals (7-8). These molecules are able to displace cholesterol during micelle formation in the intestine due to their higher hydrophobicity, reducing cholesterol absorption (9). According to Démonthy et al. (10), the daily intake of 2.5 g of phytosterols can reduce LDL-c by approximately 10%. The cardioprotective effects of n-3 FA, especially α -linolenic acid (ALA), stearidonic acid (SDA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been reported in cells culture, animals and humans studies (11-13). ALA and SDA must be first converted *in vivo* to EPA and DHA by desaturase and elongase enzymes (14). It has been reported that the conversion rate of ALA for EPA and DHA is very low, 5-10% and < 1% respectively, due to the limitation in the first step of the conversion from ALA to SDA, which is catalyzed by $\Delta 6$ -desaturase (15). Therefore, the direct dietary intake of SDA has been proposed to be another strategy to increase tissue EPA concentration (16). SDA can be found in plants, such as *Echium* (*Echium*

plantagineum), black currant seeds and other genetically modified seeds (17). Moreover, SDA does not have a fishy aftertaste and exhibit better oxidative stability than marine oils. The consumption of n-3 FA likely reduces CVD through multiple mechanisms, including reducing triacylglycerol and exerting anti-inflammatory effects, contributing to decreased atherogenesis and plaque rupture (18).

Our hypothesis is that the anti-inflammatory and hypotriglycerolemic effects of n-3 FA in combination with the hypocholesterolemic effect of phytosterols during childhood could reduce the risk of CVD in adulthood. To evaluate this hypothesis, weaning LDL-c receptor deficient mice (LDL_r knockout mice) were supplemented with one of two sources of n-3 FA (SDA/ALA or DHA), with or without addition of phytosterols, during their first 2 months of age. Following this period, dyslipidemia and oxidative stress were induced by a high-fat diet. In addition to diet, the mice received bioactive compound supplementation by gavage until the end of the trial, at which point biomarkers of atherosclerosis were evaluated.

MATERIAL AND METHODS

Oils

The n-3 FA and phytosterol esters that were used in the present study were commercial products. The algae oil containing $\approx 40\%$ DHA (DHASCO) was obtained from Martek Biosciences® (Winchester, KY, USA). The *Echium* oil containing $\approx 11.5\%$ SDA (AW39144ECH) was obtained from Oil Seed Extraction® (Ashburton, New Zealand) and the phytosterol esters (CardioAid-S) were obtained from Archer Daniels Midland Company – ADM® (Decatur, IL, USA). Fatty acids content of the algae and *Echium* oils and the phytosterol profile of CardioAid-S were analyzed by gas chromatography and are shown in **Supplemental Table 1**.

Animals and Diets

Fifty-three weaning male homozygous LDLr knockout mice (C57BL/6) (21 days) weighing about 10 g were purchased from the Faculty of Pharmaceutical Sciences, University of São Paulo (São Paulo, Brazil). The mice were housed in plastic cages (2 to 5 animals/cage) at a constant temperature ($22 \pm 2^\circ\text{C}$) and relative humidity ($55 \pm 10\%$), with a 12-h light-dark cycle. Food and water were available *ad libitum*. For 2 months, all of the animals received a control diet (Nuvilab® CR-1, Nutrivital Nutrientes Ltda) based on National Research Council recommendation (19), and were supplemented by gavage with an emulsion containing soybean oil (CON) (N = 8), *Echium* oil (ECH) (N = 10), algae oil (ALG) (N = 10), phytosterol (PHY) (N=10), *Echium* oil plus phytosterol (ECH+PHY) (N=7) or algae oil plus phytosterol (ALG+PHY) (N= 8). Following this period, the control diet was changed to a high-fat diet (**Supplemental Table 2**). In addition to this diet, the mice continued receiving supplementation by gavage for 2 additional months, for a total of 4 months of treatment. The emulsions were prepared weekly by mixing the respective oil with water using a high-pressure homogenizer (Homolab mod A-10, Alitec, São Paulo, Brazil) and kept at -80°C until the time of gavage.

The n-3 FA and phytosterol dosages were based on the recommendation of the FDA for humans (2g/d)(6). In our experiment design, the n-3 FA and phytosterol dosages were calculated on basis of mice body weight. In the groups supplemented with Alga

oil, n-3 FA was represented exclusively by DHA (0,29 mg/d/ animal). The groups who received Echium oil, the conversion rate from ALA or SDA to EPA reported by other studies was used to match the supplementation among groups. In this case, the rate of 4:1 and 0.2:1 were applied for SDA and ALA conversion to EPA, respectively (20-21). A summary of the n-3 FA and phytosterols intake during the study is presented in **Supplemental Table 3 and 4**. The supplementation dosage (equivalent to 2g/d for humans) did not taken into account the n-3 FA and phytosterols content from the diet (**Supplemental Table 3 and 4**). Thus, the total supplementation included the emulsion given by gavage and the compounds naturally present in the lipids of the diet. This level of supplementation was adopted in our study because individuals usually add functional foods or capsules to their dairy diet.

The dietary consumption was recorded daily, and the animals were weighed individually twice per week. After 4 months, the mice were deprived of food for 12 h and anaesthetized with a mixture that contained xylazine, ketamine and acepromazine. Blood samples ($\approx 700 \mu\text{L}$) were collected from the brachial plexus, immediately centrifuged ($1,600 \times g$ for 15 min at 4°C), frozen in liquid nitrogen and stored (-80°C) for future analysis. The liver was excised and weighed. Small pieces of the larger lobe were frozen for western blotting. Subsequently, the animals were perfused with a cold NaCl solution (0.9%, 240.0 mL) via their left ventricle to remove the excess of blood. Following the perfusion, the upper section of the heart was placed in an embedding medium (Tissue-Tek, Satura FineTek, Torrance, CA), frozen in liquid nitrogen and stored at -80°C . Abdominal Aorta arteries were delipidated and stored at -80°C for fatty acid profile analyses. The animal protocol was approved by the Ethics Committee for Animal Studies of the Faculty of Pharmaceutical Sciences (São Paulo, Brazil).

Determination of phytosterol

Phytosterols content of the ingredient used to prepare the emulsion was determined according to Laakso (22). The extracted sterols were derivatized with 200 μL of bis (trimethylsilyl)-trifluoroacetamid (BSTFA) containing 1% trimethylchlorosilane (TMCS) (99:1) and 100 μL of pyridine. An aliquot of 1.0 μL of derivatized sample solution as introduced into the column at 250°C . The components were separated at 300°C and detected with flame ionization detector (FID) at 280°C . The carrier gas was helium at flow rate of 1.0 ml/min. Reference standards were used to

identify the peaks. Quantification was calculated based on standard curve prepared with β -sitosterol/IS area ratio, as a linear function ($r = 0,9983$) of sterol concentration (0.5 - 5.0 mg)

Fatty acids profile

Fatty acids were isolated from the diets using the extraction methodology proposed by the Association of Official Analytical Chemists (method 996.06) (23) while oils were derivatized according method described by American Oil Chemists Society (AOCS) (Official Method Ce 1e-91) (24). Plasma and tissue homogenates samples were derivatized using direct esterification, as was described by Shirai; Suzuki; Wada (25). The composition of the samples was determined using a gas chromatograph (Agilent 7890 A GC System, Agilent technologies Inc., Santa Clara, USA). A fused silica capillary column (J&W DB-23 Agilent 122-236; 60 m x 250 mm inner diameter) was used for the injection. High-purity helium was used as the carrier gas at a flow rate of 1 ml/min, with a split injection of 50:1. The programming of the column temperature was as follows: 80°C to begin; a heating rate of 5°C/min up to 175°C followed by another gradient of 3°C/min to 230°C; maintenance at this temperature for 5 min. The temperature of the injector and detector (FID) were 250°C and 280°C, respectively. The fatty acids were identified by comparing the retention times with those of the following four purified standard mixtures of fatty acid methyl esters: 4-7801; 47085-U; 49453-U and 47885-U, all of which were from Sigma Chemical Co. (St. Louis, MO, USA). The results were expressed as % of total fatty acids.

The assessment of atherosclerosis in the aortic root

Five to six 4 μ m-sections of aortic root per animal were taken 80 μ m apart and stained with Oil Red-O to measure atherosclerosis, as previously described by Paigen et al.(26). All of the measurements were performed using Qwinimage analysis software(Leica imaging systems, UK) by an independent investigator who was unfamiliar with the experimental groups (Leica imaging systems, UK). The lesion area was expressed as the mean ($n = 5$) of the areas from the analyzed sections.

Lipoprotein measurements

The serum lipoprotein concentrations, including those of total cholesterol, very low density lipoprotein - VLDL , high density lipoprotein – HDL-c and TG, were quantified using commercial kits for enzymatic colorimetric tests from Labtest (Lagoa Santa, MG, Brazil). The LDL-c concentration were estimated using the Friedewald formula (27).

Determination of malondialdehyde concentration in liver homogenates

The malondialdehyde (MDA) concentration was determined by reverse phase HPLC, following the protocol of Hong et al. (28). The TBA-MDA conjugate derivative was injected into a Phenomenex reverse-phase C18 analytical column (250 mm x 4.6 mm; 5 mm – Phenomenex, Torrance, CA, USA) with a LC8-D8 pre-column (Phenomenex AJ0-1287) and was fluorometrically quantified at an excitation of 515 nm and an emission of 553 nm. The HPLC pump delivered the isocratic mobile phase: 60% PBS (50 mmol, pH 7.1) + 40 % methanol at a flow rate of 1.0 mL/ min. A standard curve was prepared using 1,1,3,3-tetraethoxypropano (TEP). The results were expressed as nmol MDA/mg protein and also as (nmol/mg protein)/%PUFA.

Antioxidants enzymes activity in liver homogenates

Superoxide dismutase (SOD) activity was determined according the method described by Ewing & Janero (29). The absorbance at 560 nm was continuously monitored over 5 min as an index of Nitrotetrazolium Blue chloride (NBT) reduction. A standard curve was prepared using SOD – Sigma Chemical Co. (St. Louis, MO, USA) (0.173 - 2.77 U/mg protein). The catalase activity was determined according to the method that was described by Bonaventura; Schroeder; Fang (30), with modifications. Liver homogenate that contained 0.05 µg/µL of protein (20 µL) was added to a microplate with 140 µL phosphate buffer (50 mM with 0.1 mM EDTA, pH 7.4) and 40 µL 30 mM freshly prepared hydrogen peroxide. The absorbance at 240 nm was continuously monitored over 8 min at 30°C. A standard curve was prepared using the Catalase enzyme (Sigma Chemical Co., St. Louis, MO, USA) (0.4437 - 14.17 U/mg

protein). Glutathione peroxidase (GPx) activity was determined according to the method described by Flohe & Giansler (31), with modifications. Briefly, 30 μ L liver of homogenate (with 25 μ g/ μ L of protein) was incubated with 125 μ L 0.1 M phosphate buffer with 1 mM EDTA (pH 7.4), 5 μ L freshly prepared 0.08 M reduced glutathione and 5 μ L freshly prepared Glutathione Reductase (9.6 U) at 37 °C for 5 min. Next, 30 μ L of 4 mg/mL nicotinamide adenine dinucleotide phosphate (NADPH) and 5 μ L of 0.46% tert butyl hydroperoxide were added to the reaction medium. The absorbance at 340 nm was continuously monitored over 4 min at 37 °C. A standard curve was prepared using glutathione peroxidase enzyme (Sigma Chemical Co., St. Louis, MO, USA) (2.08 - 25 U/mg protein). Glutathione Reductase (GR) activity was determined according to the method described by Torres et al. (32), with modifications. Liver homogenates that contained 4.0 μ g/ μ L of protein (20 μ L) were incubated with 180 μ L reaction medium that contained 2 mL 0.1 M phosphate buffer with 1 mM EDTA (pH 7), 1.5 mL 0.005 M EDTA, 1.5 mL milli-Q water, 10 mg glutathione disulfide and 2 mg NADPH. The absorbance at 340 nm was continuously monitored over 5 min at 37 °C. The results of this assay were expressed as percentage of NADPH oxidized. All of the enzymatic assays were performed using a plate reader (Multi-Detection microplate reader; Synergy – BIOTEK, Winooski, VT, USA) that was integrated with Gen 5 software.

Western blotting analysis

The total nuclear and cytoplasmic proteins were extracted from the frozen hepatic tissue samples using the specific commercial Kit NEPER (GE Healthcare, Little Chalfont, Bucks, UK). From the nuclear fraction, the protein expression of three transcription factors that are involved in the lipid metabolism were evaluated: peroxisome proliferator-activated receptor- α (PPAR- α), sterol regulatory element-binding proteins 1 C (SREBP1C) and liver X receptor-alpha (LXR- α). The expression of the antioxidant enzymes (catalase, GPx, GR and SOD) was evaluated from the cytoplasmic fraction. To evaluate the expression of three proteins involved in the cholesterol absorption (ABCG5, ABCG8 and Niemann-Pick C1-like protein 1 - NPC1L1), an extraction solution that contained 50 mM potassium phosphate buffer (pH 7.0), 0.3 M sucrose, 0.5 mM DL-Dithiothreitol (DTT), 1.0 mM EDTA, 0.3 mM Phenylmethanesulfonyl fluoride (PMSF), 10.0 mM NaF, phosphatase and protease

inhibitors (1:100) was applied in the small intestine samples. Next, proteins present in the supernatant of the both liver and intestine homogenates were separated on a SDS-PAGE gel and transferred to Amersham™ Hybond™-ECL™ nitrocellulose membranes (GE Healthcare UK Ltd.-Amersham Place, Little Chalfont, Buckinghamshire, UK) using a humid chamber with a buffer that contained 25 mM Tris base, 192.0 mM glycine, 0.02% um dodecyl sulfate (SDS), and 10% methanol (GE Healthcare, Little Chalfont, Bucks, UK). The membranes were then blocked with 7% skim milk powder in Tris-buffered saline and Tris buffered saline with Tween® 20 (TBST) for 2 h to prevent nonspecific binding. The membranes were incubated with the primary antibodies followed the incubation for 1 h at 4°C with a secondary antibody conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Following three washes in TBST, the immunoreactive bands were visualised using the ECL advanced detection system (Amersham Biosciences, Pittsburgh, PA, USA) using a digital detection system (IMAGE QUANT™ 400 version 1.0.0, Amersham Biosciences, Pittsburgh, PA, USA). The densitometry was quantified using the Discovery Series™ Quantity One® Analysis Software, version 4.6.3 (Bio-Rad Laboratories Inc., Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized as a positive control for all of the protein expression analyses.

Statistical Analysis

The effect of the oils and phytosterol supplementation for the six groups after 4 months was evaluated using a one-way ANOVA followed by a post-hoc Tukey test. A Hartley test was initially applied to determine the homogeneity of the variances. A probability value of 0.05 was adopted to reject the null hypothesis. All of the calculations and graphs were performed using software Statistica v.9 (Statsoft Inc., Tulsa, USA).

RESULTS

The effect of n-3 FA and phytosterols on fatty streak development in aorta artery

A high-fat diet was applied to induce inflammation, hyperlipidemic profile, oxidative stress, and consequently fatty streaks development. All of the groups that were fed a high-fat diet exhibited similar body weight (**Supplemental Table 5**). Based on the association between nutrient excess, dyslipidemia and sub-clinical inflammation, it was expected that all of the high-fat diet fed LDLr knockout mice would exhibit fatty streaks and that both phytosterols and n-3 FA would be able to reduce the lesion area. The effect of the treatments on high-fat diet-induced fatty streaks is shown in **Fig. 1**. In our study, none of the treatments reduced the formation of fatty streaks compared with CON group (**Fig. 1A**). In fact, a significant increase ($p < 0.05$) in fatty streak lesion area was observed in mice whose diets were supplemented with isolated phytosterols (**Fig. 1B**). It was also observed that this increase was reversed when n-3 FAs from either source (*Echium* or Algae oils) were added to the supplementation (**Fig. 1D and Fig. 1F**). No difference to CON was observed through supplementation with *Echium* and Algae oils (**Fig. 1C and Fig. 1E**). This result raises the question of which mechanisms would be involved in the phytosterol-induced enhancement of fatty streaks and the reversal of this effect by supplementation with n-3 FA. Two aspects of fatty streaks genesis were here investigated: lipid metabolism and oxidative stress.

The effect of n-3 FA and phytosterols on lipid profile

Figure 2 presents the effect of the treatments on plasma total cholesterol (**Fig. 2A**) and LDL-c (**Fig. 2B**). Differences in HDL-c concentration were only observed between the ALG and ALG+PHY groups (**Fig. 2C**). The expected hypocholesterolemic effect of phytosterols was not observed in the present study, since no differences in total cholesterol or LDL-c concentrations were observed for any of the groups. One of the mechanisms involved in the hypocholesterolemic action of phytosterols is the reduction in cholesterol uptake by NPC1L1 proteins (33) and the secretion of cholesterol back into the intestinal lumen via ABC transporters, such as ABCG5/ABCG8 heterodimers, which are located on the brush border of enterocytes (7). We observed no significant differences between the groups with respect to the protein expression of these three

proteins, ABCG5 (**Fig. 3A**), ABCG8 (**Fig. 3B**) and NPC1L1 (**Fig. 3C**). Similar results were previously reported by Calpe-Berdiel et al. (34) also using LDLr-deficient mice as animal model.

All groups supplemented with n-3 FA (ALG+PHY, ECH and ECH+PHY) ($p<0.05$), except group ALG ($p=0.27$), exhibited lower VLDL and TG values (**Fig. 4A and 4B**) than the group that received phytosterols (PHY) alone. Thus, based on the lipoprotein profiles, it can be suggested that n-3 FA contributed to a reduction in the isolated phytosterol-induced increase in fatty streak by reducing VLDL and TG concentration.

Table 1 presents the fatty acids found in the animal liver after 4 months of supplementation. The n-6 FA/n-3 FA ratio was lower in all of the groups that received algae and *Echium* oil, indicating that this difference was due to DHA and DPA, respectively. Our result demonstrated that after 12h of fasting, SDA from *Echium* oil was likely converted to DPA in the liver. Although the biological activity of ALA and SDA is most likely related to its conversion to EPA (14), we did not observe EPA in the liver in any of the groups. Instead of EPA, the ECH and ECH+PHY groups exhibited higher values of DHA when compared with the CON and PHY groups. Given that the gavage was conducted during the daylight hours, low content of linoleic acid (LNA) was present to compete by $\Delta 6$ -desaturases which convert ALA to SDA and tetracosapentaenoic acid (TPA) to tetracosahexaenoic acid (THA). Thus, we suggest that in animals supplemented with *Echium* oil, the “mammalian Sprecher pathway” had been favored, increasing the production of DHA from THA.

Mechanisms by which n-3 FA and phytosterols alters the plasma lipoprotein profile

To investigate the mechanism by which n-3 FA exerted its hypotriglyceridemic effect, the action of these oils was evaluated with respect to the expression of some nuclear receptors and transcription factors that are involved in the regulation of hepatic cholesterol and lipid homeostasis: PPAR- α , LXR- α and SREBP1c (**Fig. 5A - C**). PPAR- α regulates the expression of genes for enzymes associated with fatty acid oxidation and lipoprotein metabolism (14). The mice that received algae oil (ALG) or *Echium* oil + phytosterols (ECH+PHY) exhibited higher expression levels of PPAR- α (**Fig. 5A**) than all of the other groups. LXR- α modulates the expression of genes associated with fatty acids synthesis and cholesterol metabolism (35). Isolated supplementation with

phytosterols (PHY) promoted an increase in liver LXR- α expression (**Fig. 5B**). It has been observed that SREBP-1c, which up-regulates fatty acid synthase (FAS) expression and promotes fatty acids synthesis (36-37), is a target of LXRs (38). However, the effect of LXR- α on SREBP1c expression was not observed in our study (**Fig. 5C**). Other studies have also reported that LXR- α can influence fatty acids synthesis by directly increasing FAS and acetyl-CoA carboxylase- α (ACCA) expression, independent of SREBP1c (39-40).

The effect of n-3 FA and phytosterols on oxidative stress

Another factor involved in fatty streak genesis is the oxidative stress. Dietary fatty acid composition can alter the fatty acid profile of different pools (41). If these changes increase the proportion of polyunsaturated fatty acids (PUFA), tissue susceptibility to oxidation can be enhanced, contributing to oxidative stress. We evaluated fatty acid proportions in the aorta of the animals to address this possibility (**Table 2**). No differences were observed among the groups that could explain the differences in artery susceptibility to oxidation.

However, when MDA concentration by PUFA percentage in liver of animals was evaluated, we observed a very strong trend ($p=0.048$) for the groups that received *Echium* oil (ECH and ECH+PHY) to exhibit lower degree of fatty acid oxidation than the other groups (**Fig. 6A and 6B**). This effect may be due to action of naturally occurring antioxidants in the oil or due to endogenous mechanisms, such as the capacity of the supplemented bioactive compounds to modulate antioxidant enzyme activity and expression. Thus, the activities and protein expression of the four primary antioxidant enzymes were evaluated: SOD, Catalase, GPx and GR (**Fig. 7 A-D, and Supplemental Table 6**, respectively). ECH group exhibited increased catalase (**Fig. 7A**) only when compared with CON group, while ECH+PHY group showed increased GPx (**Fig. 7B**) activity when compared with all of other groups. No differences were observed in the expression levels of these two antioxidant enzymes (**Supplemental Table 6**). In addition, no differences were observed among the groups with respect to GR activity or expression (**Fig. 7C**). Catalase, GPx and GR act following the dismutation of $O_2^{\cdot-}$ to H_2O_2 , a reaction that is catalyzed by SOD. The PHY and ALG+PHY groups exhibited higher SOD activity than the ALG group (**Fig. 7D**). No differences were observed in

SOD expression between the PHY group and the ALG+PHY or ECH+PHY groups (Supplemental Table 6).

DISCUSSION

In the present study, weaning LDLr knockout mice received supplements containing two bioactive compounds (phytosterols and/or n-3 FA). The effect of this dietary intervention on fatty streak occurrence was evaluated in adulthood following the induction of dyslipidemia and oxidative stress by a high-fat diet. Instead of a reduction, isolated phytosterol supplementation increased fatty streaks lesion area; however this effect was completely reversed by co-supplementation with n-3 FA, here represented by DHA from algae oil and SDA and ALA from *Echium* oil. It is unclear why phytosterols did not reduce plasma cholesterol and increased fatty streaks in the aorta in the context of a high-fat diet. Our hypothesis is that the high dosage of phytosterols offered during the first step of the intervention, associated to the gavage, can have contributed to the higher absorption and oxidation of phytosterols, promoting the fatty streak instead of reducing LDL. According to Clifton et al. (42), high doses of phytosterols can increase their concentration in plasma without promote additional LDL reduction. Davidson et al. (43) observed an increase of campesterol absorption in individuals when the phytosterols dosage was changed from 3.0 to 9.0 g/d. It is known that the absorption of phytosterols is increased in patients with sitosterolemia. In these cases, once absorbed, phytosterols deposit in tissues and can promote atherosclerosis (44). In our study, the chronic supplementation of phytosterols associated with high dose administered in the first two months may have contributed to the greater absorption of phytosterols and their accumulation in tissues. Moreover, the supplementation was done by gavage. According Kim et al (45), this form of supplementation may contribute to increase toxic potential of the phytosterols. Thus, our data suggest that when phytosterols are not add to the diet, their physiological effects may be different, probably due to their higher absorption role. Even though taking all care, the gavage process can promote a local inflammation on animals, favoring the oxidative stress on digestive system. Liang et al. (46) observed that phytosterols oxides are better absorbed and lose their ability to reduce cholesterol concentration, contributing to the fatty streaks formation.

None of the biomarkers that were evaluated in our study differed between the PHY and CON groups, except for the increase in hepatic LXR- α expression. LXR- α is a

member of the nuclear receptor family and is activated by oxysterols that are responsive to fatty acids (47). The LXR- α and β form obligate heterodimers with retinoid X receptor and regulate genes involved in cholesterol absorption, conversion of cholesterol to bile acids, reverse cholesterol transport, lipogenesis and inflammatory response. In the present study, phytosterols acted as hepatic LXR- α agonists. Plat; Nichols and Mensink (48) showed that plant sterols and stanols from the 4-desmethylsterol family, particularly β -sitosterol and sitostanol, are activators of LXR- α . This evidence suggest that phytosterols would be able to modulate hepatic proteins, such as LXR- α , even under a low rate of absorption. According to Repa et al. (49), the activation of the LXR- α signaling pathway results in the up-regulation of ABCA1 in peripheral cells, including macrophages. This effects promotes the efflux of cholesterol from macrophages to HDL-c, which can deliver cholesterol esters to the liver by interacting with the SR-B1 receptor, via a mechanism that is referred as “reverse transport”. On basis of this mechanism, LXR- α agonists have appear to be a target for novel atherosclerosis-reducing drugs. However, rather than reverse transport, HDL-c can transfer cholesterol esters to Apo B-containing lipoproteins in a process that is mediated by cholesterol ester transfer protein (CETP) (50). Moreover, by the direct activation of genes involved in lipogenesis, LXR- α also increases fatty acids synthesis, leading to the formation of larger VLDL (47). This condition, which is characterized by a cholesterol ester-enriched HDL-c and hypertriglycemia, would promote an increase in CETP activity (51-52). However, mice do not have plasma CETP activity (53). Thus, without CETP alternative, the regeneration of lipid-poor HDL-c (pre β - HDL) via lipid exchange lipolysis is reduced, potentially resulting less cholesterol efflux via ABCA1(54), contributing to the formation of fatty streaks.

The mechanisms by which n-3 FA reversed the phytosterol-induced increase in fatty streaks involved both lipid metabolism and oxidative stress. The primary metabolic pathway that was activated by both sources of n-3 FA used in the present study (SDA from *Echium* oil and DHA from algae oil) reversed the increase in phytosterol-induced LXR- α expression and increased fatty acid oxidation via PPAR- α (**Fig. 5**), leading to a reduction in TG and VLDL concentrations (**Fig. 4**). This mechanism has been reported by other researchers to explain the hypotriglycemic effect of n-3 FA (14). Our hypothesis is that the reduction in TG and, consequently, in VLDL assembly, led to the formation of less atherogenic LDL-c (55), contributing to the reduction in fatty streaks.

Another factor involved in fatty streak genesis is the oxidative stress. We observed a trend of reduction in MDA formation between groups that received *Echium* oil either alone or in combination with phytosterols. Moreover, ECH+PHYgroup exhibited an increase of GPx activity when compared with all of other groups. Thus, this change can explain the observed trend of reduction in MDA formation in the liver of animals supplemented with *Echium* oil plus phytosterol.

In conclusion, the co-supplementation of LDLr knockout mice in the childhood with phytosterols and n-3 FA did not reduce the CDV risk in adulthood. This result could be attributed to the high dosage of phytosterols associated to their oxidation during the digestive process. However, the increase of fatty streak induced by isolated phytosterol was fully reserved by the presence of n-3 FA from both algae and *Echium* oils, via modulation of transcriptors factors involved in lipid metabolism. Thus, more studies are necessary before that functional foods containing phytosterols and n-3 FA are developed for children consumption.

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P. B. B and I. A. C designed the research; P. B. B., J. P. G, K. R. M, M.S.A conducted the research; P. B. B, A. P. L and I. A. C. analyzed the data; P. B. B and I. A. C wrote the paper; and I. A. C had primary responsibility for the final content. All authors read and approved the final manuscript. We thank Marcia K. Koike from the Department of Medicine Division of Emergency Medicine, Faculty of Medical Sciences of the University of Sao Paulo, LIM 51, SP, Brazil for her excellent technical contribution in the assessment of atherosclerosis in the aortic root.

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Figure legends

Figure 1 Lesion area (μm^2) determined in the aorta isolated from LDLr Knockout mice fed a high fat diet for 8 weeks and supplemented for 16 weeks with soy oil (CON) (A), phytosterol (PHY) (B), Algae oil (ALG) (C), algae oil plus phytosterol (ALG+PHY) (D), *Echium* oil (ECH) (E) and *Echium* oil plus phytosterol (ECH+PHY) (F). Serial sections were analysed for lipid content using Oil Red-O Stain. All data shown are means $\pm$ SE, *P<0.05.

Figure 2 Total cholesterol (A), LDL-c (B) and HDL-c (C) concentration after supplementation with soy oil (CON), phytosterol (PHY), algae oil (ALG), algae oil plus phytosterol (ALG + PHY), *Echium* oil (ECH), *Echium* oil plus phytosterol (ECH + PHY). All data shown are means $\pm$ SE, *P<0.05.

Figure 3 ABCG5 (A), ABCG8 (B), NPC1L1 (C) expression after supplementation with soy oil (CON), phytosterol (PHY), algae oil (ALG), algae oil plus phytosterol (ALG + PHY), *Echium* oil (ECH), *Echium* oil plus phytosterol (ECH + PHY). All data shown are means $\pm$ SE.

Figure 4 VLDL(A) and TG (B) concentration after supplementation with soy oil (CON), phytosterol (PHY), algae oil (ALG), algae oil plus phytosterol (ALG + PHY), *Echium* oil (ECH), *Echium* oil plus phytosterol (ECH + PHY). All data shown are means $\pm$ SE, *P<0.05.

Figure 5 PPAR- α (A), LXR- α (B), SREBP1C (C) expression after supplementation with soy oil (CON), phytosterol (PHY), algae oil (ALG), algae oil plus phytosterol (ALG + PHY), *Echium* oil (ECH), *Echium* oil plus phytosterol (ECH + PHY). All data shown are means $\pm$ SE. *P<0.05, **P<0.01.

Figure 6 MDA concentration in liver after supplementation with soy oil (CON), phytosterol (PHY), algae oil (ALG), algae oil plus phytosterol (ALG + PHY), *Echium* oil (ECH), *Echium* oil plus phytosterol (ECH + PHY). The results were expressed in nmol/mg protein (A) and nmol/ mg protein/ %PUFA (B). All data shown are means $\pm$ SE, *P<0.05.

Figure 7 Catalase (A), glutathione peroxidase (B), glutathione reductase (C) and superoxide dismutase (D) activity after supplementation with soy oil (CON), phytosterol (PHY), algae oil (ALG), algae oil plus phytosterol (ALG + PHY), *Echium* oil (ECH), *Echium* oil plus phytosterol (ECH + PHY). All data shown are means $\pm$ SE, *P<0.05; **P<0.01.

Table 1 Major fatty acids content in liver of the mice supplemented for 4 months with n-3 FA and phytosterols, submitted to a high fat diet for the last 2 months.

Fatty acids (g/100g)	CON	PHY	ALG	ALG + PHY	ECH	ECH+ PHY	P ¹
C14:0 – miristic	0.34 ± 0.04 ^{ab}	0.32 ± 0.04 ^{abc}	0.33 ± 0.03 ^{ab}	0.31 ± 0.03 ^{ac}	0.37 ± 0.03 ^b	0.28 ± 0.01 ^c	<0.01
C16:0 – palmitic	23.68 ± 0.95	24.27 ± 0.71	24.40 ± 0.61	24.25 ± 0.53	23.65 ± 1.17	24.17 ± 0.56	0.257
C16:1 n7 – palmitoleic	1.76 ± 0.60	1.66 ± 0.36	1.57 ± 0.21	1.80 ± 0.34	1.71 ± 0.32	1.20 ± 0.31	0.078
C18:0 – stearic	7.38 ± 1.72 ^a	9.17 ± 1.29 ^a	9.01 ± 1.22 ^a	8.54 ± 1.18 ^a	8.07 ± 1.62 ^a	11.50 ± 1.67 ^b	<0.01
C18:1n-9 – oleic	33.44 ± 3.98 ^a	29.74 ± 3.29 ^{ab}	29.30 ± 3.14 ^a	30.40 ± 2.72 ^{ab}	29.81 ± 3.85 ^{ab}	24.70 ± 2.17 ^b	0.002
C18:2 n-6 – linoleic (LNA)	16.38 ± 0.80 ^b	15.94 ± 0.77 ^a	16.38 ± 0.93 ^{ab}	16.75 ± 0.95 ^{ab}	17.73 ± 1.58 ^b	16.11 ± 0.63 ^a	0.012
C18:3 n-6 – γ linolenic (GLA)	0.36 ± 0.05 ^a	0.33 ± 0.01 ^a	0.37 ± 0.06 ^a	0.39 ± 0.02 ^a	0.51 ± 0.09 ^b	-	<0.01
C18:3 n-3 – α linolenic (ALA)	-	-	-	-	0.64 ± 0.16	-	-
C20:1 n-9 – eicosenoic	0.56 ± 0.12	0.52 ± 0.09	0.53 ± 0.09	0.47 ± 0.05	0.52 ± 0.06	-	
C20:4 n-6 – arachidonic (ARA)	8.18 ± 1.93 ^a	10.55 ± 1.57 ^{bc}	9.24 ± 1.24 ^{ab}	8.84 ± 1.50 ^{ab}	7.95 ± 1.83 ^a	11.78 ± 1.22 ^c	<0.01
C21:0 – Heneicosanoic	0.82 ± 0.15	0.92 ± 0.07	0.81 ± 0.15	0.73 ± 0.09	0.74 ± 0.15	1.00 ± 0.16	
C22:5 n3 – docosapentaenoic (DPA)	0.38 ± 0.00 ^a	0.32 ± 0.00 ^a	0.35 ± 0.05 ^a	0.29 ± 0.00 ^a	0.64 ± 0.07 ^b	0.62 ± 0.03 ^b	<0.01
C24:6 n3 – docosaheptaenoic (DHA)	3.98 ± 1.23 ^a	4.21 ± 0.59 ^a	5.79 ± 0.77 ^{bc}	5.38 ± 1.15 ^{ab}	5.55 ± 0.97 ^{bc}	6.93 ± 0.57 ^c	<0.01
NI ²	3.28 ± 0.69	2.69 ± 0.49	2.57 ± 0.44	2.41 ± 0.32	2.48 ± 0.47	2.02 ± 0.39	-
Saturated	32.10 ± 1.86 ^a	34.70 ± 1.56 ^{bc}	34.60 ± 1.69 ^{abc}	33.80 ± 1.44 ^{ab}	32.70 ± 2.26 ^{ab}	36.90 ± 1.63 ^c	<0.01
Monounsaturated	35.60 ± 4.53 ^a	31.70 ± 3.77 ^{ab}	31.10 ± 3.56 ^{ab}	32.50 ± 3.12 ^a	31.70 ± 4.33 ^{ab}	25.90 ± 2.43 ^b	<0.01
Polyunsaturated	28.50 ± 2.80 ^a	30.90 ± 2.84 ^a	31.60 ± 2.61 ^{ab}	31.10 ± 2.38 ^{ab}	32.50 ± 3.23 ^{ab}	35.10 ± 1.73 ^b	<0.01
n-3 FA	3.76 ± 0.90 ^b	4.40 ± 0.74 ^{bc}	5.86 ± 0.74 ^a	5.67 ± 1.02 ^{ac}	6.59 ± 0.89 ^{ad}	7.23 ± 0.77 ^d	<0.01
n-6 FA	24.80 ± 1.95	26.50 ± 2.12	25.70 ± 1.89	25.60 ± 1.60	25.90 ± 2.43	27.90 ± 1.11	0.119
n-6/n-3 FA ratio	6.80 ± 1.03 ^a	6.12 ± 0.57 ^a	4.42 ± 0.29 ^b	4.61 ± 0.57 ^b	3.96 ± 0.28 ^b	3.88 ± 0.31 ^b	<0.01

¹Probability value obtained by one-way ANOVA or Kruskal-Wallis ANOVA by Ranks. Values followed by the same upperscript

letter in the same line are not significantly different ($p < 0.05$).

² NI, not identified

Table 2 Major fatty acids content in aorta artery of the mice supplemented for 4 months with n-3 FA and phytosterols, submitted to a high fat diet for the last 2 months.

Fatty acids (g/100g)	CON	PHY	ALG	ALG+PHY	ECH	ECH+PHY	P ¹
C12:0 – lauric	1.59 ± 2.22	1.45 ± 1.72	1.92 ± 1.36	0.18 ± 0.00	1.13 ± 1.42	1.53 ± 1.19	0.912
C14:0 – miristic	1.65 ± 1.09	1.54 ± 0.52	1.25 ± 0.65	1.09 ± 0.50	1.17 ± 0.46	1.40 ± 0.35	0.557
C16:0 – palmitic	23.70 ± 10.10	33.50 ± 6.30	22.60 ± 9.20	26.3 ± 12.00	19.80 ± 8.80	26.90 ± 12.9	0.110
C16:1 n7 – palmitoleic	2.06 ± 1.12	1.44 ± 0.17	1.65 ± 0.85	1.11 ± 0.45	1.26 ± 0.62	1.34 ± 0.65	0.514
C18:0 – stearic	17.00 ± 8.68	20.5 ± 2.22	14.40 ± 5.83	16.9 ± 7.77	14.30 ± 4.52	17.80 ± 9.24	0.428
C18:1n-9 – oleic	29.30 ± 11.00 ^{ab}	19.10 ± 4.40 ^a	27.30 ± 7.60 ^{ab}	22.20 ± 8.00 ^{ab}	37.50 ± 20.60 ^b	28.50 ± 10.60 ^{ab}	0.048
C18:2 n-6 – linoleic (LNA)	15.50 ± 12.10	14.00 ± 2.60	17.90 ± 10.30	18.30 ± 8.30	14.80 ± 10.20	16.80 ± 10.9	0.922
C18:3 n-3 – α-linolenic (ALA)	2.06 ± 1.69	0.89 ± 0.28	1.48 ± 0.79	1.61 ± 0.56	1.51 ± 1.42	1.06 ± 0.39	0.809
C20:4 n-6 – arachidonic (ARA)	2.31 ± 1.74	2.85 ± 1.06	1.57 ± 0.41	1.77 ± 1.21	1.70 ± 1.17	1.79 ± 1.31	0.365
Saturated	42.56 ± 17.97	55.70 ± 11.80	38.98 ± 15.22	44.11 ± 19.68	35.76 ± 12.85	47.38 ± 22.07	0.202
Monounsaturated	30.51 ± 12.22	20.22 ± 4.54	28.41 ± 8.26	23.02 ± 8.10	38.38 ± 20.36	24.45 ± 15.72	0.194
Polyunsaturated	18.35 ± 13.23	16.71 ± 2.15	19.92 ± 10.55	20.42 ± 8.38	16.39 ± 11.23	15.72 ± 11.92	0.805
n- 3 FA	0.88 ± 1.47	0.22 ± 0.43	0.96 ± 1.04	0.81 ± 0.94	0.61 ± 1.13	0.50 ± 0.70	0.739
n- 6 FA	17.46 ± 12.06	16.48 ± 2.17	17.36 ± 9.25	19.62 ± 7.74	15.79 ± 10.24	11.26 ± 7.41	0.642
n- 6/n-3 FA ratio	12.45 ± 7.16	17.67 ± 1.74	11.42 ± 3.47	13.76 ± 2.09	16.54 ± 5.82	10.61 ± 3.88	0.436

¹Probability value obtained by one-way ANOVA or Kruskal-Wallis ANOVA by Ranks. Values followed by the same upperscript

letter in the same line are not significantly different ($p < 0.05$).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Online Supporting Material

Supplemental Table 1 Major fatty acids profile of the edible oils applied in this study.

Fatty acids (g/100g)	SOY OIL	ALGAE OIL	ECHIUM OIL
C10:0 – capric		1.12	-
C12:0 – lauric	0.04	4.92	-
C14:0 – miristic	0.11	10.19	-
C16:0 – palmitic	11.32	6.97	5.15
C18:0 – stearic	4.88	0.92	2.57
C16:1 – palmitoleic	0.11	2.82	-
C18:1 n-9 – oleic	26.75	27.48	12.77
C18:2 n-6 – linoleic (LNA)	48.26	1.17	27.52
C18:3 n-3 – α -linolenic (ALA)	5.10	-	26.75
C18:3 n-6 – γ -linolenic (GLA)	-	-	13.11
C18:4 n-3 – stearidonic (SDA)	-	-	11.13
C20:4 n-6 – arachidonic (ARA)	-	-	-
C20:5 n-3 – eicosapentaenoic (EPA)	-	-	-
C22:5 n-3 – docosapentaenoic (DPA)	-	0.38	-
C22:6 n-3 – docosaexaenoic (DHA)	-	42.89	-
NI ¹	3.43	1.14	1.0
Σ saturated fatty acids	16.35	24.12	7.72
Σ monounsaturated fatty acids	26.86	30.30	12.77
Σ polyunsaturated fatty acids	53.36	44.44	78.51
n-6 FA	48.26	1.17	40.63
n-3 FA	5.10	43.27	37.88
n-6/n-3 FA ratio	10:1	0.03:1	1:1

¹ NI, not identified

Online Supporting Material

Supplemental Table 2 Chemical composition and major fatty acids content of the two diets applied in this study.

Composition	Control (0-60days) ¹	High fat (61-120 days) ²
Moisture (g/100g)	11.49 ± 0.03	7.22 ± 0.05
Ashes (g/100g)	7.98 ± 0.17	2.94 ± 0.10
Protein (g/100g)	25.63 ± 0.72	17.99 ± 0.49
Lipids (g/100g)	4.36 ± 0.39	30.72 ± 0.57
Carbohydrate (g/100g)	50.54 ± 1.12	41.13 ± 0.33
Energy (Kcal/100g)	343.92 ± 1.89	512.96 ± 3.32
Fatty acids (g/100g total FA)		
C10:0 – capric	-	0.81 ± 0.00
C12:0 – lauric	-	1.03 ± 0.17
C14:0 – miristic	0.18 ± 0.03	1.68 ± 0.01
C16:0 – palmitic	19.92 ± 0.10	26.20 ± 0.62
C18:0 – stearic	5.03 ± 0.14	17.30 ± 0.05
C16:1 – palmitoleic	1.10 ± 0.22	0.96 ± 0.07
C18:1 n-9 – oleic	18.98 ± 0.01	32.73 ± 0.12
C18:2 n-6 – linoleic (LNA)	45.05 ± 1.10	11.94 ± 0.97
C18:3 n-3 – α-linolenic (ALA)	4.03 ± 0.15	0.60 ± 0.06
C18:3 n-6 – γ-linolenic (GLA)	2.19 ± 0.35	0.25 ± 0.06
C18:4 n-3 – stearidonic (SDA)	-	-
C20:0 – arachidic	0.29 ± 0.01	0.27 ± 0.01
C20:1 n-9 – eicosaenoic	0.34 ± 0.04	0.59 ± 0.05
C20:2 n-6 – eicosadienoic	0.44 ± 0.05	0.26 ± 0.02
C20:4 n-6 – arachidonic (ARA)	-	-
C20:5 n-3 – eicosapentaenoic (EPA)	-	-
C22:5 n-3 – docosapentaenoic (DPA)	-	-
C22:6 n-3 – docosaexaenoic (DHA)	-	-
NI ³	2.45	5.15

¹ Based on National Research Council recommendation (23)

² Based on Safwat et al. (2009) Diet was composed by 187g casein, 133,5g sucrose, 300g lard, 193,5 g corn starch, 67g cellulose, 53,5 g soybean oil, 46,5g minerals, 2,4g L-cystine, 3,3 g Choline bitartrate, and 13,35 g vitamin mix / kg diet

³ NI, not identified

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Supplemental Table 3 Fatty acids intake for each experimental group, including diet and supplementation by gavage, during the 1st step of the assay.

1st step: Control diet	CON	PHY	ALG	ALG+PHY	ECH	ECH+PHY
Diet intake (g/d)	3.54	3.76	3.71	3.86	3.60	3.56
Lipid intake from diet (mg/d) ¹	154.34	163.94	161.76	168.30	156.96	155.22
Emulsion volume (μl/d)	83.00	76.00	68.00	68.00	102.40	102.40
Oil in the emulsion (%)	1.00	1.00	1.00	1.00	10.00	10.00
Lipid intake from emulsion (mg/d) ²	0.83	0.76	0.68	0.68	10.24	10.24
Total lipid intake (mg/d)	155.17	164.70	162.44	168.98	167.20	165.46
Fatty acids (mg/d)						
C10:0 – capric	-	-	0.01	0.01	-	-
C12:0 – lauric	0.00	0.00	0.03	0.03	-	-
C14:0 – miristic	0.28	0.30	0.36	0.37	0.28	0.28
C16:0 – palmitic	30.84	32.81	32.27	33.57	31.79	31.45
C18:0 – stearic	7.80	8.28	8.14	8.47	8.16	8.07
C16:1 – palmitoleic	1.70	1.81	1.80	1.87	1.73	1.71
C18:1 - oleic (n-9)	29.52	31.26	30.89	32.13	31.10	30.77
C18:2 n-6 – linoleic (LNA)	69.93	74.20	72.88	75.83	73.53	72.74
C18:3 n-3 – α-linolenic (ALA)	6.26	6.64	6.52	6.78	9.06	8.99
C18:3 n-6 – γ- linolenic (GLA)	3.38	3.61	3.54	3.69	4.78	4.74
C18:4 n-3 – stearidonic (SDA)	-	-	-	-	1.14	1.14
C20:4 n-6 – arachidonic (ARA)	-	-	-	-	-	-
C20:5 n-3 – eicosapentaenoic (EPA)	-	-	-	-	-	-
C22:5 n-3 – docosapentaenoic (DPA)	-	-	0.00	0.00	-	-
C22:6 n-3 –docosaexaenoic (DHA)	-	-	0.29	0.29	-	-
Σ saturated fatty acids	38.92	41.39	40.65	42.29	40.23	39.80
Σ monounsaturated fatty acids	31.22	33.07	32.48	33.79	32.83	32.48
Σ polyunsaturated fatty acids	79.58	84.44	82.93	86.29	88.51	84.22
n-6 FA	73.31	77.80	76.41	79.50	78.31	77.48
n-3 FA	6.26	6.64	6.52	6.78	10.20	10.13
n6/n3	11.72	11.72	11.72	11.72	11.83	11.83
EPA from ALA and SDA ³	0.31	0.33	0.33	0.34	0.74	0.73
DHA	-	-	0.29	0.29	-	-
Phytosterols/stanols⁴(mg/d)						
Brassicasterol	-	0.01	-	0.01	-	0.01
Campesterol	0.10	0.18	0.10	0.18	0.10	0.17
Campestanol	-	0.00	-	0.00	-	0.00
Stigmasterol	0.09	0.15	0.10	0.15	0.09	0.14
Cholesterol	-	0.00	-	0.00	-	0.00
Beta-sitosterol	0.27	0.42	0.28	0.42	0.27	0.40
Beta-sitostanol	-	0.00	-	0.00	-	0.00
Others	-	0.01	-	0.01	-	0.01
Total EPA + DHA (mg/d)	0.31	0.33	0.62	0.63	0.74	0.73
Total phytosterols (mg/d)	0.45	0.77	0.47	0.78	0.46	0.74
EPA + DHA - Human equivalence (g/d)	2.17	2.30	4.32	4.41	5.17	5.14
Phytosterol -Human equivalence (g/d)	3.16	5.39	3.32	5.48	3.22	5.21

¹Total lipid content of the Control diet (4.36%)

²This amount was based on 10.0g of body weight at the beginning of the intervention, and it was increased according to the body weight gain of each animal.

³It was adopted the following conversion rates: 5% from ALA to EPA and 25% from SDA to EPA (20-21).

⁴Phytosterol content of the soybean oil used to prepare the diet were estimated based on USDA (Nutrient data for 04044); while phytosterols and phytostanols content of the source used to prepare the emulsions were obtained by GC analysis.

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Supplemental Table 4 Fatty acids intake for each experimental group, including diet and supplementation by gavage, during the 2nd step of the assay.

2 nd step: High Fat diet	CON	PHY	ALG	ALG+PHY	ECH	ECH+PHY
Diet intake (g/d)	2.26	2.3	2.26	2.21	2.24	2.42
Lipid intake from diet (mg/d) ¹	694.27	706.56	694.27	678.91	688.13	743.42
Emulsion volume (μl/d)	83.00	76.00	68.00	68.00	102.40	102.40
Oil in the emulsion (%)	1.00	1.00	1.00	1.00	10.00	10.00
Lipid intake from emulsion (mg/d) ²	0.83	0.76	0.68	0.68	10.64	10.64
Total lipid intake (mg/d)	694.27	706.56	694.27	678.91	688.13	743.42
Fatty acids (mg/d)						
C10:0 – capric	5.62	5.72	5.63	5.51	5.57	6.02
C12:0 – lauric	7.15	7.28	7.18	7.03	7.09	7.66
C14:0 – myristic	11.66	11.87	11.73	11.48	11.56	12.49
C16:0 – palmitic	181.99	185.20	181.95	177.92	180.82	195.30
C18:0 – stearic	120.15	122.27	120.12	117.46	119.31	128.88
C16:1 – palmitoleic	6.67	6.78	6.68	6.54	6.01	7.14
C18:1 – oleic (n-9)	227.46	231.46	227.42	222.39	226.53	244.63
C18:2 n-6 – linoleic (LNA)	83.30	84.78	82.90	81.07	84.98	91.58
C18:3 n-3 – α-linolenic (ALA)	4.21	4.28	4.17	4.07	6.87	7.20
C18:3 n-6 – γ-linolenic (GLA)	1.74	1.77	1.74	1.70	3.06	3.20
C18:4 n-3 – stearidonic (SDA)	-	-	-	-	1.14	1.14
C20:4 n-6 – arachidonic (ARA)	-	-	-	-	-	-
C20:5 n-3 – eicosapentaenoic (EPA)	-	-	-	-	-	-
C22:5 n-3 – docosapentaenoic (DPA)	-	-	0.00	0.00	-	-
C22:6 n-3 – docosahexaenoic (DHA)	-	-	0.29	0.29	-	-
Σ saturated fatty acids	326.58	332.35	326.61	319.39	324.35	350.35
Σ monounsaturated fatty acids	234.12	238.24	234.11	228.93	233.14	251.77
Σ polyunsaturated fatty acids	89.24	90.77	89.10	87.14	96.05	103.12
n-6 FA	85.03	86.50	84.64	82.77	88.04	94.78
n-3 FA	4.21	4.28	4.46	4.37	8.01	8.34
n6/n3	20.32	20.32	18.98	18.95	20.43	20.43
EPA from ALA and SDA ³	0.21	0.21	0.21	0.20	0.63	0.64
DHA	-	0.00	0.29	0.29	0.00	0.00
Phytosterols/stanols⁴ (mg/d)						
Brassicasterol	0.00	0.01	0.00	0.01	0.00	0.01
Campesterol	0.00	0.08	0.00	0.08	0.00	0.08
Campestanol	0.00	0.00	0.00	0.00	0.00	0.00
Stigmasterol	0.00	0.05	0.00	0.05	0.00	0.05
Cholesterol	0.00	0.00	0.00	0.00	0.00	0.00
Beta-sitosterol	0.00	0.13	0.00	0.13	0.00	0.13
Beta-sitostanol	0.00	0.00	0.00	0.00	0.00	0.00
Others	0.00	0.01	0.00	0.01	0.00	0.01
Total EPA + DHA (mg/d)	0.21	0.21	0.50	0.50	0.63	0.64
Total phytosterols (mg/d)	0.00	0.29	0.00	0.29	0.00	0.29
EPA + DHA - Human equivalence (g/d)	1.46	1.48	3.50	3.50	4.39	4.51
Phytosterol -Human equivalence (g/d)	0.00	2.03	0.00	2.03	0.00	2.03

¹ Total lipid content of the High fat diet (30.72%)

² This amount was based on 10.0g of body weight at the beginning of the intervention, and it was increased according to the body weight gain of each animal.

³ It was adopted the following conversion rates: 5% from ALA to EPA and 25% from SDA to EPA (20-21).

⁴ Phytosterol content of the soybean oil used to prepare the diet were estimated based on USDA (Nutrient data for 04044); while phytosterols and phytosterols content of the source used to prepare the emulsions were obtained by GC analysis.

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Supplemental Table 5 Body weight of the animals during the trial.

	CON	PHY	ALG	ALG+PHY	ECH	ECH+PHY	P ¹
Initial Body Weight (g)	11.96 ± 1.21	12.20 ± 1.77	11.79 ± 1.64	13.24 ± 3.16	12.12 ± 3.04	12.00 ± 1.29	0.782
BW after 60 days (g)	23.39 ± 1.33	24.18 ± 1.19	23.86 ± 1.22	24.19 ± 1.41	23.48 ± 0.93	23.38 ± 0.84	0.495
BW after 120 days (g)	26.02 ± 1.11	27.09 ± 1.42	26.42 ± 1.44	27.02 ± 1.89	26.38 ± 1.61	26.52 ± 0.87	0.629

¹Probability value obtained by one-way ANOVA.

Online Supporting Material

Supplemental Table 6 Protein expression of antioxidants enzymes

Protein expression (INT/mm²)	CON	PHY	ALG	ALG+PHY	ECH	ECH+PHY	P¹
SOD	2.76 ± 0.15 ^c	0.97 ± 0.26 ^{ab}	0.94 ± 0.17 ^{ab}	1.28 ± 0.48 ^a	1.47 ± 0.38 ^a	0.60 ± 0.16 ^b	<0.01
GPx	1.01 ± 0.72	1.26 ± 1.08	2.26 ± 1.71	1.21 ± 0.72	1.81 ± 0.77	1.51 ± 1.14	0.223
CAT	0.96 ± 0.37	1.05 ± 0.47	1.21 ± 0.74	0.97 ± 0.45	1.18 ± 0.68	0.81 ± 0.31	0.734
GR	0.92 ± 0.20	0.74 ± 0.20	0.73 ± 0.28	0.71 ± 0.29	0.75 ± 0.26	0.78 ± 0.09	0.711

¹Probability value obtained by one-way ANOVA.

CAPÍTULO 3

Atividades Extras

- 1- Estágio de 2 meses (Outubro/novembro 2012) no Departamento de Ciências dos Alimentos, Laboratório de Química dos Lipídios da Universidade de Bologna na Itália, sob supervisão da Profa. Dr. Maria Tereza Rodriguez-Estrada (Anexo 1)
- 2- Validação da metodologia de quantificação do MDA (Apêndice 1)

Anexo 1 - Estágio de 2 meses (Outubro/novembro 2012) no Departamento de Ciências dos Alimentos, Laboratório de Química dos Lipídios da Universidade de Bologna na Itália, sob supervisão da Profa. Dr. Maria Tereza Rodriguez-Estrada

DIPARTIMENTO DI SCIENZE E TECNOLOGIE AGRO-ALIMENTARI

Bologna, November 26th, 2012.

Subject: Certificate of attendance of Ms. Patrícia Borges Botelho, for research purposes, at the Lipid Chemistry Laboratory of the Department of Agricultural and Food Sciences of the Alma Mater Studiorum-Università di Bologna (Italy)

This letter certifies that Ms. Patrícia Borges Botelho, from the University of São Paulo (Brazil), did a 2-month stage (from October 2012 to November 2012) at the Lipid Chemistry laboratory of the Department of Agricultural and Food Sciences of the Alma Mater Studiorum-Università di Bologna. During this period, she participated in a research project about the oxidative stability of phytosterols in confectionary products under different storage conditions, supervised by Dr. Maria Teresa Rodriguez-Estrada. In particular, she learnt the analytical methodologies to evaluate the sterol oxidation products in phytosterol-enriched and non-enriched chocolates, by using different extraction/purification methodologies (lipid extraction and cold saponification) and chromatographic techniques (solid-phase microextraction (SPE) and gas chromatography-mass spectrometry (GC-MS)). A manuscript with the most relevant results will be written and sent to a high IF international journal.

Sincerely yours,

Dr. Maria Teresa Rodriguez Estrada
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Alma Mater Studiorum-Università di Bologna
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40127 Bologna (Italia)
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01

Quantificação de MDA em plasma e homogenato de tecidos

Referência:
Hong et al (2000). Clinical Biochemistry 33:619-25.

1. Fundamento

A peroxidação lipídica é um processo de degradação de ácidos graxos poliinsaturados mediado por radicais livres, que leva à formação de hidroperóxidos lipídicos. A decomposição destes hidroperóxidos dá origem a uma grande variedade de compostos carbonílicos, como o MDA, um dos principais produtos secundários da oxidação.

Vários compostos como o MDA

Portanto o MDA, da é um marcador da última etapa da oxidação lipídica e deve ser avaliado na presença de marcadores das fases anteriores.

Neste método a {MDA} é medida através da sua reação com o ácido tiobarbitúrico (TBA).

1 mol de MDA reage com 2 mols de TBA em meio ácido formando um aduto (TBA-MDA) que absorve a 532 nm.

2. Reagentes

- BHT	- TBA
- TCA	- Metanol
- Tampão fosfato de potássio 50mM - NaOH	-Acetonitrila
	- Butanol (grau HPLC)

3. Material

- Vortex
- Banho-maria
- Balança semi-analítica
- Banho de ultrassom
- Centrífuga
- Filtro Millex
- Micropipetas de 20 a 200 μ L, de 100 a 1000 μ L e de 0,5 a 5mL

4. Segurança

- Atenção na manipulação do TCA, pois é corrosivo.
- Colocar tubos contaminados com fluidos biológicos e tecidos de molho com Lisoform ou água sanitária ao final do procedimento.

5. Preparo das soluções

NaOH 10N (PM = 40g /mol)

Vol. Final: 50 mL

- Pesar 20g de hidróxido de sódio em um béquer de 50 ml.
- Dissolve-lo em aproximadamente 25 ml de água no béquer, com a ajuda de um bastão de vidro em banho de gelo
- Transferir para um balão volumétrico de 50mL e completar com água.
- Armazenar em frasco plástico em temperatura ambiente.
- Antes de usar uma solução, observar se não há sólido branco em suspensão, que indica a carbonatação do hidróxido, que pode ocorrer com o tempo

TCA 7,2%

Vol. Final: 500 mL

- Pesar cerca de 36 g de TCA e transferir para um béquer de 250mL.
- Completar para 500 mL com água milli-Q.
- A reação é exotérmica.
- Armazenar em frasco de vidro na geladeira.

TBA 0,6%**Vol. Final: 25 mL**

- Pesar 150 mg de TBA em uma barquinha
- Diluir em água milli-Q com auxílio de uma pipeta de pasteur
- Colocar no ultrassom até sua diluição completa
- Completar o balão para 25 mL com água milli-Q.

TCA 7,2% + KI 1%**Vol. Final: 50 mL**

- Pesar 500 mg de KI em uma barquinha
- Diluir em TCA 7,2% com auxílio de uma pipeta de pasteur
- Completar o balão para 50 mL com TCA 7,2%.

BHT 0,2%**Vol. Final: 100 mL**

- Pesar 0,2g de BHT
- Diluir em etanol e completar para 100 mL com etanol.

6. Curva padrão

- Pese 8,8 mg TEP em um tubo falcon e adicione 10 mL HCL 0,1N.
- Leve ao banho-maria a 95°C por 8 min.
- Transferir para um balão volumétrico e completar para 2 L com água milli-Q- (Solução 20µM MDA)

- Realizar as seguintes diluições:

15µM: 750µL da solução de 20 µM + 250 µL de água milli-Q
10 µM: 500 µL da solução de 20 µM + 500 µL de água milli-Q
7 µM: 350 µL da solução de 20 µM + 650 µL de água milli-Q
5 µM: 250 µL da solução de 20 µM + 750 µL de água milli-Q
3,5 µM: 175 µL da solução de 20 µM + 825 µL de água milli-Q
2,5 µM: 125 µL da solução de 20 µM + 875 µL de água milli-Q
1,5 µM: 75 µL da solução de 20 µM + 925 µL de água milli-Q
0,5 µM: 25 µL da solução de 20 µM + 975 µL de água milli-Q

6. Procedimento

50 μ L amostra* / branco/padrão + 12,5 μ L BHT 0,2% + 6,25 μ L NaOH 10N

* Plasma – sem diluição

Homogenato de fígado de camundongos – diluído 15 vezes (Considerar essa diluição no momento do cálculo).

7. Condições cromatográficas

- Detector de fluorescência: λ 515 nm para excitação
 λ 553 nm para emissão
- RT: 4,9-5,3
- Detector DAD: 532 nm
 - $\left\{ \begin{array}{l} \text{BW: 20} \\ \text{Ref: 360} \\ \text{BW: 20} \end{array} \right.$
- Fase móvel: $\left\{ \begin{array}{l} \text{A: 60\% Tampão Fosfato de Potássio (50 mM; pH 7,4) – Filtrado em} \\ \text{membrana 0.22}\mu\text{m} \\ \text{B: 40\% metanol - Filtrado em membrana 0.45}\mu\text{m} \end{array} \right.$
- Fluxo: 1 ml/min
- Coluna: Phenomenex Reverse-phase C18 (250 mm x 4,6 mm ; 5 μ m)
- Pré-coluna: LC8-DB
- Volume de injeção: 50 μ L
- Post run: 5 min
- Tempo de corrida: 8 min

9. Exemplo de cromatogramas e cálculo

Amostra	Área	Área - B	[]: (77-4,5362)/8,3453	X fator diluição (x15)	[] = 130,2/27607,06
15	95	77	8,68	130,2	0,0047
Branco(B)	18				

[] de ptn da amostra acima 27,61 mg/mL

9. Valores encontrados na literatura

Para Homogenato de fígado:

Food and Chemical Toxicology 2008, 46:2267: 0,005-0,05 $\mu\text{mol/mg}$ de ptn

Experimental Gerontology; 2002; 37:1031: 0,001 $\mu\text{mol/mg}$ de ptn

Food and Chemical Toxicology 2009; 47:496: 0,001-0,0025 $\mu\text{mol/mg}$ de ptn

Journal of ethnopharmacology 2010; 131:478-484: 0,003 $\mu\text{mol/mg}$ de ptn

Food and Chemical Toxicology 2012 in press: 0,0015 $\mu\text{mol/mg}$ de ptn

Plasma humano :

Hong et al. Clinical Biochemistry. 2000; 33:619-25: 1,54 $\mu\text{mol/L}$

Volpe. J. Chromatograph B. 713:433-437: 0,906-1,246 $\mu\text{mol/L}$

Templar. Nephrol Dial Transplant. 1999; 14:946-951:0,11-0,32 $\mu\text{mol/L}$

Cheng. Analytica Chimica Acta 2008;616:230-234: 0,15-2,43 $\mu\text{mol/L}$

8. Descarte do resíduo

- TBA e TCA em outros
- Butanol e fase móvel – não clorados
- Sempre anotar na ficha de descarte a composição e volume do descarte.

9. Redação e Revisões

- Padronizado e validado por Patrícia em junho/2012
- Redigido por Patrícia em junho/2012
- Foram testados os seguintes métodos:

Formação do aduto com TBA

- Hong et al 2000. Clinical Biochemistry. 33:619-25
- Volpe. J Chromatograph B 713: 433-437
- Templar. Nephrol Dial Transplant. 1999; 14:946-951
- Cheng. Analytica Chimica Acta 2008;616:230-234

Formação do aduto com DNPH

- Antunes et al. Brazilian Journal of Pharmaceutical Sciences.2008.
- Pilz et al. J. Chromatography B. 2000;742:315-325

Destes os que apresentaram picos mais definidos foram:

Hong et al 2000 e Antunes et al 2008.

Analisando a porcentagem de recuperação dos dois métodos selecionados, observamos que o método desenvolvido por Hong et al (2000) apresentou maior porcentagem de recuperação ($\approx 94,24\%$) quando comparado ao método que utilizava a derivatização com DNPH ($\approx 81\%$).

10. Validação da metodologia

1 - Linearidade: $r : 0,995$

2- % de recuperação

Amostra	Valor observado(Área)	Valor esperado	% de recuperação
Homogenato de fígado A (50 microlitros)	134,4		
Homogenato de fígado A (25 microlitros) adicionado de TEP 10 μ M (25 microlitros)	152,9	158,25	96,62
Homogenato de fígado B (50 microlitros)	275,2		
Homogenato de fígado B (25 microlitros) adicionado de TEP 10 μ M (25 microlitros)	205,5	228,65	89,88
Homog de fígado C (50 microlitros)	76,9		
Homogenato de fígado C (25 microlitros) adicionado de TEP 10 μ M(25 microlitros)	124,6	129,5	96,22
TEP 10 μ M (50 microlitros)	182,1	91,05	

3- Repetibilidade:

Amostra	1º dia	2º dia	3º dia	4º dia	Média	DP	CV
plasma	1,68	1,71	1,79	1,69	1,72	0,05	3,03
Padrão 10µM	12,92	10,85	12,02	10,83	11,66	1	8,65

4 - Limite de detecção e quantificação

Dia	Coeficiente angular (a)	Coeficiente linear (b)
1º dia	8,35	4,54
2º dia	9,87	2,06
3º dia	11,07	5,45
4º dia	12,62	0,58
5º dia	10,77	0,53
6º dia	11,36	1,46
DP	1,45	2,08
média	10,67	2,44
<i>LD</i>	<i>0,64 µM</i>	
<i>LQ</i>	<i>1,95 µM</i>	

CONCLUSÕES

Na primeira etapa deste estudo, a suplementação com três fontes de N-3 FA foi avaliada usando modelos Knockout LDLr com inflamação induzida pela dieta hiperlipídica. Os animais suplementados com óleo de *Echium* apresentaram os maiores índices de redução de triacilgliceróis e VLDL, além de inibição da esteatose causada pela dieta hiperlipídica. Entretanto, diferente dos óleos marinhos, tais efeitos não envolveram ativação de fatores de transcrição envolvidos no metabolismo lipídico, como PPAR α e LXR α hepáticos.

Tais resultados foram confirmados no segundo experimento, onde o óleo de *Echium* promoveu melhor perfil lipídico e reduziu estresse oxidativo, contribuindo para reverter a formação de estrias gordurosas promovida pelos fitosteróis. Isoladamente não foi capaz de alterar a expressão de PPAR- α e LXR- α . No entanto, quando associado aos fitosteróis, induziu o aumento da expressão de PPAR- α e reduziu o aumento da expressão de LXR- α .

Portanto, a melhor alternativa para suplementação de crianças, objetivando reduzir o risco de desenvolver aterosclerose foi oferecida pelo N-3 FA, especialmente o óleo de *Echium*. Mais estudos são necessários para esclarecer o efeito dos fitosteróis no desenvolvimento das estrias gordurosas, quando associado à dieta rica em gordura.

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n-3 Fatty acids from fish or fish-oil supplements, but not linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review¹⁻³

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Ofício CEUA/FCF/34/2010

CERTIFICADO

A Comissão de Ética no Uso de Animais da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo Certifica que o Projeto **"Efeito do consumo de um alimento infantil contendo ácidos graxos ômega 3 e fitoesteróis sobre o estresse oxidativo e a gênese da aterosclerose"** (Protocolo nº 258), de responsabilidade do(a) pesquisador(a) **Patrícia Borges Botelho** sob a orientação do(a) Profa. Dra. Inar Alves de Castro, está de acordo com as normas do Conselho Nacional de Controle de Experimentação Animal - CONCEA e foi **APROVADO** em reunião de 10 de maio de 2010.

São Paulo, 11 de maio de 2010.

Prof. Dr. Marco Antonio Stephano
Coordenador da Comissão de Ética no Uso de Animais
CEUA/FCF/USP