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RAQUEL DAL SASSO FREITAS

AVALIAÇÃO DOS EFEITOS *IN VIVO* E *IN VITRO* DE ÁCIDOS GRAXOS POLI-INSATURADOS ÔMEGA-3 SOBRE OS EFEITOS DO QUIMIOTERÁPICO, CICLOFOSFAMIDA.

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Dissertação apresentada como requisito para obtenção do grau de Mestre pelo Programa de Pós-Graduação em Medicina e Ciências da Saúde, Área de Concentração em Farmacologia Bioquímica e Molecular, da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientador: Profa. Dra. Maria Martha Campos

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Dedico este trabalho à minha família

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“Science, my boy, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth.”

— Jules Verne, Journey to the Center of the Earth

RESUMO

A suplementação com ômega-3 é bastante utilizada como coadjuvante no tratamento quimioterápico. Os efeitos benéficos das dietas ricas em ômega-3 estão principalmente relacionados com a presença do ácido eicosapentaenoico (EPA) e do ácido docosahexaenoico (DHA). A cistite hemorrágica é um efeito colateral que ocorre em até 40% dos pacientes que utilizam o agente antitumoral, ciclofosfamida. Este trabalho teve por objetivo avaliar os efeitos do ômega-3 na cistite hemorrágica induzida por ciclofosfamida, *in vivo*, em camundongos. Ademais, o presente estudo também investigou a influência da incubação *in vitro* com DHA sobre os efeitos antitumorais da ciclofosfamida em células humanas de câncer de mama. Três tratamentos diferentes foram realizados previamente à aplicação de uma única dose de ciclofosfamida (300 mg/kg i.p.) em camundongos machos *Swiss*: (i) suplementação com óleo de peixe rico em ômega-3 (10% e 20%) por 21 dias, aplicação aguda (ii) i.p. e (iii) i.t de DHA, 1 hora e 15 minutos antes, respectivamente. As dietas consideradas controle foram suplementadas com óleo de milho nas mesmas concentrações. Animais tratados com solução salina, por via i.p. ou i.t., foram utilizados como controles para o tratamento com DHA. A ciclofosfamida causou nocicepção espontânea e alodínia mecânica na pata traseira e no abdômen, sendo esses comportamentos revertidos pela suplementação de óleo de peixe 20 % e pelo DHA, administrado por ambas as vias. A inflamação vesical induzida pela ciclofosfamida, caracterizada por edema e hemorragia marcantes, não foi revertida por nenhum dos tratamentos testados, corroborando com os resultados negativos encontrados em relação à atividade da mieloperoxidade (MPO) em todos os grupos. Em relação à contagem total de leucócitos, a ciclofosfamida aumentou consideravelmente o número de neutrófilos, levando à redução do número de linfócitos. Entretanto, tanto a suplementação com ômega-3, como o tratamento com DHA (i.p.) reverteu parcialmente esse perfil leucocitário, após aplicação da ciclofosfamida. A administração de ciclofosfamida resultou em aumento significativo dos níveis séricos de interleucina-6 (IL-6) e da proteína quimiotática de monócitos-1 (MCP-1), sendo que o tratamento com DHA produziu apenas uma leve redução da produção de IL-6. Em relação à ativação de células da glia na medula espinhal, viu-se que a ciclofosfamida não foi capaz de ativar as células da micróglia; porém, estimulou a ativação de astrócitos. O tratamento com DHA sistêmico mostrou uma tendência em diminuir essa ativação, mas de maneira não significativa. No que diz respeito à expressão do receptor

GPR40/FFAR1 na medula espinhal, a ciclofosfamida foi capaz de diminuir de forma significativa sua expressão, sendo este parâmetro revertido pelo tratamento com DHA i.p. De forma interessante, a incubação com DHA, de forma isolada, causou redução significativa da viabilidade de células humanas de câncer de mama da linhagem MDA-MB231. Além disso, o DHA potencializou o efeito antitumoral da ciclofosfamida, quando testado em esquemas de combinação. Os resultados apresentados demonstram que o ômega-3, tanto na forma de suplementação ou, administrado por via parenteral ou central parece representar um tratamento coadjuvante promissor durante as terapias anti-câncer. Ademais, os dados obtidos indicam que o DHA pode melhorar os efeitos antitumorais de fármacos quimioterápicos, incluindo a ciclofosfamida.

Palavras-chaves: cistite hemorrágica, ciclofosfamida, ômega-3, nocicepção, inflamação, GPR40, DHA

ABSTRACT

This study investigated the effects of the long-term dietary fish oil supplementation or the acute administration of the omega-3 fatty acid docosahexaenoic acid (DHA) in the mouse hemorrhagic cystitis (HC) induced by the anti-cancer drug cyclophosphamide (CYP). HC was induced in mice by a single CYP injection (300 mg/kg i.p.). Animals received four different diets containing 10% and 20% of corn or fish oil, during 21 days. Separated groups received DHA by i.p. (1 µmol/kg) or i.t. (10 µg/site) routes, 1 h or 15 min before CYP. The behavioral tests (spontaneous nociception and mechanical allodynia) were carried out from 1 h to 6 h following CYP injection. Bladder inflammatory changes, blood cell counts, and serum cytokines were evaluated after euthanasia (at 6 h). Immunohistochemistry analysis was performed for assessing spinal astrocyte and microglia activation, or GPR40/FFAR1 expression. *In vitro* studies were conducted to determine the potential effects of DHA on CYP-induced cytotoxicity in human breast cancer cells. Either fish oil supplementation or DHA treatment (i.p. and i.t.) markedly prevented visceral pain, without affecting CYP-evoked bladder inflammatory changes. Moreover, systemic DHA significantly prevented the neutrophilia/lymphopenia caused by CYP, with a partial effect on serum IL-6 levels. DHA also modulated the spinal astrocyte activation and the GPR40/FFAR1 expression. Finally, the pre-incubation of DHA increased the anti-tumor effects of CYP in human breast cancer cells.

The supplementation with omega-3 fatty acids-enriched fish oil or parenteral DHA might be interesting nutritional approaches for cancer patients under chemotherapy schemes with CYP.

Keywords: hemorrhagic cystitis, cyclophosphamide, omega-3, nociception, inflammation, GPR40, DHA

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIAÇÕES

AP-1 – Fator de transcrição ativador de proteína-1

NF-KB – Fator de transcrilção nuclear KB

TNF – Fator de necrose tumoral

IL-1β – Interleucina-1 β

IL-4 - Interleucina-4

IL-13 – Interleucina-13

COX-2 – Ciclooxigenase-2

5-LOX – Lipoxigenase-5

PGE_2 – Prostaglandina E2

LTB4 – Leucotrieno-B4

PUFA – Ácidos Graxos Poli-Insaturados

EPA – Ácido Eicosapentaenoico

DHA – Ácido Docosahexaenoico

SUMÁRIO

INTRODUÇÃO

Cistite hemorrágica

A cistite hemorrágica é um processo inflamatório com etiologia variada, tendo como causa principal, o tratamento quimioterápico e radioterápico em regiões próximas à bexiga. A cistite hemorrágica é caracterizada por hematúria, dor na região púbica, frequência, urgência, incontinência e noctúria. Estes sintomas podem ser observados durante o tratamento com doses baixas ou altas de quimioterápicos e podem persistir por anos após o fim do tratamento. A cistite hemorrágica secundária à quimioterapia é causada pelo grupo das oxazosforinas, mais precisamente, a ciclofosfamida e a ifosfamida (Batista *et al*, 2006; Yoshida *et al*, 2008; Emadi *et al*., 2009; Korkmaz *et al*, 2012; Ribeiro *et al*, 2012). As medidas preventivas para pacientes que apresentam cistite hemorrágica recebendo ciclofosfamida são diversas, como a co-administração de 2-mercaptoetanosulfonato de sódio (Mesna), hiper-hidratação com diurese forçada e, utilização de cateter Foley para diminuir a exposição do urotélio ao agente tóxico. Em casos mais graves, pode-se realizar uma cistectomia (McCarville *et al*, 2000).

A ciclofosfamida foi introduzida em 1958 como um agente antitumoral e, desde então, é um dos fármacos mais utilizados no tratamento de diversos tipos de câncer. Além disso, também é utilizada como imunossupressor, podendo ser administrada por via endovenosa ou oral. É comumente empregada no tratamento de tumores sólidos, leucemias, linfomas, neuroblastomas, retinoblastomas e carcinomas, juntamente com outros agentes quimioterápicos. Também é utilizada no tratamento de doenças autoimunes ou após transplantes. (de Jonge *et al*, 2005; Emadi *et al*., 2009)

A ciclofosfamida é ativada no fígado pelo sistema citocromo P450, sendo transformada em 4-hidroxiciclofosfamida e seu tautômero, a aldofosfamida. Estes são transportados pela corrente sanguínea até as células tumorais, onde a aldofosfamida sofre clivagem e produz o metabólito ativo antitumoral mostarda de fosforamida e o agente tóxico acroleína (de Jonge *et al.*, 2005).

A acroleína é um dos aldeídos α-β-insaturados mais reativos que existem cujo principal mecanismo de toxicidade é sua rápida ligação e depleção a glutationa. É considerada tanto um produto, como um causador de peroxidação lipídica (Kehrer, 2000; Korkmaz *et al*., 2007). O ser humano está exposto à acroleína por diferentes formas: este composto é usado como herbicida, está presente em alimentos, no vapor formado pelo óleo de cozinha superaquecido e, em diferentes formas de fumaça, incluindo a fumaça do cigarro (Kehrer *et al.*, 2000). A cisitite hemorrágica ocorre em função do contato direto da acroleína com o urotélio, causando o aumento da produção de espécies reativas de oxigênio no epitélio da bexiga, induzindo a expressão de alguns fatores de transcrição intracelulares, tais como NF-κB e AP-1 e, assim levando à produção de citocinas pró-inflamatórias, como TNF e IL-1β. Porém, as reais causas da urotoxicidade da acroleína não estão totalmente elucidadas (Batista *et al.,* 2006; Korkmaz *et al.,* 2007). A toxicidade provocada pela acroleína pode ser prevenida com Mesna, que forma um composto que é eliminado pela urina sem danificar o urotélio (Brunton *et al.*, 2010).

O processo inflamatório

A inflamação é um processo iniciado quando há lesão tecidual ou infecção por algum microorganismo, sendo caracterizada por quatro sinais clássicos: tumor, calor, rubor e dor. Estes sinais são decorrentes do aumento do fluxo sanguíneo, do aumento da permeabilidade vascular – permitindo que moléculas maiores migrem da circulação para o sítio inflamatório – e, do deslocamento de leucócitos para o tecido. Didaticamente, a inflamação é dividida em duas fases principais: aguda e crônica, sendo a segunda, consequência da primeira. Durante a fase de instalação da resposta inflamatória, as primeiras células a serem recrutadas são os neutrófilos e os macrófagos, que liberam citocinas pró-inflamatórias (IL-1β e TNF-α), eicosanoides, óxido nítrico, entre outros, atraindo mais células do mesmo tipo para o local. Os neutrófilos também são responsáveis pelo processo de fagocitose no sítio inflamatório, liberando espécies reativas de oxigênio. Normalmente, quando os monócitos atingem o local da inflamação e se diferenciam em macrófagos, os neutrófilos entram em apoptose. Os mediadores inflamatórios produzidos anteriormente podem diminuir a velocidade do processo de

apoptose dos neutrófilos (Serhan, 2014; Serhan, 2010). Os macrófagos ativados expressam citocinas pró-inflamatórias, quimiocinas e enzimas envolvidas na produção de espécies reativas de oxigênio e de óxido nítrico. As citocinas IL-4 e a IL-13 liberadas pelos macrófagos ativam a via da lipoxigenase em outros macrófagos, estimulando a produção de mediadores anti-inflamatórios, como as lipoxinas, protectinas, resolvinas e maresinas (Ariel *et al.*, 2012; Buckley *et al*., 2014).

As prostaglandinas e os leucotrienos são eicosanoides predominantemente pró-inflamatórios derivados do ácido araquidônico pelas vias da ciclooxigenase (COX-2) e da lipoxigenase (5-LOX). A produção de prostaglandinas e leucotrienos, mais especificamente da prostaglandina E2 (PGE_2) e do leucotrieno B4 (LTB4), ocorre no início do processo inflamatório pelos neutrófilos, sendo potentes mediadores da inflamação. A PGE_2 é essencial para o controle do fluxo sanguíneo e da dilatação do endotélio vascular, sendo necessária para a adesão dos leucócitos à parede dos vasos e, para a diapedese (Serhan *et al.*, 2005). Sabe-se que a PGE_2 também exerce funções anti-inflamatórias, como inibição da produção de leucotrienos da série 4 e produção de lipoxinas. Por outro lado, o LTB4 é somente pró-inflamatório, aumentando a permeabilidade vascular, induzindo a liberação de espécies reativas de oxigênio pelos neutrófilos, estimulando a produção de TNF-α, IL-1β e IL-6. (Calder, 2006)

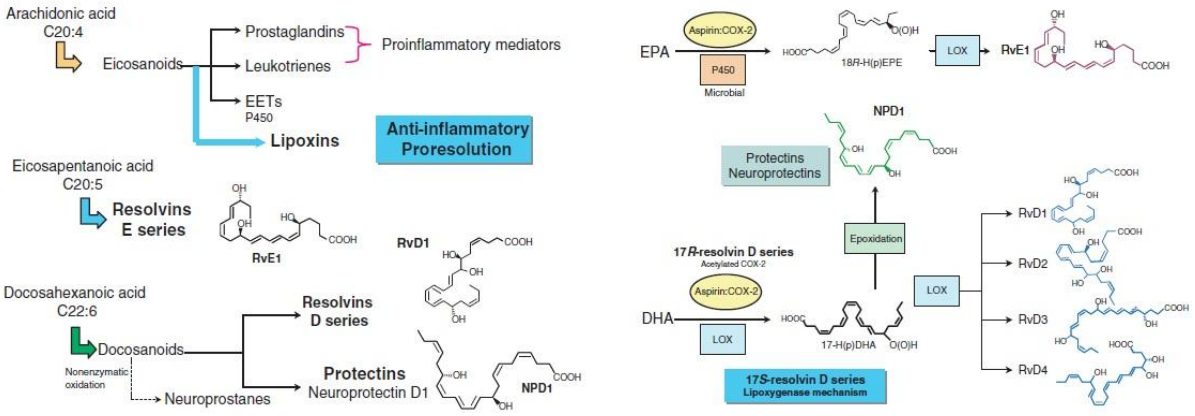

Figura 1. Síntese de mediadores lipídicos pró-inflamatórios e anti-inflamatórios. (retirado de Sehran *et al*., 2005).

As lipoxinas foram os primeiros mediadores lipídicos anti-inflamatórios a serem descobertos, sendo produzidos a partir do ácido araquidônico. Resolvinas e protectinas também são mediadores lipídicos prórresolução e são produzidos a partir dos ácidos graxos poli-insaturados n-3, ácido eicosapentaenoico (EPA) e ácido docosahexaenoico

(DHA), tendo sido descobertos posteriormente. Existem também as maresinas, mediadores anti-inflamatórios derivados do DHA com produção exclusiva nos macrófagos. A principal função deles é a ativação de macrófagos, cujo papel é de fagocitar os neutrófilos em apoptose, levando à resolução do processo inflamatório. Os mesmos macrófagos são responsáveis pela produção de mediadores lipídicos anti-inflamatórios; aparentemente, cada família de mediadores atua em diferentes momentos da resolução da inflamação, embora todos sejam coletivamente responsáveis pelo bloqueio da entrada de neutrófilos no sítio de inflamação (Serhan, 2005; Serhan *et al.*, 2008).

As resolvinas foram descobertas em função da identificação das lipoxinas e de uma série de estudos com n-3, que mostraram resultados positivos relacionados à resolução da inflamação. As resolvinas são produzidas a partir do EPA e do DHA pela via da lipoxigenase, sendo as da série E a partir do EPA e, as da série D a partir do DHA. As protectinas e as neutroprotectinas (estas recebem o prefixo *neuro* em função da localização no organismo) são derivadas apenas do DHA. Quando estes mediadores forma testados experimentalmente, foi observada uma redução de neutrófilos no sítio de inflamação pelo bloqueio da sua migração via endotélio vascular e o controle do deslocamento de leucócitos. Também foi observada inibição da produção de citocinas e quimiocinas pró-inflamatórios e, indução do processo de fagocitose feito pelos macrófagos em neutrófilos apoptóticos (Sehran *et al.*, 2008; Stables *et al.*, 2011).

Um dos principais sinais do processo inflamatório é o edema, que se deve ao aumento da permeabilidade vascular. Quando esse processo ocorre, há liberação dos ácidos graxos n-3, EPA e DHA, aumentando a disponibilidade para a produção de protectinas e resolvinas. Altos níveis de EPA e DHA foram identificados em apenas 1 hora após o início do processo inflamatório, se mantendo elevados por até 48h. Ocorre também, nos próprios neutrófilos, uma mudança das vias metabólicas que provoca o fim da produção de leucotrienos e prostaglanidas e, inicia a produção de lipoxinas a partir do ácido araquidônico, deflagrando a resolução da inflamação. Enquanto os macrófagos fagocitam os neutrófilos apoptóticos, citocinas e quimiocinas produzem mais resolvinas, protectinas e lipoxinas, estabelecendo um mecanismo de *feedback* negativo, a fim de diminuir a permeabilidade vascular e o edema. Em função da importância da presença de ácidos graxos n-3, observou-se que existia uma competição entre o ácido

araquidônico e os ácidos graxos n-3 pelas mesmas enzimas responsáveis pelas reações de dessaturação e alongamento das moléculas, tendo uma maior afinidade com o n-3. Logo, havendo maior quantidade de n-3 no organismo, menor é a produção de mediadores lipídicos pró-inflamatórios derivados do ácido araquidônico, elevando a produção de EPA e DHA e, consequentemente, de mediadores anti-inflamatórios, estimulando a resolução da inflamação (Serhan *et al.*, 2005; Calder, 2006; Serhan, 2010).

Ácidos graxos poli- insaturados e dieta

Os ácidos graxos essenciais poli-instaturados (PUFAs) são uma classe de moléculas que não são sintetizadas pelo organismo, assim precisam ser obtidas através da alimentação. Os dois grupos mais importantes são o n-3 e o n-6. Dos n-3, os mais importantes são o ácido α-linolênico, o EPA e o DHA. Dos n-6, os mais bem caracterizados são o ácido linoleico e o ácido araquidônico. As principais fontes desses ácidos graxos são os peixes de água fria, óleos de linhaça (n-3) e óleos vegetais, como de milho, soja, girassol e canola (n-6) (Weylandt *et al.*, 2012).

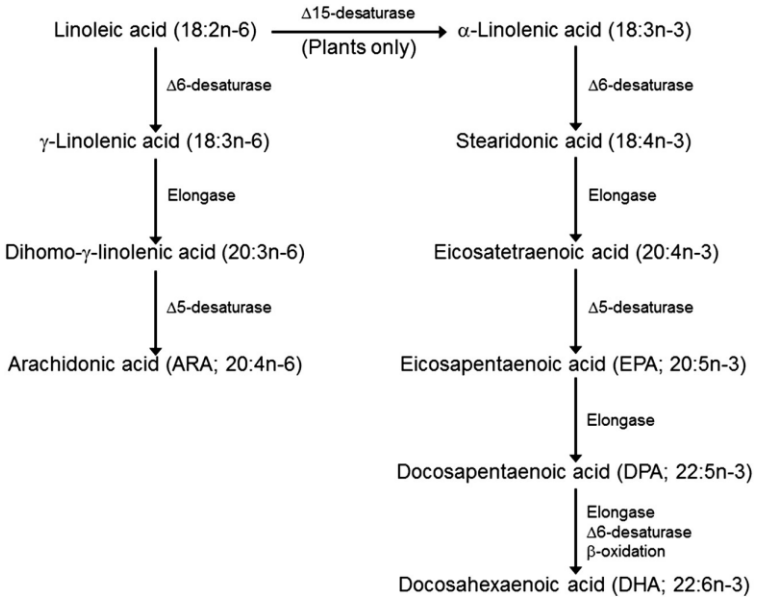

Figura 2. Via de conversão dos ácidos graxos n-6 e n-3 (retirado de Calder, 2014)

O n-3 chamou atenção em estudos feitos com esquimós da Groelândia, em que foi observada uma baixa prevalência de doenças cardiovasculares, asma, artrite reumatoide e outras doenças autoimunes. Foi feito um estudo com amostras dos alimentos consumidos por esquimós da Groelândia, sendo observado que os ácidos graxos predominantes na alimentação eram os poli-insaturados, principalmente os da classe n-3. A inflamação, sendo o denominador comum destas doenças, levou a pensar em um efeito anti-inflamatório do n-3, em função da alimentação desta população ser rica em ácidos graxos (Bang *et al.*, 1980; Weylandt *et al.*, 2012).

Os ácidos graxos n-3 provenientes dos vegetais, como a linhaça, estão na forma de ácido α-linonênico, tendo que ser convertidos em EPA e DHA no organismo. Por outro lado, no caso da suplementação com óleo de peixe, não há necessidade deste processo (Calder, 2014).

Sabe-se que a suplementação alimentar com n-3 está relacionada com a prevenção e melhora de doenças cardiovasculares, alterações inflamatórias crônicas, doenças autoimunes e câncer (Laviano *et al*., 2013; De Caterina *et al*., 2011; Vaughan *et al*., 2013). Existem evidências demonstrando os benefícios da suplementação desses ácidos graxos durante o tratamento oncológico, pois além de potencializarem os efeitos antitumorais das medicações quimioterápicas, auxiliam no aparecimento de algumas reações adversas relacionadas à quimioterapia (Bourgnoux *et al*.,2009; Murphy *et al*., 2012; Laviano *et al*, 2013). Além do mais, demonstrou-se que a suplementação diária de n-3 previne o aparecimento da síndrome da anorexia-caquexia relacionado ao câncer, proporcionando a manutenção do peso corporal, melhorando os parâmetros inflamatórios e protegendo contra o estresse oxidativo (Murphy *et al*., 2011; Finnochiaro *et al*., 2012; Hajjaji *et al*., 2012).

É possível que estas estratégias possam ser úteis como medidas preventivas para pacientes submetidos a esquemas de quimioterapia e/ou radioterapia, evitando ou amenizando os quadros de cistite hemorrágica após ou durante o tratamento com ciclofosfamida.

OBJETIVOS

Objetivos Gerais

Avaliar os efeitos de ácidos graxos poli-insaturados n-3 sobre os efeitos adversos e antitumorais do quimioterápico, ciclofosfamida, através de modelos *in vivo* e *in vitro*.

Objetivos Específicos

- Avaliar o efeito da suplementação com n-3 de óleo de peixe e da administração de DHA sistêmico e espinhal, sobre as alterações inflamatórias vesicais (edema e hemorragia), após a aplicação de ciclofosfamida em camundongos.
- Avaliar o efeito da suplementação com n-3 de óleo de peixe e da administração de DHA sistêmico e espinhal, sobre o comportamento nociceptivo após a aplicação de ciclofosfamida em camundongos.
- Verificar o efeito da suplementação com n-3 de óleo de peixe e da administração de DHA sistêmico, sobre os níveis de citocinas periféricas após a aplicação de ciclofosfamida em camundongos.
- Verificar o efeito da suplementação com n-3 de óleo de peixe e da administração de DHA sistêmico, sobre a migração de neutrófilos na bexiga após a aplicação de ciclofosfamida em camundongos.
- Analisar o efeito da suplementação com n-3 de óleo de peixe e da administração de DHA sistêmico, sobre a contagem total de leucócitos circulantes após a aplicação de ciclofosfamida em camundongos.
- Analisar o efeito da suplementação com n-3 de óleo de peixe e da administração de DHA sistêmico sobre a expressão do mRNA do receptor GPR40/FFAR1 após aplicação de ciclofosfamida em camundongos.
- Verificar o efeito da suplementação com n-3 de óleo de peixe e da administração de DHA sistêmico, sobre a ativação das células da glia (GFAP, Iba-1) na medula espinhal após a aplicação de ciclofosfamida em camundongos.

- Verificar o efeito da suplementação com n-3 de óleo de peixe e da administração de DHA sistêmico, sobre a presença do receptor GPR40/FFAR1 na medula espinhal após a aplicação de ciclofosfamida em camundongos.
- Analisar o efeito do DHA e da ciclofosfamida sobre a linhagem de célula tumoral de mama MDA-MB-231.

CONSIDERAÇÕES FINAIS

O n-3 é utilizado como suplemento para pacientes oncológicos em função da sua capacidade de diminuir o crescimento de tumores e de aumentar a eficácia de tratamentos quimioterápicos. Também é utilizado para prevenir o aparecimento da síndrome de anorexia-caquexia, prejudicando o tratamento do paciente de uma forma global (Silva, 2006; Bougnoux *et al*. 2009).

A ciclofosfamida é amplamente utilizada na prática clínica como quimioterápico e como imunossupressor em terapias pós-transplantes, tendo diversos efeitos colaterais que prejudicam a qualidade de vida do paciente. Um destes efeitos é a cistite hemorrágica, um processo inflamatório crônico, que pode ser prevenido pelo uso do Mesna, mesmo não tendo 100% de efetividade (de Jonge *et al.*, 2006).

Os dados apresentados neste trabalho reveleram outros efeitos exercidos pela suplementação de óleo de peixe rico em n-3 ou pela administração parenteral de DHA, em relação à analgesia em dor visceral associada à CYP. Surpreendentemente, nenhum dos tratamentos propostos neste trabalho foi capaz de alterar a inflamação na bexiga causada pela CYP.

Analizando os possíveis mecanismos dos efeitos analgésicos dos ácidos graxos n-3 no nosso modelo experimental, podemos sugerir que há o envolvimento de modulação periférica e central. Em relação às alterações periféricas, foram observadas modificações nas contagens de neutrófilos e linfócitos circulantes no tratamento com DHA. De forma intrigante, a ração que era, até então, considerada como controle – rica em n-6 – também alterou as taxas de neutrófilos e linfócitos, de maneira semelhante à dieta rica em n-3. Já na modulação central, viu-se a ativação de astrócitos e a regulação da expressão do receptor GPR40/FFAR1 na medula espinhal. Este último resultado, em relação ao receptor GPR40/FFAR1, abriu novas portas para essa investigação, já que Nakamoto *et al.* (2012) demonstrou que esse receptor está diretamente relacionado com analgesia e que ele está expresso de forma constitutiva em diversas porções do sistema nervoso central.

Além de resultados interessantes ao nível de dor visceral, nosso trabalho também gerou evidências demonstrando um efeito benéfico do DHA no tratamento

quimioterápico, aumentando a citoxicidade provocada pela CYP em uma linhagem de células de tumor de mama.

De modo geral, nossos resultados apliam os efeitos benéficos da suplementação de n-3 quando associada ao tratamento antitumoral.

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ANEXOS

ANEXO A - CARTA DE APROVAÇÃO CEUA

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA, INOVAÇÃO E DESENVOLVIMENTO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 012/13 – CEUA

Porto Alegre, 08 de março de 2013.

Senhor Pesquisador:

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 12/00303, **"Efeitos da suplementação com ômega-3 de óleo de peixe no modelo de cistite hemorrágica sub-crônica induzida por ciclofosfamida em camundongos".**

Sua investigação está autorizada a partir da presente data.

Lembramos que é necessário o encaminhamento de relatório final quando finalizar esta investigação.

Atenciosamente,

Profa. Dra. Anamaria Gonçalves Feijó
Coordenadora da CEUA/PUCRS

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ANEXO B - MANUSCRITO DO TRABALHO EXPERIMENTAL

Os resultados do presente trabalho foram submetidos à revista *The Journal of Nutritional Biochemistry*, fator de impacto 4.592 (JCR 2013).

Omega-3 fatty acids are able to modulate the painful symptoms associated to cyclophosphamide-induced-hemorrhagic cystitis in mice

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Abstract

This study investigated the effects of the long-term dietary fish oil supplementation or the acute administration of the omega-3 fatty acid docosahexaenoic acid (DHA) in the mouse hemorrhagic cystitis (HC) induced by the anti-cancer drug cyclophosphamide (CYP). HC was induced in mice by a single CYP injection (300 mg/kg i.p.). Animals received four different diets containing 10% and 20% of corn or fish oil, during 21 days. Separated groups received DHA by i.p. (1 μmol/kg) or i.t. (10 μg/site) routes, 1 h or 15 min before CYP. The behavioral tests (spontaneous nociception and mechanical allodynia) were carried out from 1 h to 6 h following CYP injection. Bladder inflammatory changes, blood cell counts, and serum cytokines were evaluated after euthanasia (at 6 h). Immunohistochemistry analysis was performed for assessing spinal astrocyte and microglia activation, or GPR40/FFAR1 expression. *In vitro* studies were conducted to determine the potential effects of DHA on CYP-induced cytotoxicity in human breast cancer cells. Either fish oil supplementation or DHA treatment (i.p. and i.t.) markedly prevented visceral pain, without affecting CYP-evoked bladder inflammatory changes. Moreover, systemic DHA significantly prevented the neutrophilia/lymphopenia caused by CYP, with a partial effect on serum IL-6 levels. DHA also modulated the spinal astrocyte activation and the GPR40/FFAR1 expression. Finally, the pre-incubation of DHA increased the anti-tumor effects of CYP in human breast cancer cells. The supplementation with omega-3 fatty acids-enriched fish oil or parenteral DHA might be interesting nutritional approaches for cancer patients under chemotherapy schemes with CYP.

Keywords: hemorrhagic cystitis, cyclophosphamide, omega-3, mice, GPR40, DHA

1. Introduction

The beneficial prophylactic effects of fish oil-derived omega-3 fatty acids, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been extensively demonstrated in metabolic disorders and cardiovascular diseases [1, 2, 3]. As a consequence, the dietary supplementation with omega-3 fatty acids for the general adult population has been widely recommended during the last decade [4]. Compelling evidence also indicates that omega-3 fatty acids might be useful for cancer patients, even by enhancing the anti-tumor effects of chemotherapy agents, or by preventing the related side effects [1, 5, 6]. Furthermore, it has been shown that dietary daily intake of supplements enriched with EPA/DHA prevents cancer-induced cachexia and/or sarcopenia, providing body weight maintenance in affected individuals [3]. For instance, Murphy *et al*. [7] demonstrated that supplementation with 2.2-g fish oil per day prevented the losses of body weight and muscle mass in patients with non-small cell lung cancer under chemotherapy. In addition, Finnochiaro *et al*. [8] presented similar results in a multi-center study conducted with the same cancer type, revealing a reduction of inflammatory and oxidative parameters in patients receiving omega-3 fatty acids. Interestingly, it was demonstrated that DHA-rich diet displayed protective effects on the body weight loss in rats treated with the chemotherapy drug doxorubicin [9].

Hemorrhagic cystitis (HC) is an adverse effect of chemotherapy or radiotherapy on the pubic region. The most common symptoms are dysuria, frequency, nocturia, urgency, intense suprapubic pain and gross hematuria [10, 11]. Cyclophosphamide (CYP) is an alkylating agent employed in chemotherapy schemes for a series of different types of cancer, such as non-Hodgkin lymphoma, leukemia, breast cancer, among other solid tumors [12]. Despite the potential anti-tumor effects of CYP, its use is highly associated to the occurrence of HC, due to the generation of the urotoxic

metabolite acrolein, affecting 2 to 40 % of the treated patients. Preventive approaches, including intense bladder irrigation and the co-administration of sodium-2-mercaptoethane sulphonate (Mesna) have been used, although these strategies are not totally effective in clinics, especially after long-term exposure to high doses of CYP [13]. Therefore, it is reasonable to propose that omega-3 rich diets could be useful in preventing CYP-induced HC. In fact, it was previously demonstrated that repeated treatment with EPA (100 to 300 mg/kg) was able to reduce CYP-elicited genotoxicity and oxidative stress in mice, although the inflammatory or painful urological alterations have not been assessed in this study [14]. Notably, high levels of omega-3 fatty acids have been positively correlated with disease remission rates in patients with non-Hodgkin's lymphoma that had been treated with different chemotherapy agents, including CYP [15].

In the light of literature data, the present study investigated to what extent the long-term supplementation with marine omega-3 fatty acids or the acute treatment with DHA, might prevent the collateral effects associated with HC induced by CYP in mice, aiming to assess the mechanisms related to the protective effects of omega-3 in this *in vivo* experimental model. Efforts have also been made to evaluate whether DHA interferes with the *in vitro* anti-tumor effects of CYP in a human breast cancer cell line.

2. Methods

2.1. Animals

Male Swiss mice (25 – 30g; total number of 200 mice) obtained from the Universidade Federal de Pelotas (UFPEL) were used throughout the study. The animals were housed in groups of five per cage and maintained in controlled temperature (22 ± 2°C) and humidity (60–70%), under a 12 h light–dark cycle, with food and water *ad*

libitum. Experiments were conducted in accordance with current guidelines for the care of laboratory animals, ethical guidelines for the investigation of experimental pain in conscious animals and the ARRIVE Guidelines Checklist [16, 17]. All the experimental procedures were approved by the Animal Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul (RS) (Protocol Number: CEUA 12/00303). The experiments were performed between 8:00 and 12:00 AM to minimize the potential circadian variations in the behavioral responses. The number of animals and the intensity of noxious stimuli were the minimum necessary to demonstrate the consistent effects of the treatments.

2.2. Drugs

The following drugs were used: cyclophosphamide (CYP; Genuxal - Baxter Oncology GmbH; Halle/Westfalen, Germany) was purchased at Medilar (Porto Alegre, Brazil), being diluted in distilled water. Docosahexaenoic acid in 1% ethanol (DHA) was purchased from Cayman Chemicals (Michigan, USA) and was diluted in phosphate buffered saline (PBS) until the desired concentration. The final ethanol concentration never exceeded 0.1%.

2.3. Diets and treatments

Four different diets were prepared using Nuvilab® CR-1 chow, with the addition of corn oil (50% of omega-6 fatty acids) or concentrated fish oil (55% of omega-3 fatty acids); each oil was added at two distinct concentrations, 10% and 20%. The detailed composition of diets is provided in the Supplementary Table 1. Dietary scheme started 1 month after birth, lasting 21 days, being HC induced at the 22^{th} day [18]. In a separate series of experiments, the animals received DHA at different schedules of

administration. DHA was acutely dosed, 1 h or 15 min before the induction of HC, at 1 µmol/kg (intraperitoneal; i.p.) or 10 µg/site (intrathecal; i.t.), respectively [19, 20]. DHA-treated groups received only regular chow during all the experimental periods.

2.4. Induction of cystitis and nociception assessment

HC was induced by a single i.p. administration of CYP (300 mg/kg). Immediately after the i.p. injection of CYP, mice were housed in individual plastic cages, without sawdust bedding, and the spontaneous nociception behavior was measured for 2 min, every 30 min, over a total period of 4 h. The behavioral alterations were scored according to the following scale: 0 = normal; 1 = piloerection; 2 = strong piloerection; 3 = labored breathing; 4 = licking of the abdomen; or 5 = stretching and contractions of the abdomen, and the activity (walking, grooming, and rearing) was recorded in seconds [21].

At the end of the 5th h, Von Frey test was conducted to evaluate the mechanical allodynia in the lower abdominal area. For this experimental set, 6-10 mice/group (supplementation-treated animals) or 8 mice/group (DHA i.p. and i.t.) were used. Mice were placed individually in clear Plexiglas boxes (9 x 7 x 11 cm) on elevated wire mesh platforms to allow access to the abdomen. The withdrawal response frequency was measured after 10 applications (duration of 1 s each) of 0.4 g von Frey hair (VFH) (Stoelting, Chicago, IL), obtaining the percentage of frequency responses. The following reactions were considered as a positive withdrawal response: sharp retraction of the abdomen, immediate licking, scratching at the site of filament application, and/or jumping [22].

In separate experimental groups, to evaluate the mechanical allodynia during all the 6-h period after HC induction, the 0.4-g VFH was applied below to the plantar

surface of the right hind paw (to assess referred pain), or to the lower abdomen area, as described by Meotti *et al*. [23], with slight modifications. The nociceptive responses were evaluated at different time-points (1, 2, 4, 6 h) following CYP injection. The 0.4 g VFH filament application to the hind paw and to the lower abdomen area produces a mean withdrawal frequency of about 10% and 30%, respectively, which are adequate values for the measurement of mechanical hypersensitivity. For this series of experiments, 5-6 mice or 9-11 mice/group (10% and 20% dietary lipid concentration, respectively), or 7-8 mice/group (DHA i.p. and i.t.) were used. In all the cases, after 6 h of the CYP injection, the animals were killed by deep inhalation of sevoflurane for further evaluation of inflammatory parameters.

2.5. Determination of bladder inflammatory parameters

This method was based on criteria established by Gray *et al.* [24]. Following euthanasia (6 h after CYP application), all the bladders were dissected free from connecting tissues, and transected at the bladder neck. Each bladder was macroscopically evaluated, by an examiner unaware of the treatment groups. The edema formation was categorized as severe (3), moderate (2), mild (1) or absent (0). Edema was considered severe when fluid was seen externally in the walls of the bladder, as well as internally. When edema was confined to the internal mucosa, it was reported as moderate; when it was between normal and moderate, the edema was defined as mild. Bladders were also examined for hemorrhage and categorized into four classes, depending on the presence of intravesical clots (3), mucosal hematomas (2), dilatation of the bladder vessels (1), or normal aspect (0). As an additional measure of bladder edema, the wet weight of each bladder was recorded and expressed as mg per 100 g of animal [24]. For this purpose, animals from the first set of behavioral tests were used.

2.6. Hematological parameters

After euthanasia, a small drop of blood was collected for the smear evaluation, using Giemsa staining [25]. Differential cell counts (neutrophils, eosinophils, basophils, lymphocytes, monocytes and immature cells) were estimated under an x40 objective, by counting 100 cells [26]. Representative pictures were captured. For this analysis, the animals from the second set of mechanical allodynia experiments were used.

2.7. Myeloperoxidase (MPO) activity

Neutrophil recruitment to the urinary bladder was measured by means of tissue MPO activity, according to the method described by Martins *et al.* [21], with some modifications. After euthanasia following 6 h of CYP injection, the bladders were removed and stored at -80 °C. The tissues were homogenized in 5% (w/v) EDTA/NaCl buffer (pH 4.7) and centrifuged at 4,000 rpm for 25 min, at 4°C. The pellet was resuspended 0.5% hexadecyltrimethyl ammonium bromide buffer (pH 5.4), and the samples were re-centrifuged (4,000 rpm, 25 min, 4°C). Twenty-five microliters of the supernatant were used for the MPO assay. The enzymatic reaction was assessed with 1.6 mM tetramethylbenzidine, 80 mM $NaPO_4$, and 0.3 mM hydrogen peroxide. The absorbance was measured at 595 nm, and the results are expressed in optical density (OD) per milligram of tissue. For MPO assay, 4-6 animals/groups were used for the 10% and 20% dietary lipid supplementation groups, whereas 4 animals/group were used for DHA treatment group.

2.8. Analysis of GPR40/FFAR1 expression by quantitative real time RT-PCR (RT-qPCR)

The spinal cords were collected 6 h after induction of HC by CYP. The total RNA was isolated with Trizol® reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The total RNA was quantified (A260, A280, A230) with the Quantifier spectrophotometer L-quant (Loccus Biotecnologia) and after treated with Deoxyribonuclease I (Invitrogen) to eliminate genomic DNA contamination in accordance with the manufacturer's instructions. The cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from 1 µg total RNA, following the manufacturer´s instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 µL using 12.5 µL of diluted cDNA, containing a final concentration of 0.2x SYBR® Green I (Invitrogen), 100 µM dNTP, 1x PCR Buffer, 3 mM MgCl2 0.25 U Platinum® Taq DNA Polymerase (Invitrogen), 0.5 M of betaine (for *Ffar-1*), and 200 nM of each reverse and forward primers (Supplementary Table 2) The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 to 99 °C and showed in all cases one single peak. *Hprt1*, *Ppia* and *Tbp* were used as reference genes for normalization. Relative expression levels were determined with 7500 Fast Real-Time Systems Software v.2.0.6 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 2012.3 Software (http://LinRegPCR.nl). Relative mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method. For this assay, the number of animals was 4-5 animals/group (i.p. saline/i.p. saline; i.p. saline/i.p. CYP, and i.p. DHA/i.p. CYP).

2.9. Evaluation of glia activation and GPR40/FFAR1 expression by immunohistochemistry

The technique was performed as described by Maciel *et al*. [27], with adaptations. After euthanasia at 6 h, the lumbar spinal cords (L3–L6 region) were collected for immunohistochemistry analysis. Immunopositivity for activated astrocytes or microglia, and GPR40/FFAR1 expression was assessed on paraffin tissue sections (3-µm) by using the monoclonal rabbit anti-GFAP (1:250, Cat. #04-1062; Lot #2145973; Merck Millipore, Darmstadt, Germany), monoclonal mouse anti-Iba1/AIF1 (1:300; Cat. #MABN92; Lot #2172784; Merck Millipore, Darmstadt, Germany), and polyclonal rabbit anti-GPR40/FFAR1 (1:100; Item no. 10007205; Cayman Chemicals, Michigan, USA). High-temperature antigen retrieval was performed by immersion of the slides in a water bath at 98–100 °C in 10 mM trisodium citrate buffer, pH 6.0 (anti-Iba-1), Tris-EDTA buffer pH 9.0 (anti-GFAP and anti-GPR40) for 40 min. The peroxidase was blocked by incubating the sections with perhidrol 5% for 30 min. The nonspecific protein binding was blocked with milk serum solution 5% for 30 min. After overnight incubation at 4 °C with primary antibodies, the slides were washed with PBS and incubated with the secondary antibody HRP conjugate (Invitrogen), ready-to-use, for 20 min at room temperature. The sections were washed in PBS, and the visualization was completed by using 3,3′-diaminobenzidine (Dako Cytomation) in chromogenic solution and counterstained lightly with Harris's Hematoxylin solution. Images were examined with a Zeiss AxioImager M2 light microscope (Carl Zeiss, Gottingen, Germany). For each section, 4-5 images were taken, in order to contemplate most areas of the spinal cord. The images were captured in x200 magnification, and evaluated by using the Image NIH Image J 1.36b Software (NIH, Bethesda, MD, USA). The number of GFAP-

positive astrocytes and Iba1-positive microglia cells was quantified by two independent examiners in a blinded manner, in the following regions of the lumbar spinal cord: right dorsal horn (RDH), left dorsal horn (LDH), right ventral horn (RVH), left ventral horn (LVH) and central canal (CC). For the quantification of GPR40/FFAR1 positive neurons, digitized 8-bit images were transferred to a computer, and the average pixel intensity was calculated by using NIH ImageJ 1.36b Software, by analyzing the following regions of the spinal cord: right ventral horn (RVH), left ventral horn (LVH), right to the central canal (RCC), left to the central canal (LCC). For immunohistochemistry analysis, 7–8 animals per group were used (i.p. saline/i.p. saline; i.p. saline/i.p. CYP, and i.p. DHA/i.p. CYP).

2.10. Flow cytometry for serum cytokines

As a parameter of peripheral inflammation, the CBA mouse inflammation kit (BD Biosciences) was used to determine the serum levels of interleukin-6 (IL-6), IL-10, monocyte chemoattractant protein-1 (MCP-1), interferon-γ (IFN-γ), tumor necrosis factor (TNF), and IL-12p70. The samples were analyzed using a flow cytometer (FACSCanto II, BD Biosciences, San Jose, CA, USA) with a 488 nm laser and fitted with a high-throughput sampler, as described previously by Nicoletti *et al.* [28]. Sample data were acquired using BD FACSDiva V6.1.3 (BD Biosciences), and the results were analyzed using FCAPArray v1.0.1 (BD Biosciences/Soft Flow HungaryLtd.) analysis software. The data are expressed in pg/ml. For these experiments, 4-5 animals per group were used (i.p. saline/i.p. saline; i.p. saline/i.p.CYP, and i.p. DHA/i.p. CYP).

2.11. Analysis of DHA effects on CYP-induced cytotoxicity

MDA-MB-231 human breast cancer cell lines were from American Type Culture Collection (ATCC-Rockville, Maryland, USA). The cells were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS) at a temperature of 37°C, a minimum relative humidity of 95 %, and an atmosphere of 5 % CO2 in air. The number of cells with metabolically active mitochondria was determined based on the mitochondrial reduction of a tetrazolium bromide salt (MTT [3-(4,5-imethylthia-zol-2-yl)-2,5-diphenyltetrazolium bromide] assay), according to the method described by Gehring *et al.* [29]. In a first series of experiments, the cells were treated with 4 different concentrations of CYP (0.1 mM, 1 mM, 10 mM, 50 mM) or DHA (25 µM, 50 µM, 75 µM, 100 µM) when incubated alone [30,31]. Secondly, the effects of a subliminal and an effective concentration of DHA (75 µM and 100 µM) and CYP (CYP 1 mM and 10 mM) were tested in combination, in order to determine whether or not DHA might affect the anti-tumor effects of CYP. In this case, DHA was added 30 min before CYP incubation, and the cell viability was assessed as described previously.

2.12. Statistical analysis

Results are presented as mean ± standard error mean (SEM). The statistical comparison of the data was performed by one-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc test. The areas under the curves (AUC) were calculated in the time-course experiments to evaluate hind paw or abdominal allodynia. *P* values less than 0.05 ($P< 0.05$) were considered as indicative of significance (GraphPad Prism 5.0, La Jolla, CA, USA).

3. Results

3.1. Effects of 21-day lipid dietary supplementation on painful and inflammatory parameters in CYP-induced HC model

Extending previous literature data [32, 33], the present results show that acute administration of CYP caused marked bladder inflammatory alterations, accompanied by reduced locomotor activity, spontaneous nociception and regional mechanical allodynia (Fig. 1-5). In this experimental model, the long-term dietary supplementation with 10 % or 20 % fish oil produced a significant inhibition of the spontaneous nociceptive behavior, when compared to the groups that received 10 % and 20 % of corn oil diet ($P < 0.05$, $P < 0.01$, respectively; Fig. 1a). Furthermore, the diet supplementation with both concentrations of fish oil (10 % and 20 %) also resulted in a significant reduction of abdominal mechanical allodynia, in comparison to the same concentrations of corn oil, when evaluated at the 5^{th} h ($P < 0.05$; Fig. 1b). Regarding the inflammatory parameters, either 10 % or 20 % fish oil diet supplementation failed to significantly alter the hemorrhage score (Fig. 1e) or the increased bladder wet weight induced by CYP (Fig. 1f). Nevertheless, a slight inhibition of the edema score was obtained with the 20 % fish oil diet supplementation, when compared to the 20 % corn oil group ($P < 0.05$; Fig. 1d). Finally, the CYP-induced locomotor deficits were not altered by any of the lipid dietary concentrations (Fig. 1c; $P > 0.05$).

3.2. Effects of 21-day lipid dietary supplementation on mechanical allodynia throughout 6-h period

Concerning the time-course evaluation of abdominal and hind paw mechanical allodynia, 10% fish oil dietary supplementation was not able to reduce the hypersensitivity, when VFH was applied to the right hind paw (Fig. 2a, b) or to the

lower abdomen (Fig. 2c, d), in comparison to the 10 % corn oil diet groups ($P > 0.05$), according to the analysis of the AUC. In contrast, 20% fish oil dietary supplementation significantly inhibited the abdominal mechanical allodynia, in relation to the 20% corn oil diet group ($P < 0.01$; Fig. 2g,h), as indicated by the AUC evaluation. However, the hind paw mechanical response in CYP-treated animals was not altered when compared to the saline groups, with no difference between both lipids ($P > 0.05$; Fig. 2e, f).

3.3. Effects of i.p. and i.t. DHA on painful and inflammatory parameters in CYP-induced HC model.

Based on earlier literature data demonstrating marked analgesic effects for DHA in rodents [34], and also on the results described above for fish oil supplementation, we decided to assess the effects of DHA administration, dosed by i.p. or i.t. routes, on the adverse effects elicited by CYP (Fig 3-5). The systemic treatment with DHA (1 μmol/kg, i.p.), given 1 h before, significantly inhibited the spontaneous nociceptive behavior caused by CYP ($P < 0.01$; Fig. 3a). In addition, the DHA i.p. treatment also reduced the abdominal mechanical allodynia on the 5^{th} h after CYP injection ($P < 0.05$; Fig. 3b). Conversely, the i.p. administration of DHA failed to inhibit the edema ($P > 0.05$; Fig. 3d), the hemorrhage ($P > 0.05$; Fig. 3e), the increased bladder wet weight ($P > 0.05$; Fig. 3f), or the reduced locomotor activity, when compared to the control CYP-treated groups ($P > 0.05$; Fig. 3c).

The effects of DHA administration were also tested when this fatty acid was injected spinally. The i.t. administration of DHA (10 μg/site), given 15 min before CYP administration, significantly lessened the spontaneous nociceptive behavior induced by CYP (Fig. 4a; $P < 0.05$). Similarly, the abdominal mechanical hypersensitivity at the 5^{th} h was also reduced by the spinal application of DHA (Fig. 4b; $P < 0.05$). In relation to

the inflammatory changes, the i.t. application of DHA was not capable of altering the edema (Fig. 4d; $P > 0.05$), or the hemorrhage (Fig. 4e; $P > 0.05$) associated to CYP toxicity. However, the spinal treatment with DHA slightly inhibited CYP-induced increase of the bladder wet weight (Fig. 4f; $P < 0.05$), whereas it did not affect CYP-dependent reduced locomotor activity (Fig. 4c; $P > 0.05$).

3.4. Effects of i.p. and i.t. DHA on mechanical allodynia throughout 6-h period

The systemic administration of DHA displayed marked analgesic effects when VFH was applied to the right hind paw (Fig. 5a,b; $P < 0.05$) or to the lower abdomen (Fig. 5c,d; $P < 0.05$), according to the analysis of AUC. Nevertheless, the spinal treatment with DHA caused a modest, although not significant reduction of hind paw (Fig. 5e, f; $P > 0.05$) or abdominal mechanical allodynia elicited by CYP (Fig. 5g, h; $P > 0.05$), as indicated by the AUC analysis.

3.6. Effects of 21-day lipid dietary supplementation or systemic DHA administration on total blood cell counts.

Confirming the literature data [26], our results showed that saline-treated animals display a predominance of lymphocytes. In contrast, the application of CYP induced a marked reduction of lymphocyte counts, associated to a significant increase of neutrophils in animals with a regular chow, an effect that was significantly reversed by the i.p. administration of DHA (Fig. 6c, $P < 0.05$). On the other hand, the dietary supplementation with 10 % (Fig. 6b) and 20 % (Fig. 6c) of either corn or fish oil led to visible changes of lymphocyte/neutrophil rates in CYP-treated mice, and therefore the statistical comparison for this set of experiments was not possible. Representative images for hematological analysis are provided in the Supplementary Fig. 1.

3.7. Effects of 21-day lipid dietary supplementation or systemic DHA administration on peripheral cytokine levels and on urinary bladder MPO activity.

Our data show that CYP injection was associated to increased MPO levels, but this was not significantly changed by fish oil supplementation at 10 % (Fig. 6d) or 20 % (Fig. 6e), or even by the i.p. treatment with DHA (Fig. 6f) ($P > 0.05$).

The urinary bladder inflammation induced by CYP involves the production of local inflammatory cytokines, as demonstrated by Silva *et al*. [33] Nevertheless, in the current study, we decided to evaluate the levels of circulating cytokines, based on the differences of circulating leukocyte counts, as described above. In our experimental model, the levels of IFN-γ and IL-12p70 were undetectable. Additionally, there was no difference among the experimental groups when comparing the serum levels of TNF (Fig. 7c) or IL-10 (Fig. 7d) ($P > 0.05$). The production of MCP-1 was significantly augmented by CYP administration ($P < 0.01$), but the i.p. treatment with DHA did not modify the levels of this cytokine (Fig. 7b). Regarding the serum production of IL-6, the injection of CYP induced a marked increase of this cytokine ($P > 0.01$), and the systemic DHA treatment was able to decrease IL-6 levels, but not in a significant manner (Fig. 7a).

3.8. Effects of systemic administration of DHA on glial cells activation in the lumbar spinal cord

Previous evidence demonstrates that administration of some chemotherapy agents is associated with the spinal activation of glial cells in rats [35, 36]. Herein, the administration of CYP led to astrocyte activation throughout distinct regions of the mouse lumbar spinal cord, with significant differences at the right dorsal horn, in relation to the saline control groups (Fig. 8a; $P < 0.05$). In this protocol, the i.p.

treatment with DHA caused a slight reduction in the number GFAP-positive astrocytes, although this effect was not significant ($P > 0.05$). The total number of activated astrocytes was calculated, showing no significant difference among the experimental groups (Fig. 8b; $P > 0.05$). Representative images of the right dorsal horn of the lumbar spinal cord are provided, indicating the presence of GFAP-positive astrocytes in the following experimental groups: saline i.p. (Fig. 8c), CYP 300 mg/kg i.p. + saline i.p. (Fig. 8d), and CYP 300 mg/kg i.p. + DHA 1 µmol/kg i.p (Fig. 8e). The microglial activation was also evaluated by determining the positive immunolabelling for Iba1/AIF1 in the mouse spinal cord. However, the immunopositivity for this marker was sparsely observed throughout the different experimental groups (Supplementary Fig. 2a-c).

3.9. Effects of systemic administration of DHA on GPR40/FFAR1 immunolabeling in the lumbar spinal cord

The activation of the fatty acid receptor GPR40/FFAR1 by DHA binding has been associated to analgesic effects in rodent models of pain [37]. Initially, we decided to employ RT-qPCR to assess GPR40/FFAR1 expression in the mouse spinal cord. However, it was not possible to detect transcripts in either experimental group (a representative amplification plot is provided in the Supplementary Fig. 2d). Two sets of primers were constructed and tested using 1µg of total RNA template per RT reaction, and the effects of PCR-enhancing agents (betaine and DMSO) were also evaluated, without successful amplification. Of note, all the reference genes used (Ppia, Tbp and Hprt1) worked well under the conditions adopted, indicating that this method was not suitable for GPR40/FFAR1 detection in our experiments. Therefore, an immunohistochemistry analysis for GPR40/FFAR1 detection was carried out, and

analyzed throughout four different anatomical regions of the lumbar mouse spinal cord. The administration of CYP led to a visible reduction of immunolabelled GPR40/FFAR1 positive neurons, when compared to the saline control group, an effect that was brought to the control values by the i.p. treatment with DHA, with significant effects at the right ventral horn (Fig. 9a; $P < 0.05$). The scatter dot plots did not reveal any significant difference for the total quantification of positive GPR40/FFAR1 neurons among the experimental groups, although it is possible to observe a similar profile with a reduction of GPR40/FFAR1 immunopositivity in CYP-treated animals, and a reversion to the saline control values in the DHA-treated group (Fig 9b, $P > 0.05$). Representative images of the right ventral horn of the lumbar spinal cord show positive immunolabelled neurons for GPR40/FFAR1, with evident dark-brown staining in the three experimental groups (Fig. 9c-e).

3.10. In vitro effects of DHA treatment on CYP-induced cytotoxicity in MDA-MB-231 human breast cancer cells.

Initially, we analyzed different concentrations of CYP and DHA separately. The treatment with CYP (10 mM and 50 mM) or DHA (100 µM) was able to reduce the viability of MDA-MB-231 human breast cancer cells in a significant manner (Fig. 10a; $P < 0.01$). The other tested concentrations of CYP (0.1 mM and 1mM) or DHA (25 µM, 50 µM, 75 µM) failed to alter the cell proliferation (Fig. 10a; $P > 0.05$). Next, we assessed the effects of DHA and CYP, when these agents were tested in combination, indicating that cytotoxic effects of CYP were not impaired by DHA incubation (Fig. 10b). Of note, the calculation of inhibition rates demonstrated that pre-incubation of a subliminal concentration of DHA (75 µM) failed to potentiate the cytotoxic effects of a

low concentration of CYP (1 mM). However, additive significant effects were observed by the treatment with DHA (100 µM) plus CYP (10 mM) (Fig. 10c; $P < 0.01$).

4. Discussion

The supplementation with fish oil-derived omega-3 fatty acids is frequently used during chemotherapy, mostly to prevent the development of cancer cachexia [3]. HC is a major adverse effect of the treatment with the antitumor agent CYP. HC is characterized by a marked inflammation of the urinary bladder, causing severe lower abdominal pain, among other symptoms [38, 39]. As an initial aim, this study evaluated the protective effects of the dietary supplementation with fish oil-derived omega-3 fatty acids on CYP-induced HC. Subsequently, we assessed the effects of the systemic or spinal treatment with DHA in the same *in vivo* experimental parameters of CYP-caused HC [40]. In addition, on the basis of the clinical trial carried out by Bougnoux *et al*. [5], the effects of DHA were also tested in combination with CYP, by using an *in vitro* assay for human breast cancer cell viability.

As demonstrated before, the acute administration of CYP is capable of inducing marked spontaneous visceral nociception in rodents [41, 42]. In the current study, we demonstrated an inhibition of the CYP-induced spontaneous nociceptive behavior in the animals that received fish oil-enriched diets (10 and 20 %) during 21 days, when compared to the corn oil diet groups. Of note, this inhibition was dependent on the tested concentration of fish oil added to the chow. Supporting our data, a previous study conducted by Nobre *et al.* [43]demonstrated marked analgesic effects for low doses of fish oil-derived omega 3 fatty acids in rodent models of spontaneous nociception, including the visceral pain elicited by acetic acid in mice. Furthermore, a meta-analysis

study published in 2007 suggested that the supplementation with fish oil derived-omega-3 fatty acids represents an interesting adjunctive treatment for joint pain associated with rheumatoid arthritis, inflammatory bowel diseases and dysmenorrhea [44].

On the basis of recent literature data, it is tempting to suggest that analgesic effects of fish-oil rich diets are likely related to the presence of DHA [45, 46]. Therefore, we decided to evaluate the effects of the systemic treatment with DHA in our *in vivo* experimental paradigm. Notably, the single i.p. administration of DHA produced a marked reduction of the nociceptive spontaneous behavior in the model of HC induced by CYP in mice. Supporting these results, it was previously demonstrated that oral administration of DHA was able to prevent either thermal or chemical nociception in mice [47].

Altogether, our first series of data clearly indicate that either fish oil dietary supplementation or systemic DHA display favorable analgesic effects in the mouse model of CYP-evoked HC. This evidence prompted us to further assess the analgesic effects of both approaches. As mentioned before, visceral pain is a major symptom of CYP-induced HC [38, 39], and abdominal or hind paw mechanical sensitivity is considered as indicative of this type of pain [32, 48]. In fact, Bon *et al.* [42] demonstrated increased abdominal hypersensitivity at the 4^{th} h after dosing CYP (300 mg/kg), which is the same dose of CYP used in our experimental protocol, in different mouse strains. In addition, a marked and time-related reduction of the abdominal or hind paw mechanical threshold in CYP-treated rats was demonstrated before [23]. In the present study, we also evaluated the abdominal mechanical allodynia at the 5^{th} h, after the final assessment of spontaneous behavior, or in separate time-course experiments from 1 to 6 h after CYP injection. In this case, only the 20 % fish oil

dietary supplementation reduced the abdominal mechanical allodynia, whereas the i.p. administration of DHA inhibited both the abdominal and the hind paw mechanical hypersensitivity, demonstrating the ability of this omega-3 fatty acid in preventing referred pain.

It was previously demonstrated that spinal DHA was able to increase the paw withdrawal threshold in the carrageenan-induced inflammatory pain or in CFA-evoked heat hyperalgesia [19, 49]. To gain insights on the possible site of action of DHA, we decided to assess the same set of behavioral tests as presented above, when DHA was dosed i.t., 15 min before CYP. Our data indicate that DHA analgesic effects are, at least partly, mediated by the modulation of spinal pathways related to pain transmission. Accordingly, the i.t. administration of DHA displayed similar inhibitory actions on either the spontaneous nociception or the mechanical allodynia, when compared to the analgesic effects observed after the systemic DHA treatment, or the 21-day fish oil dietary supplementation. Additionally, the i.t. treatment with DHA partially inhibited the abdominal and the hind paw mechanical allodynia, when evaluated from 1 to 6 h after CYP.

CYP-induced urinary bladder inflammation is characterized by the presence of the severe edema and hemorrhage, accompanied by bladder neutrophil migration [21, 33, 50]. Although the intake of omega-3 fatty acids has been frequently associated with anti-inflammatory effects [51], in our study, either the dietary supplementation with fish oil or the treatment with DHA failed to display anti-inflammatory effects in the model of HC caused by CYP. In fact, only a slight reduction of bladder edema was seen in the groups that received 20 % fish oil or i.t. DHA. Thus, it is possible to conclude that analgesic effects of marine-derived omega-3 fatty acids observed by us are not dependent on the modulation of bladder inflammation.

A previous study demonstrated that repeated administration of low doses of CYP (25 mg/kg) for three days resulted in a marked reduction of blood lymphocytes and erythrocytes in mice, an effect that was prevented by the oral administration of *Aloe vera* gel [52]. Extending this evidence, the present data revealed that a single i.p. injection of a high dose of CYP (300 mg/kg) elicited a marked decrease of circulating lymphocytes, associated to a significant increase of neutrophils, when compared to saline-treated negative groups. Of note, the systemic administration of DHA modified the lymphocyte/neutrophil rates in CYP-treated mice towards the saline control values. Intriguingly, CYP-evoked alterations of lymphocytes and neutrophils were similarly recovered by the dietary supplementation with omega-3-enriched fish oil, or even with omega-6-containg corn oil. At this moment, we cannot explain this effect, but the beneficial effects of corn oil supplementation in our model might be attributed to omega-6-derived pro-resolution mediators, but this remains to be evaluated.

CYP-induced HC is accompanied by increased production of bladder or circulating pro-inflammatory cytokines [21, 33, 53]. Considering the beneficial effects observed for DHA on hematological parameters, we decided to investigate its actions on serum production of cytokines. Our data revealed that CYP administration led to a marked elevation of IL-6 and MCP-1 in the mouse serum, whereas the systemic treatment with DHA caused a partial reduction of IL-6 levels. Thus, the favorable effects on DHA on CYP-induced neutrophilia/lymphopenia could be explained, to some extent, by the modulation of IL-6 production.

The use of chemotherapy agents has been associated with the development of neuroinflammation, with marked activation of glia cells, such as astrocytes and microglia [54]. In addition, both acute and chronic pain states are related to the stimulation of glia cells in the central and peripheral nervous system [55]. Herein, we

show that a single injection of CYP was able to increase the number of activated astrocytes in the right dorsal horn of the spinal cord, and the pretreatment with DHA partially reversed this activation. Notwithstanding, it was not possible to detect any change of microglia activation, in all the evaluated experimental groups. Previous reports described a similar profile of astrocytic activation, without alteration of microglia, in the neuropathic pain induced by the chemotherapy drugs, namely paclitaxel, oxaliplatin or bortezomib in rats [35, 36]. Allied to literature data, our results allow suggesting that analgesic effects displayed by DHA on CYP-induced visceral pain are dependent on the modulation of astrocyte activation in the spinal cord.

It has been suggested that long-chain fatty acids exert their effects by interaction with the G protein-coupled receptor GPR40/FFAR1 [56, 57]. Accordingly, convincing evidence demonstrated that analgesic effects of DHA in the mouse model of pain induced by global cerebral ischemia or by CFA injection involve the central activation of GPR40/FFAR1 [45, 58]. The results of the present study extend this notion by clearly showing that acute administration of CYP was associated with reduced immunopositivity for GPR40/FFAR1 throughout neurons of the mouse spinal cord, whilst the i.p. pre-treatment with DHA reversed this effect to the saline control levels. Hence, the analgesic effects of DHA in CYP-treated mice probably rely on the neuronal modulation of GPR40/FFAR1 receptors in the mouse spinal cord.

Our data extend the notion on the protective effects of omega-3 fatty acids in chemotherapy-related adverse effects. In the case of the present study, we showed marked analgesic effects for fish oil supplementation, and mainly DHA administration against visceral pain associated to CYP-induced HC. To support our *in vivo* results, we evaluated whether DHA might affect the cytotoxic effects of CYP. For this purpose, we employed the MTT viability assay and the MDA-MB-231 human breast cancer cell

lineage. In this regard, it has been suggested that marine-derived omega-3 fatty acids, such as DHA, are able to improve the effectiveness of cancer chemotherapy [59, 60]. For instance, it was demonstrated that DHA increased the cytotoxic *in vitro* effects of the chemotherapy agent doxorubicin in MDA-MB-231 cells [61]. Herein, we provide evidence indicating that isolated treatment with DHA or CYP led to concentration-dependent reduction of MDA-MB-231 cell viability. Of note, the pre-incubation of DHA resulted in increased effectiveness of antitumor effects displayed by CYP in this cell line.

The supplementation with fish oil-derived omega-3 fatty acids is frequently employed for cancer patients under chemotherapy, particularly to control cancer cachexia. Our data shed new lights on the effects of omega-3 fatty acids, by revealing the ability of fish oil-enriched diet or DHA administration to prevent the visceral pain associated to CYP-induced HC. Surprisingly, either fish oil dietary supplementation or parenteral DHA failed to alter the bladder inflammation caused by CYP. An analysis of the possible mechanisms underlying the analgesic effects of omega-3 fatty acids in our experimental model suggests the involvement of either peripheral or central mechanisms, especially via modulation of circulating neutrophils, besides astrocytic activation and GPR40/FFAR1 expression at the spinal level. One might suppose that DHA would impair the anti-cancer effects of CYP; nonetheless, we also bring evidence on the ability of this omega-3 fatty acid to sensitize human breast cancer cells to the cytotoxic effects of CYP. Altogether, our results extend the notion about the beneficial effects of omega-3 enriched diets to prevent the adverse effects caused by chemotherapy drugs, or to improve their anti-tumor effects.

Conflict of interest

The authors declare that they have no conflict of interest.

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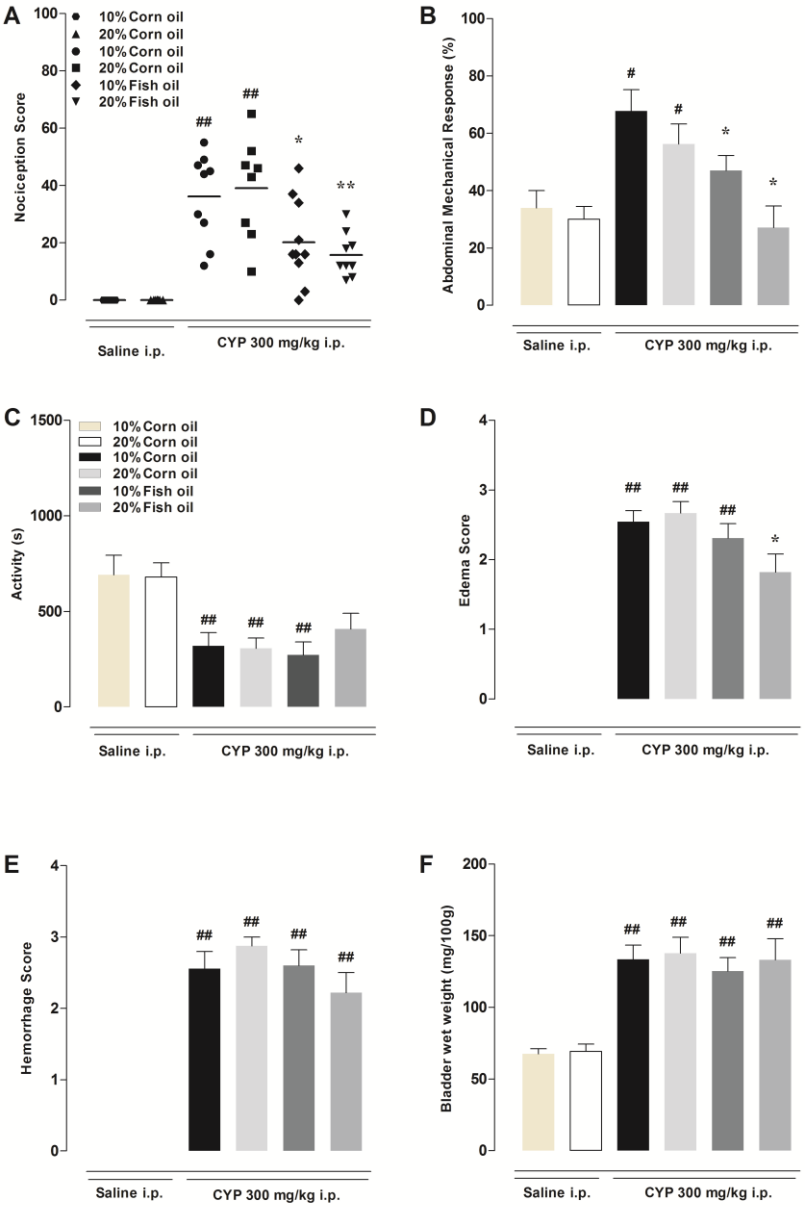


Fig. 1. Effects of dietary supplementation with corn oil or fish oil (10 % and 20 %), during 21 days, on painful and inflammatory parameters in the model of CYP-induced HC. (A) Cumulative 4-h nociception score; (B) abdominal mechanical allodynia 5 h after CYP injection; (C) cumulative 4-h locomotor activity; (D) edema and (E) hemorrhage scores at the 6th h; (F) bladder wet weight in grams. Each column represents the mean ± SEM of 6 to 12 mice/group. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ significantly different from saline-treated negative groups; $^{**}P < 0.01$, $^{*}P < 0.05$ significantly different from the respective corn oil CYP-treated group.

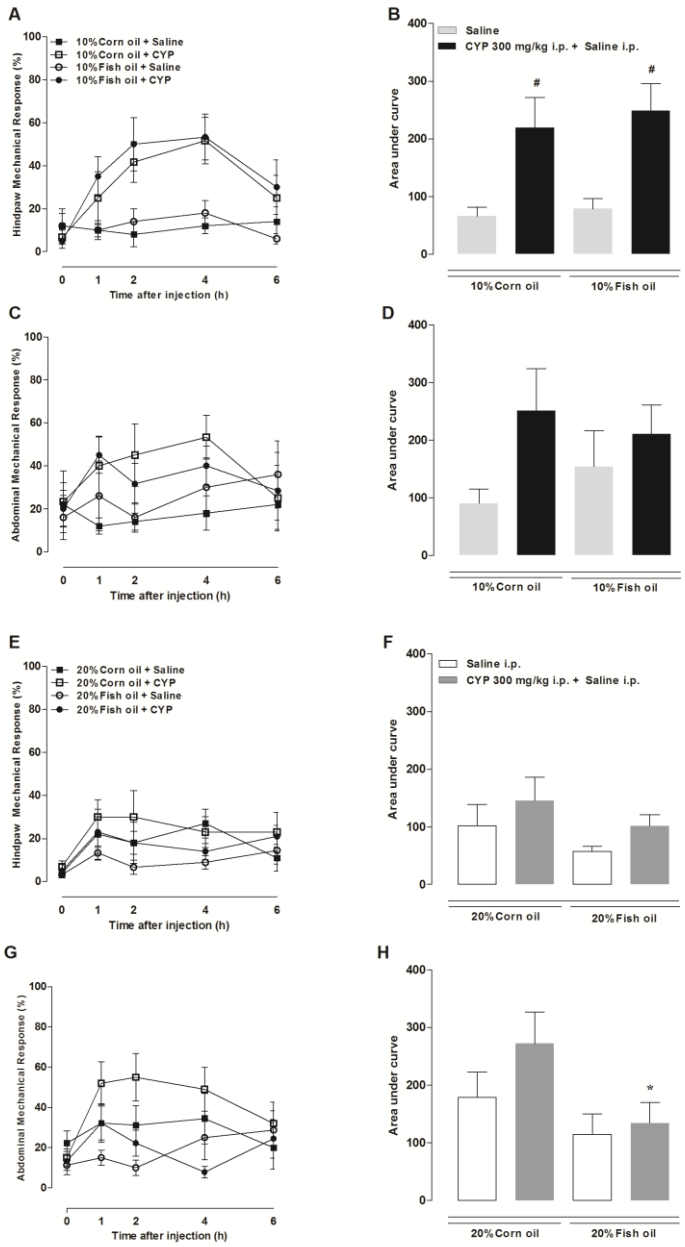


Fig. 2. Effects of dietary supplementation with corn oil or fish oil (10 % and 20 %), during 21 days, on (A and E) hind paw or (C and G) abdominal mechanical allodynia throughout 6-h period after CYP administration. The AUC for (B and F) hind paw or (D and H) abdominal allodynia were calculated for both oils at 10 % and 20 %, respectively. Each point of the curve or each column of the AUC graphs represents the mean ± SEM of 6 to 11 mice/group. $^{\#}P < 0.05$ significantly different from saline-treated negative groups; $^{*}P < 0.05$ significantly different from the respective corn oil CYP-treated group.

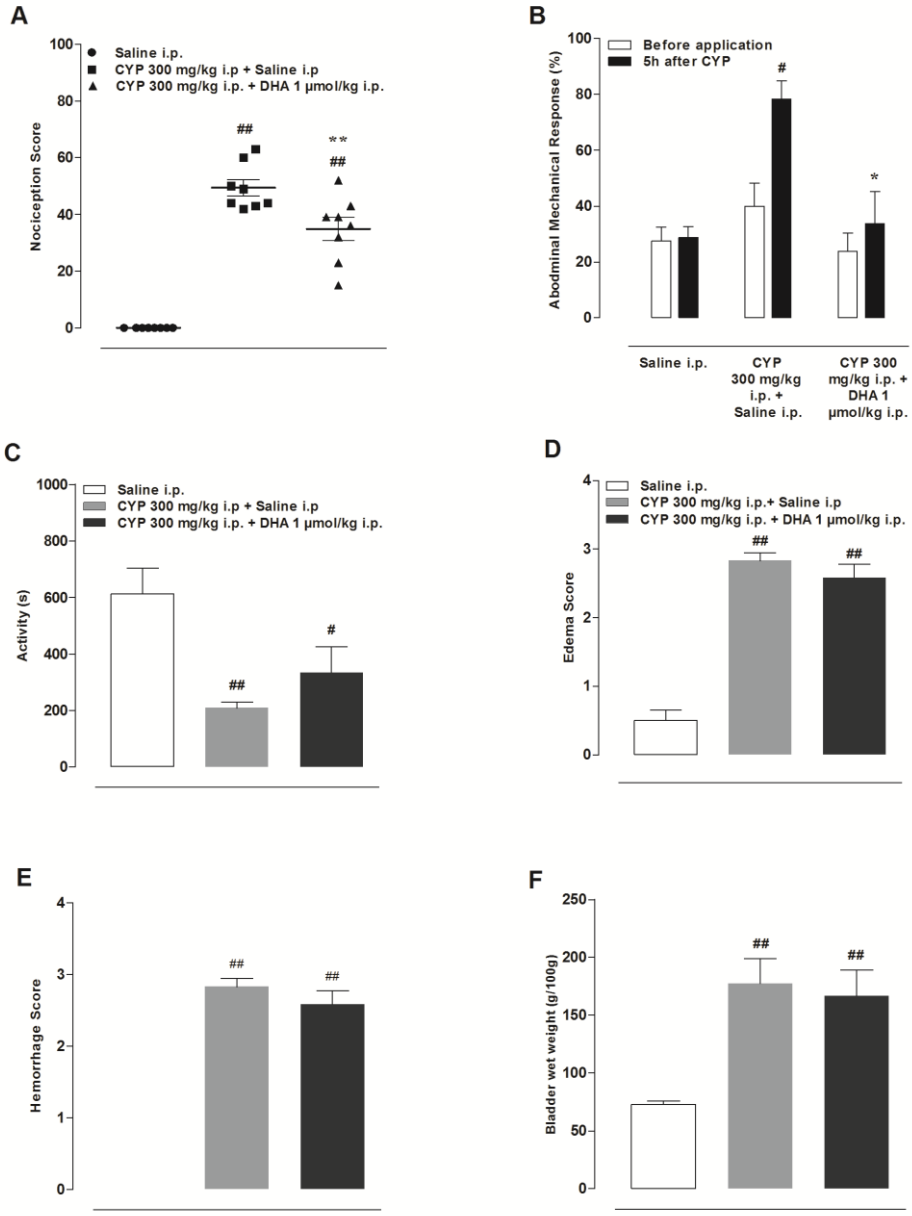


Fig. 3. Effects of acute intraperitoneal administration of DHA (1 µmol/kg), dosed 1 h before, on painful and inflammatory parameters in the model of CYP-induced HC. (A) Cumulative 4-h nociception score; (B) abdominal mechanical allodynia 5 h after CYP injection; (C) cumulative 4-h locomotor activity; (D) edema and (E) hemorrhage scores at the 6th h; (F) bladder wet weight in grams. Each column represents the mean ± SEM of 8 to 12 mice/group. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ significantly different from saline-treated negative groups; $^{**}P < 0.01$, $^{*}P < 0.05$ significantly different from CYP-treated groups.

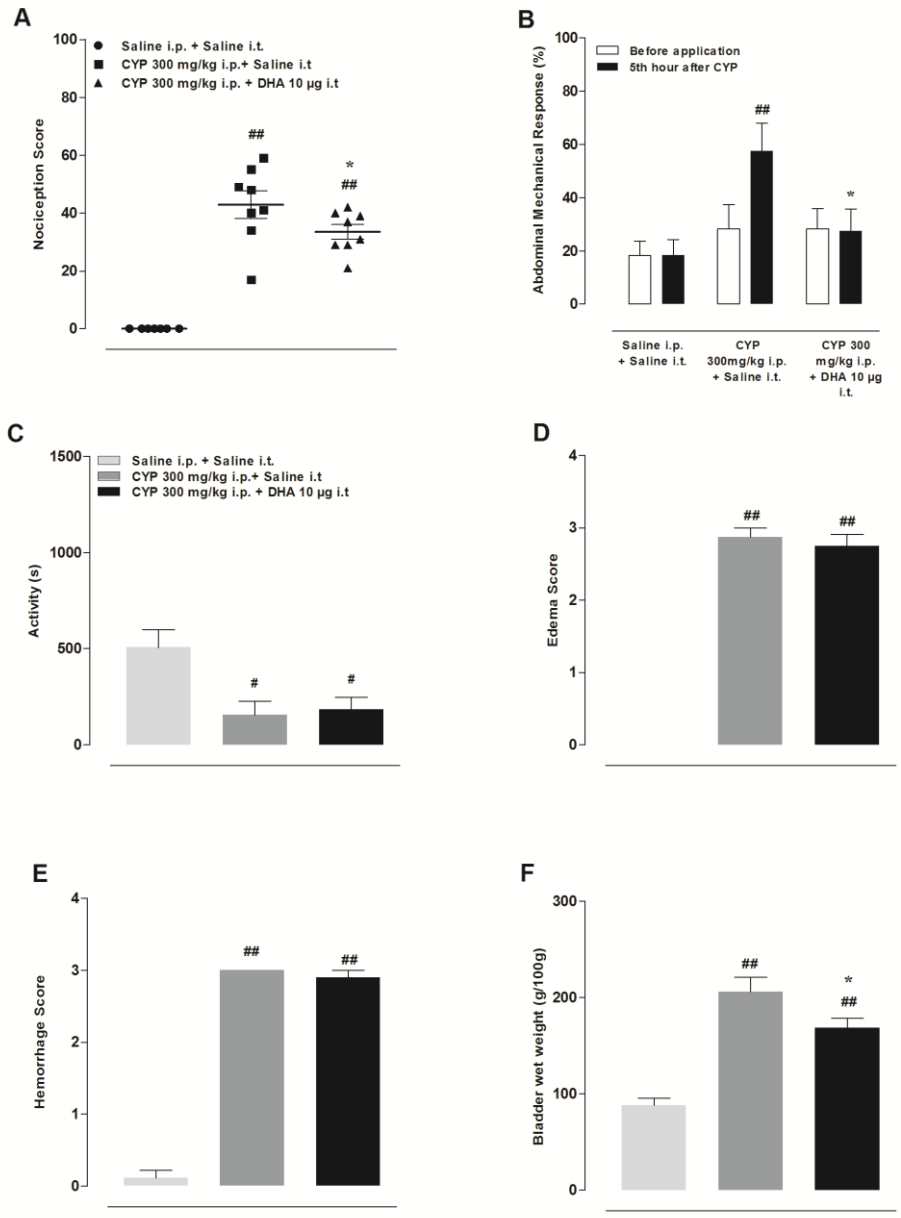


Fig. 4. Effects of acute spinal administration of DHA (10 µg/site), injected 15 min before, on painful and inflammatory parameters in the model of CYP-induced HC. (A) Cumulative 4-h nociception score; (B) abdominal mechanical allodynia 5 h after CYP injection; (C) cumulative 4-h locomotor activity; (D) edema and (E) hemorrhage scores at the 6th h; (F) bladder wet weight in grams. Each column represents the mean ± SEM of 8 to 12 mice/group. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ significantly different from saline-treated negative groups; $^{*}P < 0.05$ significantly different from CYP-treated groups.

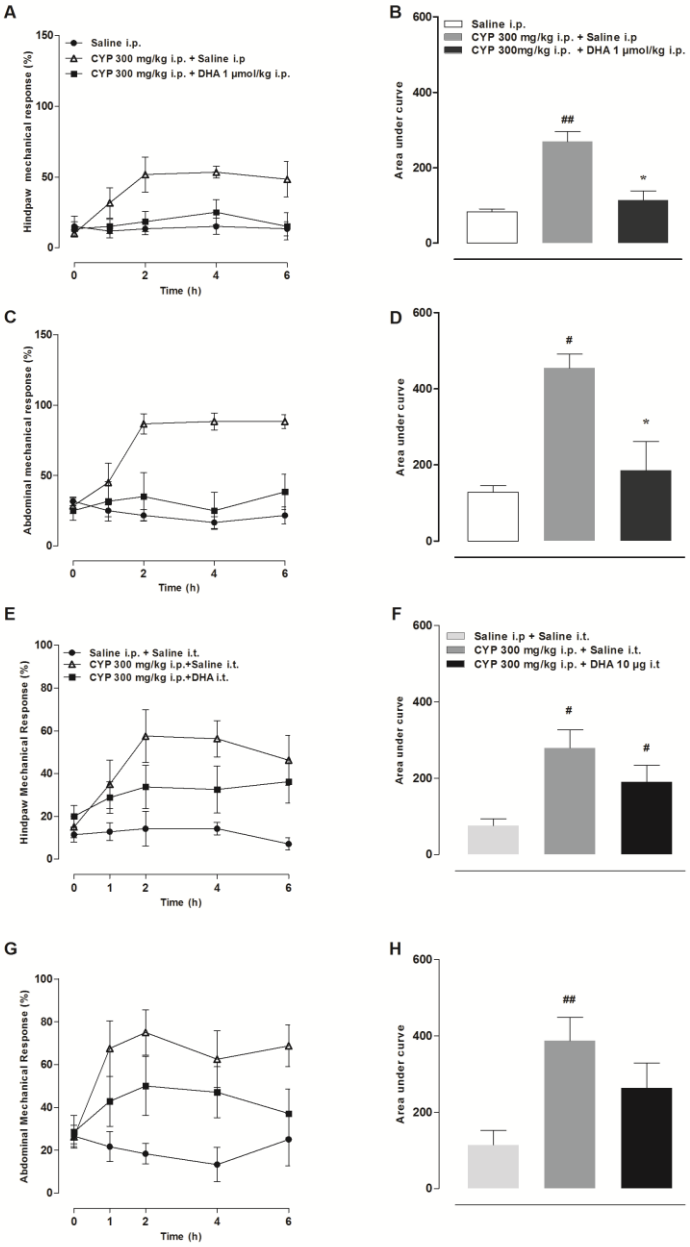


Fig. 5. Effects of (A, B, C and D) intraperitoneal administration of DHA (1 µmol/kg, dosed 1 h before), or (E, F, G, H) spinal administration of DHA (10 µg/site, injected 15 min before), on (A and E) hind paw or (C and G) abdominal mechanical allodynia throughout 6-h period after CYP administration. The AUC for (B and F) hind paw or (D and H) abdominal allodynia were calculated for i.p. and i.t., respectively. Each point of the curve or each column of the AUC graphs represents the mean ± SEM of 7 to 8 mice/group. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ significantly different from saline-treated negative groups; $^{*}P < 0.05$ significantly different from CYP-treated groups.

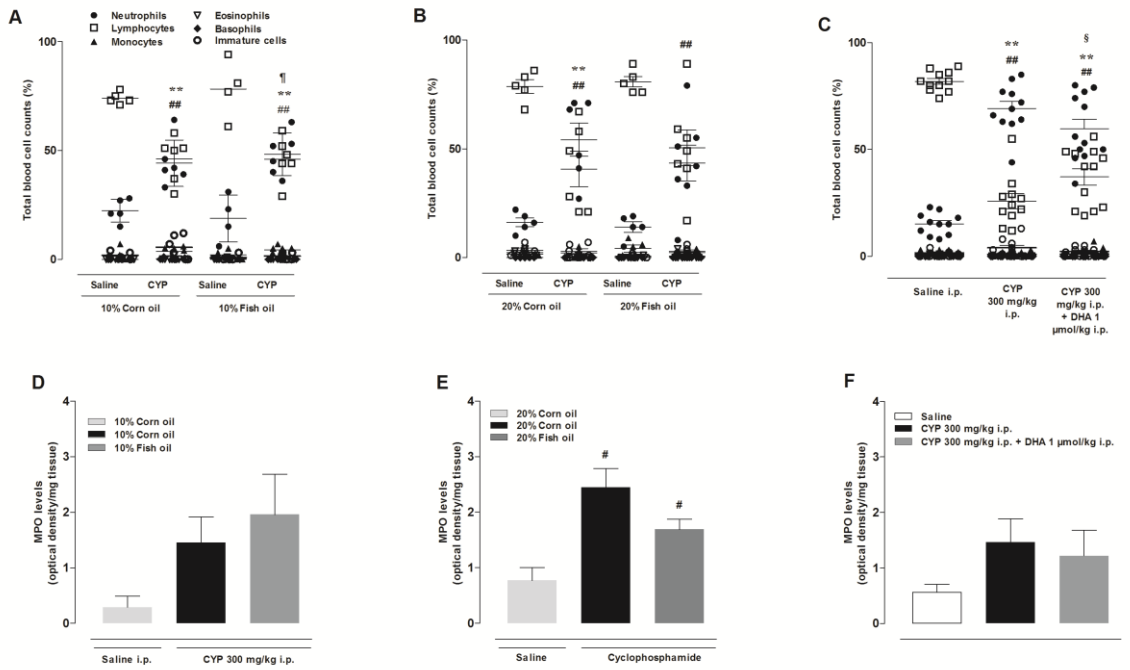


Fig. 6. Effects of 21-day dietary supplementation with corn oil or fish oil (10 % and 20 %), or effects of intraperitoneal administration of DHA (1 µmol/kg, dosed 1 h before), on (A, B and C) total blood cell counts or (D, E and F) bladder MPO activity. The scatter dot plots or the columns represent the mean ± SEM of 5 to 11 mice/group in blood cell counts and 4 mice/group in MPO activity. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ neutrophil counts or MPO activity significantly different from saline-treated negative groups; $^{**}P < 0.01$ lymphocyte counts significantly different from saline-treated negative groups; $^{¶}P < 0.05$ immature cells significantly different from saline-treated negative groups; $^{§}P < 0.05$ lymphocyte counts significantly different from CYP-treated groups.

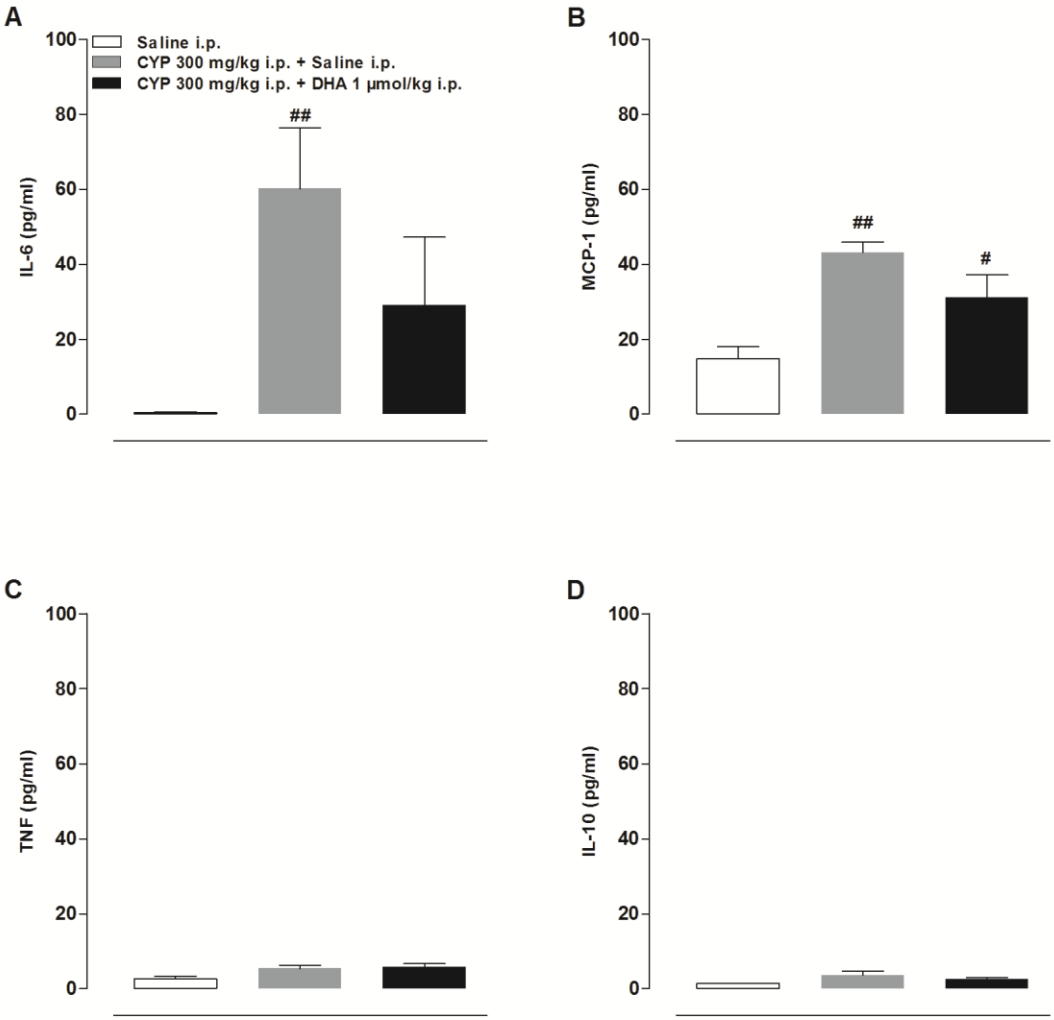


Fig. 7. Effects of acute intraperitoneal administration of DHA (1 µmol/kg), dosed 1 h before, on serum levels of (A) IL-6; (B) MCP-1; (C) TNF; (D) IL-10. Each column represents the mean ± SEM of 4 mice/group. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ significantly different from saline-treated negative groups.

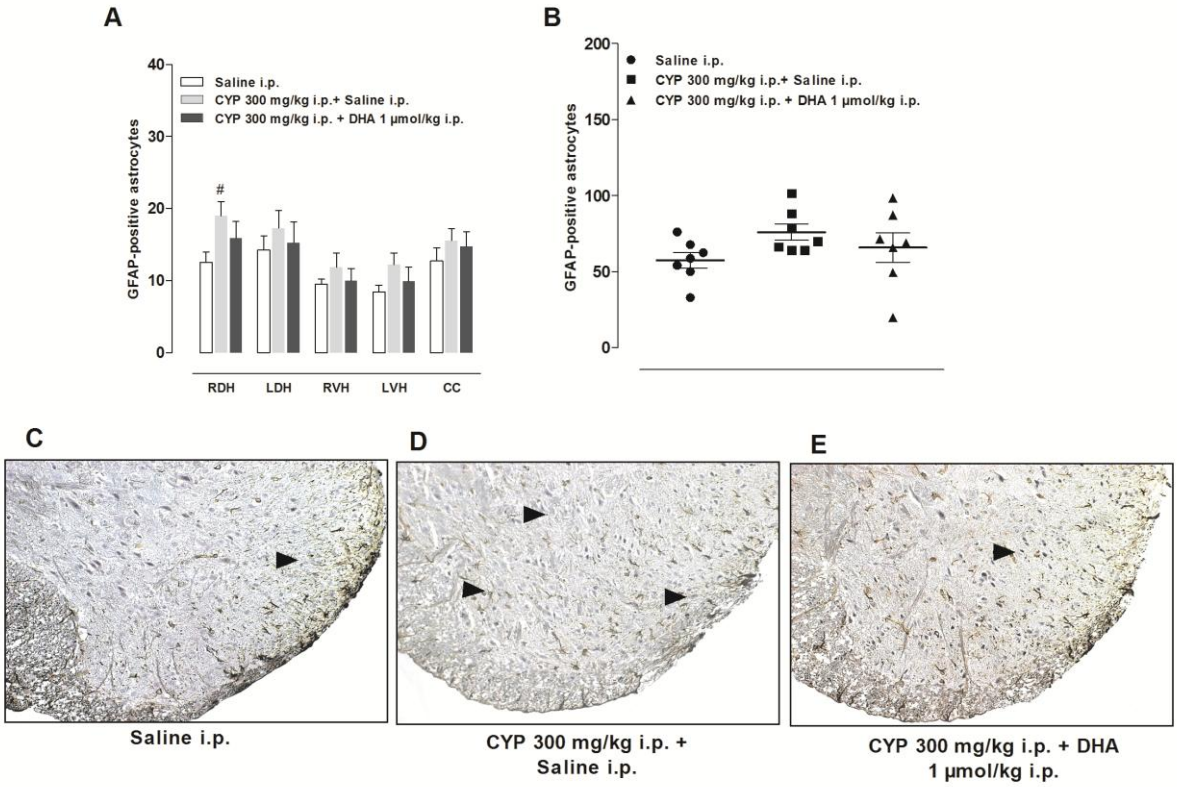


Fig. 8. Effects of acute intraperitoneal administration of DHA (1 µmol/kg), dosed 1 h before, on the number GFAP-positive astrocytes (A) throughout distinct spinal cord regions: right dorsal horn (RDH), left dorsal horn (LDH), right ventral horn (RVH), left ventral horn (LVH) and central canal (CC); (B) scatter dot plot graph showing the total number GFAP-positive astrocytes. The scatter dot plots or the columns represent the mean ± SEM of 7 mice/group. $^{\#}P < 0.05$ significantly different from saline-treated negative groups. Representative images of RDH in: (C) saline-treated negative group; (D) CYP-treated group; (E) CYP plus DHA group.

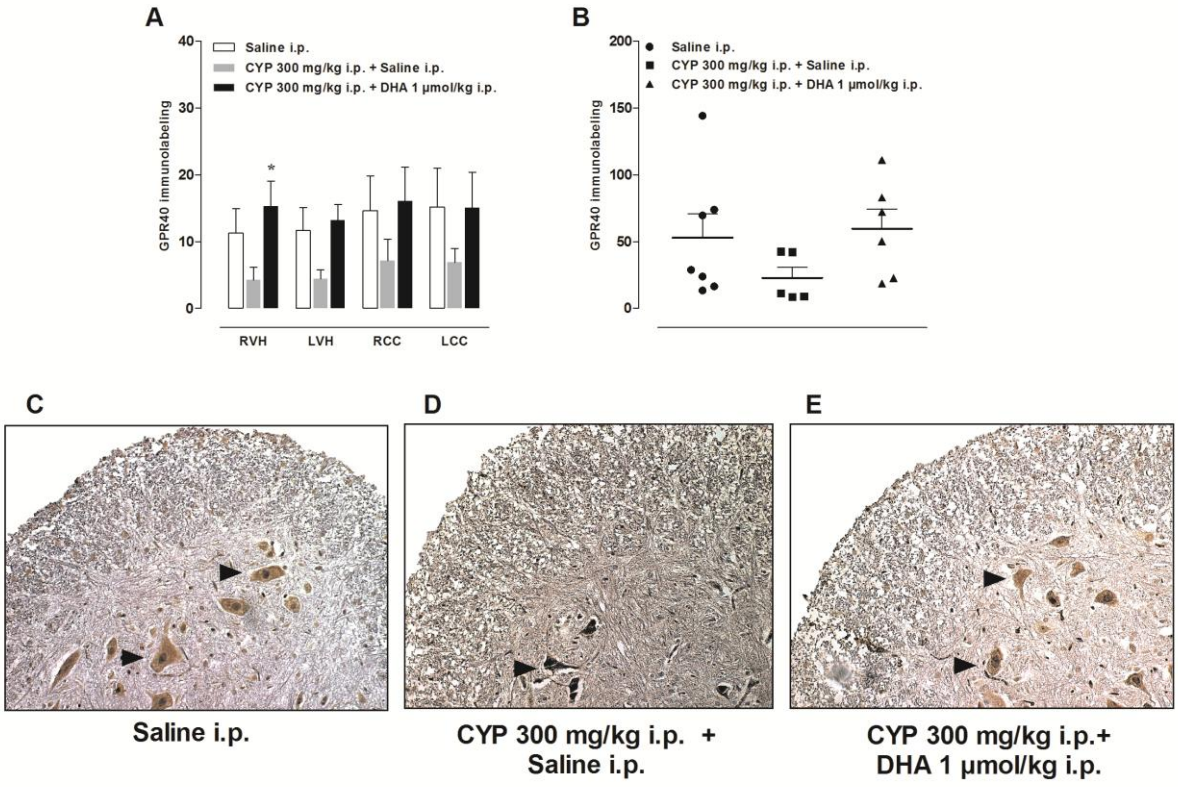


Fig. 9. Effects of acute intraperitoneal administration of DHA (1 µmol/kg), dosed 1 h before, on the positive GPR40/FFAR1 immunolabelling (A) throughout distinct spinal cord regions: right ventral horn (RVH), left ventral horn (LVH), right to the central canal (RCC), left to the central canal (LCC); (B) scatter dot plot graph showing the positive GPR40 immunolabelling. The scatter dot plots or the columns represent the mean ± SEM of 5 to 7 mice/group. $^{*}P < 0.05$ significantly different from CYP-treated groups. Representative images of RVH in: (C) saline-treated negative group; (D) CYP-treated group; (E) CYP plus DHA group.

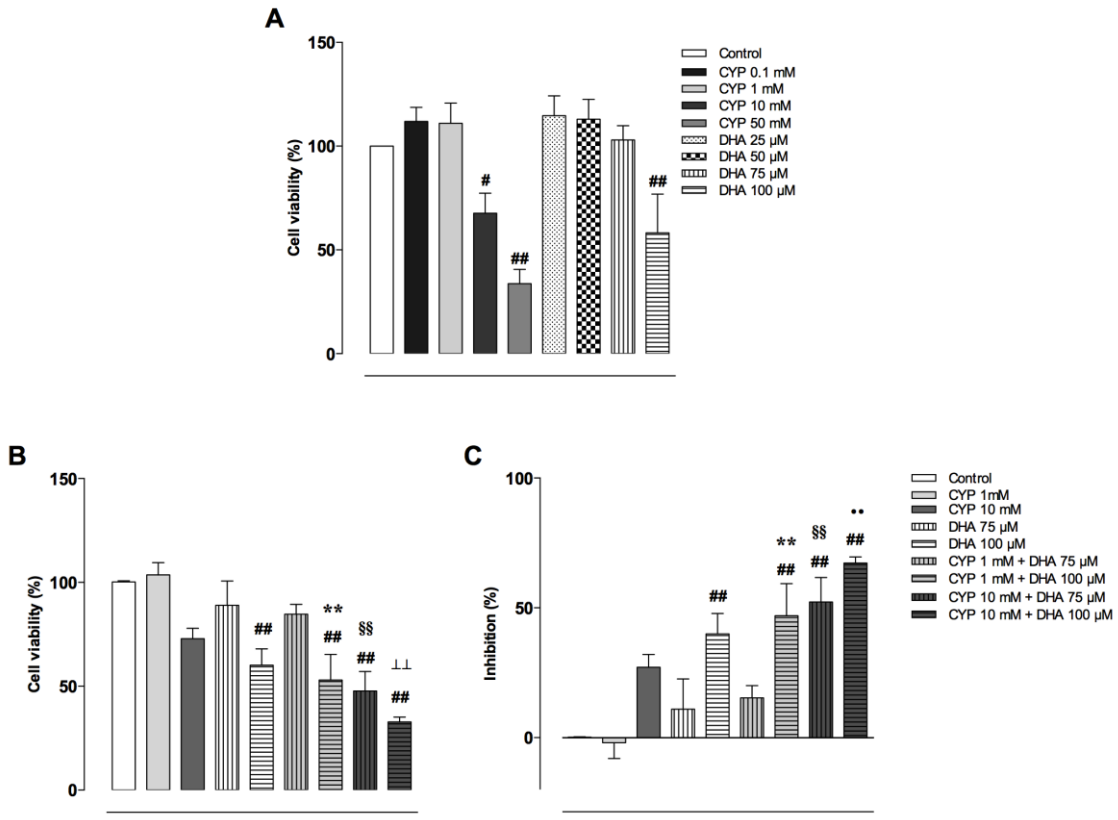


Fig. 10. *In vitro* effects of DHA treatment on CYP-induced cytotoxicity in MDA-MB-231 human breast cancer cells. (A) MTT cell viability assay with four different concentrations of CYP (0.1 mM, 1 mM, 10 mM, 50 mM) or DHA (25 µM, 50 µM, 75 µM, 100 µM) after 48 h of incubation. (B) MTT cell viability assay with the pre-incubation with DHA for 30 min (75 µM, 100 µM) followed by CYP (1 mM, 10 mM) incubation, totalizing 48 h. (C) Inhibition rates in the MTT cell viability assay for the combination protocols as described above. The columns represent the mean ± SEM of four independent experiments carried out in triplicate. $^{\#}P < 0.05$ compared to the control; $^{\#\#}P < 0.01$ compared to the control; $^{**,\S\S,\cdot\cdot}P < 0.01$ comparison between: CYP 1 mM vs. CYP 1 mM plus DHA 100 µM; CYP 10 mM vs. CYP 10 mM plus DHA 100 µM; DHA 75 µM vs. CYP 10 mM plus DHA 75 µM, respectively.

Supplementary Table 1. Diet composition by weight and percentage of macronutrients

Constituents[1]	Regular chow	10% CO	20% CO	10% FO	20% FO
			g/kg		
Protein	220	220	220	220	220
Total Fat	40	50	60	50	60
Fiber	80	80	80	80	80
Mineral mix*	100	100	100	100	100
Carbohydrates	560	560	560	560	560

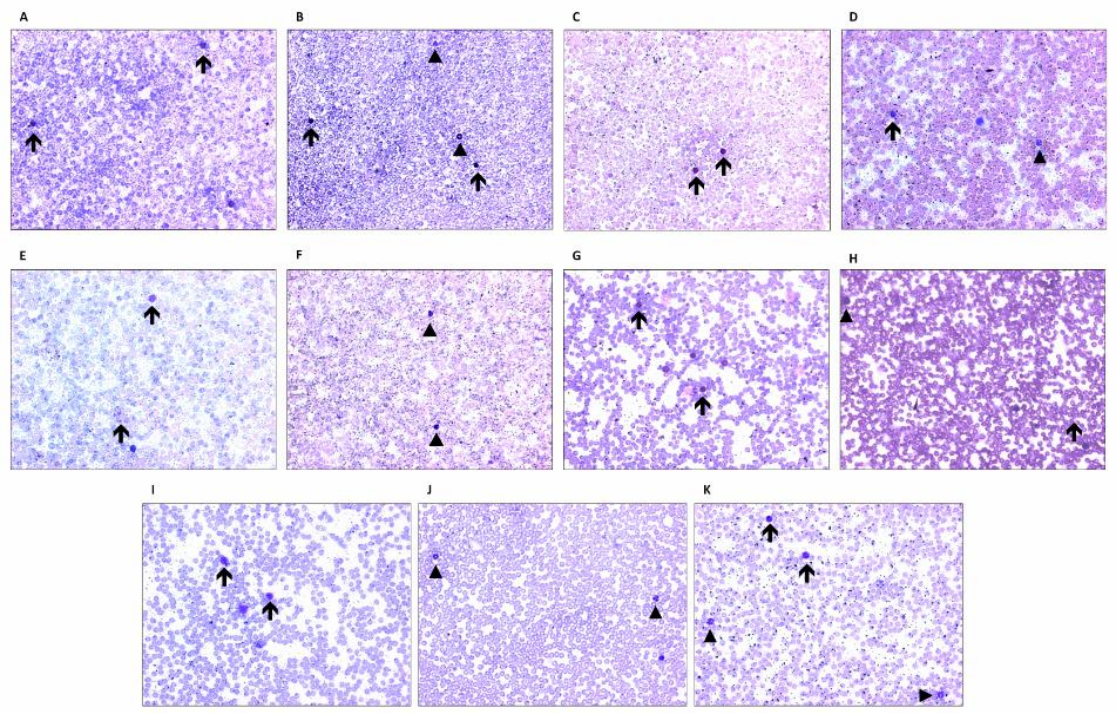
*****Mineral mix: Iron 50 mg; zinc 60 mg; copper 10 mg; iodine 2 mg; manganese 60 mg; selenium 0.05 mg; cobalt 1.50 mg.

Supplementary Table 2. Reverse and forward primers

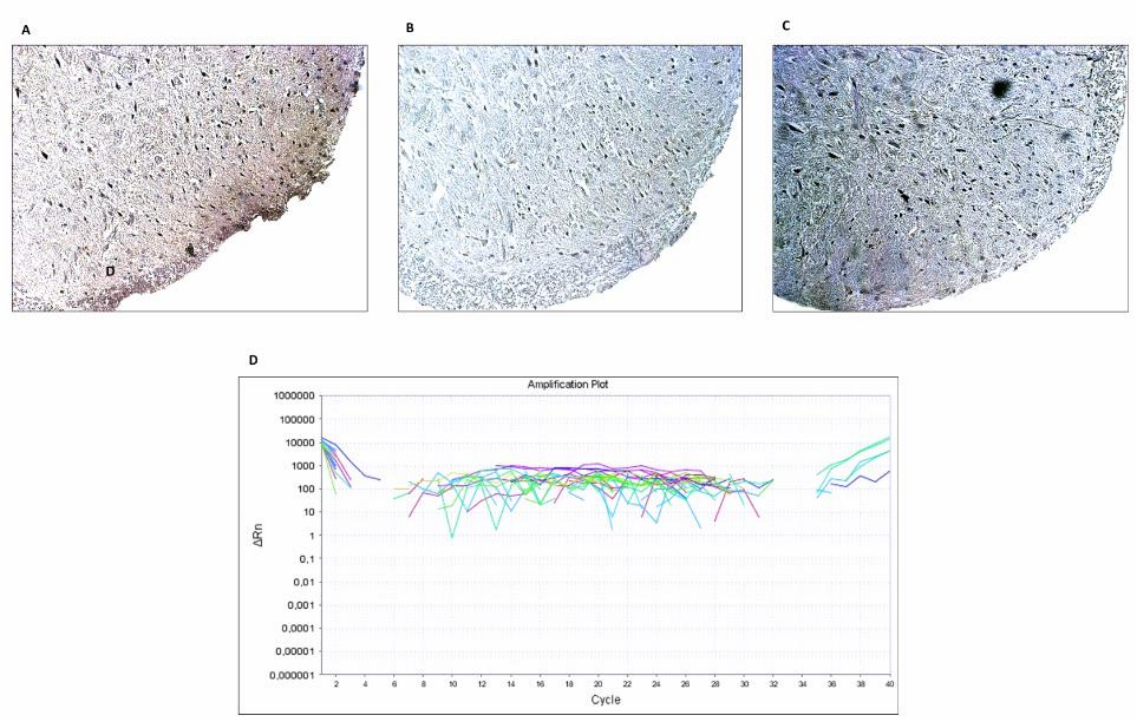
Gene	Primer sequences (5'-3')	Accession number (mRNA)	Amplicon size (bp)
*Hprt1**	F-CTCATGGACTGATTATGGACAGGAC R-GCAGGTCAGCAAAGAACTTATAGCC	NM_013556	123
*Tbp**	F-CCGTGAATCTTGGCTGTAAACTTG R-GTTGTCCGTGGCTCTCTTATTCTC	NM_013684	118
*Ppia**	F-TATCTGCACTGCCAAGACTGAATG R-CTTCTTGCTGGTCTTGCCATTCC	NM_008907	127
*Ffar-1***	F-GCTCAGGCCTGAGCCACAAACG R-AGTACCACACTCCAGGCCCCTGTG	NM_194057.2	181

* According to Pernot et al. (2010).

** Designed by authors.



Supplementary Fig. 1. Representative images of the total blood cell counts. (A) 10 % corn oil plus saline; (B) 10 % corn oil plus CYP; (C) 10 % fish oil plus saline; (D) 10 % fish oil plus CYP; (E) 20 % corn oil plus saline; (F) 20 % corn oil plus CYP; (G) 20 % fish oil plus saline; (H) 20 % corn oil plus CYP; (I) saline i.p. ; (J) CYP i.p. plus Saline i.p.; (K) CYP i.p. plus DHA i.p. Head arrows indicated the neutrophils, and arrows indicated the lymphocytes.



Supplementary Fig. 2. Immunohistochemistry for microglial activation, and RT-qPCR for GPR40/FFAR1. Representative images of immunohistochemistry analysis showing the absence of positive immunolabelling for Iba1/AIF1 in the right dorsal horn (RDH) of the lumbar spinal cord in: (A) saline-treated negative group; (B) CYP-treated group; (C) CYP plus DHA group. (D) Amplification plot of the expression of GPR40/FFAR1 receptor for mouse spinal cord samples from different experimental groups.

ANEXO C - COMPROVANTE DE SUBMISSÃO DO ARTIGO

Dear Dr. Raquel Freitas,

You have been listed as a Co-Author of the following submission:

Journal: Journal of Nutritional Biochemistry
Corresponding Author: Maria Martha Campos
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