Universidad Autónoma del Estado de México
Facultad de Enfermería y Obstetricia

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“Suplementación con ácidos grasos EPA y DHA extraídos a partir de microalgas y su efecto sobre los marcadores de estrés oxidante e inflamación en ratones db/db”

TESIS

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Presenta:
M. en C. S. Laura Elisa Gutiérrez Pliego

Comité Tutorial:
Tutor Académico: Dra. en C. Roxana Valdés Ramos
Tutor Interno: Dra. en I. M. Beatriz Martínez Carrillo
Tutor externo: Dr. En C. B. C Aldo Arturo Reséndiz Albor

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Resumen

La diabetes mellitus (DM) es una enfermedad crónica no transmisible de etiopatogenia multifactorial que se caracteriza por un defecto en la secreción de insulina, en su acción o ambos. Esta condición favorece la formación de productos finales de la glicación avanzada (AGES); considerados los principales causantes de las complicaciones de la diabetes, uniéndose a sus receptores en la célula, desencadenando procesos celulares que incluyen el aumento de estrés oxidante y de la producción de citosinas proinflamatorias. Una de las estrategias para combatir o minimizar los daños ocasionados por los estados de oxidación e inflamación en la diabetes, es el aumento en el consumo de ácidos grasos omega-3 específicamente EPA y DHA. Hasta ahora las fuentes dietéticas principales de EPA y DHA son de origen marino: peces, crustáceos, mariscos y algunos mamíferos. Por lo tanto, la búsqueda de fuentes adecuadas, sustentables y de bajo costo de estos ácidos grasos representa un desafío en nutrición y en el desarrollo biotecnológico. Recientemente se ha planteado el uso de microalgas como fuente sustentable y renovable de ácidos grasos omega 3 específicamente EPA y DHA.

El objetivo del presente proyecto es evaluar el efecto de la suplementación con ácidos grasos EPA y DHA extraídos a partir de microalgas sobre los marcadores de estrés oxidante e inflamación en ratones db/db. Para ello, se realizó un estudio analítico experimental aleatorizado. Se utilizaron 60 ratones macho de 8 semanas de edad, de dos cepas: 30 ratones db/db y 30 ratones CD1. Para ambas cepas se formó un grupo inicial (BL) y 4 grupos de estudio (n=6): i) Control (RC); ii) Croqueta con EPA+DHA (MD); iii) Liofilizado de ácidos grasos (LY) y iv) Grasa Saturada (CO). El grupo LY fue suplementado con liofilizado de EPA y DHA extraídos de microalgas a una dosis diaria de 1mg/g de peso diluido en agua destilada y administrado con pipeta por vía. El grupo MD fue alimentado con una formulación de croquetas igual a las del consumo de los otros grupos de estudio a excepción en su contenido de EPA y DHA (2.0% vs. 0.2%) proveniente de microalgas. El grupo CO fue suplementado con aceite de coco como fuente de grasa saturada a una dosis diaria de 1 mg/g de peso. La suplementación se llevó a cabo por 8 semanas, a partir de la 8ª semana de vida hasta la 16ª semana de vida.

Al final de la 16ª semana de vida para los grupos CL, CR, LI, GSAT y 8ª semana de vida del grupo BL, se procedió al sacrificio del animal. Posteriormente se obtuvieron: 1) Sangre total para citometría de linfocitos frescos para la fenotipificación y determinación de citocinas pro y antiinflamatorias. 2) Plasma para la determinación de Insulina, Péptido C, Glucagon, Adiponectina, Resistina y Leptina, así como marcadores de estrés oxidante (MDA y capacidad antioxidante). Del presente trabajo se obtuvieron como resultado la publicación de dos artículos científicos.

Introducción

La diabetes mellitus (DM) es una enfermedad crónica no transmisible de etiopatogenia multifactorial que se caracteriza por un defecto en la secreción de insulina, en su acción o ambos. Esta condición favorece la formación de productos finales de la glicación avanzada (AGES); considerados los principales causantes de las complicaciones de la diabetes, uniéndose a sus receptores en la célula, desencadenando procesos celulares que incluyen el aumento de estrés oxidante y de la producción de citosinas proinflamatorias. Una de las estrategias para combatir o minimizar los daños ocasionados por los estados de oxidación e inflamación en la diabetes, es el aumento en el consumo de ácidos grasos omega-3 específicamente EPA y DHA. Hasta ahora las fuentes dietéticas principales de EPA y DHA son de origen marino: peces, crustáceos, mariscos y algunos mamíferos. Por lo tanto, la búsqueda de fuentes adecuadas, sustentables y de bajo costo de estos ácidos grasos representa un desafío en nutrición y en el desarrollo biotecnológico. Recientemente se ha planteado el uso de microalgas como fuente sustentable y renovable de ácidos grasos omega 3 específicamente EPA y DHA.

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1. Antecedentes

1.1 Diabetes mellitus

La diabetes mellitus es una enfermedad crónica no transmisible de origen multifactorial que se caracteriza por estados de hiperglucemia como resultado de defectos en la secreción de insulina, su acción o ambas circunstancias[1]. En México, según datos de la Encuesta Nacional de Salud y Nutrición 2016 Medio Camino (ENSANUT 2016 MC), el 9.4% de la población adulta ha recibido un diagnóstico de diabetes[2] y representa la primer causa de complicaciones de salud como infarto al corazón, ceguera, falla renal, amputaciones y muerte prematura[3].

La mayoría de los casos de diabetes en el mundo se agrupan en dos categorías: Por un lado, la diabetes tipo 1 cuya causa es una deficiencia en la secreción de insulina como resultado de la destrucción autoinmune de las células β pancreáticas y que ocurre en 5-10% de los casos[1]. Por otro lado, la diabetes tipo 2, cuya causa es una combinación entre la resistencia a la acción de la insulina por parte de las células y una inadecuada respuesta compensatoria de secreción de la misma. Ésta última categoría abarca el 90-95% de los casos de diabetes y se encuentra asociada a estados de sobrepeso, obesidad y elevado porcentaje de grasa corporal a nivel central[4]. Según los criterios de la Organización Mundial de la Salud (OMS), el diagnóstico de diabetes sobreviene cuando las concentraciones de glucosa en ayunas superan los 126.11 mg/dl (7.0 mmol/L)[5], sin embargo, se sabe que el deterioro en la tolerancia a la glucosa es progresivo e inicia aproximadamente 7 años antes de ser diagnosticado.

1.1.1 Fisiopatología de la diabetes mellitus

Los islotes pancreáticos están constituidos por cuatro tipos de células β, α, δ, y PP o F, los cuales sintetizan y liberan hormonas como insulina, glucagon, somatostatina y el polipéptido pancreático, respectivamente[6]. La liberación de insulina ocurre como respuesta al aumento de glucemia como consecuencia al consumo de alimentos[7].

Tras un aumento de glucosa circulante, los transportadores de glucosa GLUT2, que se encuentran en células de hígado, riñón y β pancreáticas, permiten el ingreso de glucosa a éstas células; a su vez, el GLUT2 en las células β pancreáticas regulan la secreción de

insulina; es decir, sólo permiten el transporte de glucosa cuando las concentraciones plasmáticas alcanzan los 70 mg/dL[6]. Cuando la insulina se une a su receptor en las células del músculo, se inician las vías de señalización que permiten la translocación del transportador GLUT4 localizado en vesículas hacia la membrana plasmática para permitir la transportación de la glucosa en sangre al interior de la célula y ser utilizada como fuente de energía[8].

Cuando hay alteraciones en la captación de glucosa por parte de los tejidos específicamente hígado, músculo y tejido adiposo se denomina resistencia a la insulina. Lo anterior estimula la producción pancreática de insulina (hiperinsulinemia) pero cuando no se puede contrarrestar la insulinorresistencia, aparece la hiperglucemia. Si la hiperglucemia se mantiene, se produce glicolipotoxicidad sobre la célula β, lo que afecta a la secreción de insulina y aumenta el estado de insulinorresistencia favoreciendo la evolución de la diabetes[9].

1.1.1.1. Productos finales de glicación avanzada (AGEs)

La hiperglucemia crónica es la principal causa de complicaciones a largo plazo en la diabetes[10]. La consecuencia más importante de la hiperglucemia crónica es la unión excesiva de un grupo carbonilo del azúcar al grupo amino de las proteínas, mejor conocido como glucosilación no enzimática o reacción de Maillard, cuyo producto es un compuesto poco estable llamado base de Schiff. Si no hay disminución en las concentraciones de glucosa, entonces estas bases se transforman en compuestos más estables llamados productos de Amadori, este proceso ocurre en el transcurso de varios días. Después, a través de algunos procesos de deshidratación, condensación y oxidación (semanas o meses) se forma un grupo de productos fluorescentes irreversibles llamados productos finales de glicación avanzada (AGEs por sus siglas en inglés) (Fig.1)[11].

Estos productos se acumulan en el interior de las células que no dependen de insulina como en el cristalino y los nervios, pero también se acumulan de manera extracelular uniéndose a proteínas de membranas plasmáticas, circulantes y estructurales. Un ejemplo claro de esto es la formación de hemoglobina glucosada dentro de los eritrocitos[12].

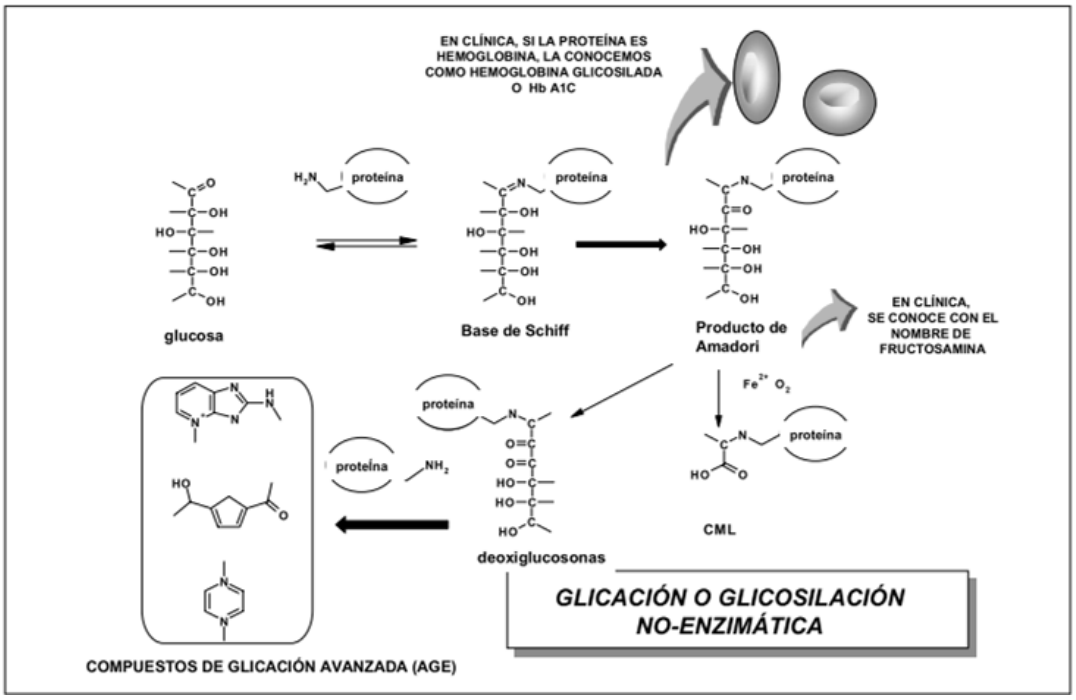

Fig. 1. Glicación no enzimática[11]

Aunque los principales sustratos de los AGEs son las proteínas, también puede ocurrir la glicación con otras moléculas como los lípidos y ácidos nucleicos[13]. La unión de los AGEs a sus receptores en las células permite su degradación[14] y dependiendo de la célula, ésta unión se asocia a quimiotaxis, angiogénesis, estrés oxidante e inflamación[15].

1.1.2 Diabetes mellitus y estrés oxidante

Los radicales son especies químicas con un electrón desapareado en su orbital más externo, lo que le confiere inestabilidad y por tanto una alta capacidad reactiva con otras moléculas[16]. Estas especies se producen de manera normal como parte de las reacciones orgánicas de oxidación y reducción permitiendo procesos fisiológicos como la activación de genes y enzimas, síntesis de prostaglandinas y fagocitosis entre otros. Los radicales del oxígeno, así como sus precursores o moléculas intermedias se denominan especies reactivas de oxigeno o (EROS)[17].

Cuando se produce un aumento de EROS, el riesgo de dañar diversas macromoléculas y por tanto su función se ve incrementada; ante esta situación existen mecanismos que en conjunto se conocen como sistema antioxidante. Sin embargo, cuando la capacidad antioxidante se ve sobrepasada por la producción de EROS, se desencadena un estado llamado estrés oxidante[18].

Durante la formación de AGEs, es decir en todas las reacciones de glicación no enzimática, se generan diferentes tipos de EROS[19]; también, la unión de los AGEs a sus receptores en las células promueven la producción intracelular de EROS y contribuye a disminuir las concentraciones de antioxidantes[18]. La hiperglucemia también conduce a un aumento de las vías de auto-oxidación y de la cadena respiratoria que conllevan a la producción del radical $O_2\bullet^-$. El aumento de EROS genera un aumento en la actividad de la proteína cinasa C (PKC), activando señales intracelulares de estrés metabólico y oxidativo, lo que finalmente contribuye a incrementar la actividad de las NADPH y NADH oxidasas[20]. El incremento de la actividad de estas enzimas produce un aumento adicional de O_2 dando por resultado un aumento en el radical peroxinitrito ($ONOO^-$) que ocasiona daño oxidativo a lípidos, proteínas y DNA. La suma de éstos procesos da como resultado la generación de daños vasculares, ateroesclerosis, nefropatías, neuropatías y retinopatías[20].

1.1.3. Diabetes mellitus e inflamación

El aumento del estrés oxidante *per se*, favorece el estado de inflamación crónica de bajo grado en diabetes. Como se mencionó anteriormente, la unión de AGEs a su receptor activa el factor de transcripción nuclear NF-Kb, que deriva en la activación de diversos genes involucrados en la inflamación, inmunidad y ateroesclerosis, entre ellas el TNF-α y β, interleucinas 1, 6 y 8 (IL-1, IL-6, IL-8) interferón-γ y moléculas de adhesión celular (Fig.2)[21–23].

La activación de los mecanismos efectores inflamatorios implica múltiples vías de transducción de señal intracelular incluyendo las vías p21ras, MAP cinasas, P13 cinasas, Jack/STAT, NADPH oxidasa[22–25].

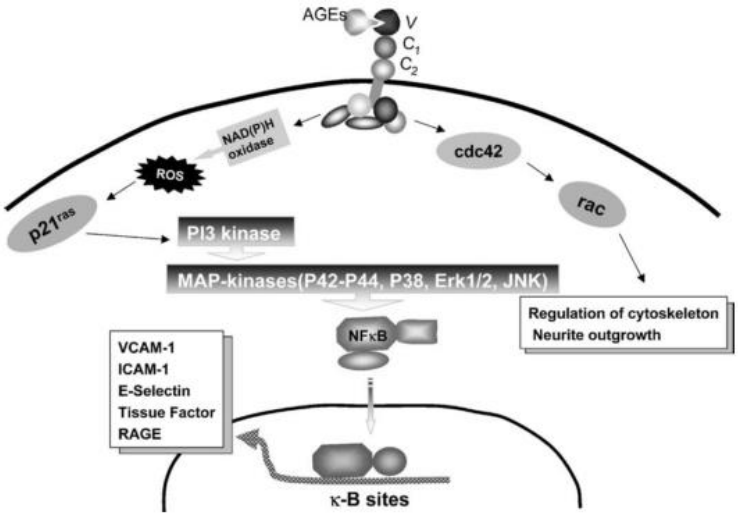

Fig. 2 Activación de NF-kB por la activación de receptores de AGEs [57]

1.2 Ácidos grasos

Las grasas, al igual que los hidratos de carbono, contienen carbono, hidrógeno y oxígeno; son compuestos insolubles en agua y solubles en solventes químicos. Con el término lípido, se incluyen todas las grasas (sólidos) o aceites (líquidos) comestibles y que están presentes en la alimentación humana[26].

Para una persona adulta, el consumo de grasas y aceites en la dieta no debe exceder el 35% de la ingestión diaria de energía[27]. La digestión de las grasas da inicio gracias a la lipasa lingual en la boca; al llegar al estómago, la emulsión formada pasa por el píloro al duodeno donde los ácidos biliares y la lipasa pancreática favorecen una microemulsión transformando todos los triacilglicéridos en ácidos grasos libres y 2-monoacilglicéridos. Además, el jugo pancreático contiene otras enzimas que hidrolizan otros lípidos de la dieta como fosfolípidos y ésteres de colesterol. Todos los productos de la digestión forman agregados moleculares que atraviesan la membrana de los enterocitos de la mucosa intestinal. En el citoplasma de los enterocitos, se da la reconversión de los ácidos grasos y 2-monogliéridos en triacilglicéridos. A éstos lípidos resintetizados se les incorpora una proteína específica, apolipoproteína B-48 hasta formar quilomicrones. Éstos se segregan a los capilares linfáticos del plexo mesentérico para pasar a los vasos linfáticos y finalmente al

torrente sanguíneo. En sangre, en los quilomicrones se produce la lipolisis de los triacilglicéridos liberando ácidos grasos para su metabolismo[26,28].

Los ácidos grasos son nutrimentos esenciales que junto con las proteínas e hidratos de carbono constituyen la base de la dieta humana[29]. Tienen una estructura generalmente linear conformada por un grupo carboxilo (HOOC-) en un extremo y en el otro un grupo metilo (CH_3-), mientras que el resto de la molécula es una cadena hidrocarbonada[26,28]. De acuerdo a la longitud de su cadena pueden clasificarse en: ácidos grasos de cadena corta (4-6 carbonos), de cadena media (8-12 carbonos), de cadena larga (14-18 carbonos) y de cadena muy larga (20 o más carbonos)[28].

Por otra parte, según la presencia de dobles enlaces en la molécula, estos pueden ser ácidos grasos saturados (AGS) cuando no presentan dobles enlaces, e insaturados cuando presentan dobles enlaces. Los ácidos grasos insaturados se dividen en: monoinsaturados (AGM) por tener un doble enlace y poliinsaturados (AGPI) al presentar dos o más dobles enlaces en su estructura[26,30].

La mayoría de los alimentos de origen animal terrestre (carnes, mantequillas, huevo) contienen ácidos grasos saturados; por otra parte, las grasas provenientes de fuentes vegetales y el pescado tienen mayor contenido de ácidos grasos insaturados, específicamente poliinsaturados, sin embargo, existen algunas excepciones como el aceite de coco que contiene una gran cantidad de ácidos grasos saturados. El consumo excesivo de grasas saturadas tiene implicaciones en el desarrollo de enfermedades crónico-degenerativas y cardiovasculares; en contraste, los ácidos grasos poliinsaturados ejercen una función protectora[26].

La nomenclatura para los ácidos grasos indica el número de átomos de carbono en la cadena, el número de dobles enlaces y la posición del primer doble enlace a partir del grupo metilo; por ejemplo 18:2n-6 indica que es un ácido graso conformado por 18 carbonos, y dos dobles enlaces, empezando el primero en el 6º carbono a partir del extremo N-terminal[31]

1.2.1 Ácidos grasos poliinsaturados (AGPI)

En las paredes internas de los capilares sanguíneos la enzima lipoproteína lipasa (LPL) se encarga de hidrolizar los triacilglicéridos presentes en las lipoproteínas de los quilomicrones, liberando así ácidos grasos incluyendo AGPIs[32] Los AGPI libres se incorporan en los triacilglicéridos del tejido adiposo e inhiben la expresión génica de enzimas involucradas en la lipogénesis; en el músculo incrementan la oxidación de ácidos grasos y reducen la acumulación de triacilglicéridos[33], en la glándula mamaria se utilizan para la síntesis de lípidos de la leche,[34] y en el hígado suprimen la síntesis de lípidos y estimulan la oxidación de ácidos grasos[35]. Otra función de los AGPI es controlar la composición de las membranas, por tanto, su actividad enzimática, la unión entre moléculas y sus receptores, la interacción intracelular y el transporte de nutrimentos[36]. Además, son necesarios para la síntesis de importantes fosfolípidos de membrana y mensajeros intracelulares (prostaglandinas)[30].

Dentro de los AGPI, existen dos que el organismo no puede sintetizar y por tanto deben obtenerse de la dieta: el ácido linoleico (AL) y alfa-linolénico (ALA). Este tipo de ácidos grasos son considerados como esenciales y su indispensabilidad se debe a que los mamíferos carecen de enzimas necesarias para insertar dobles enlaces en los átomos de carbono que están más allá del carbono 9 a partir del carboxilo terminal. Éstas dos familias pertenecen a la familia n-6 o n-3 también conocidos como ω-6 y ω-3 respectivamente, indicando la posición del primer doble enlace[32].

En el hígado, el AL y ALA pueden formar AGPI de cadena larga (AGPI-CL) mediante procesos de elongación y desaturación dependientes de malonil coenzima A (CoA). El AL puede convertirse a ácido araquidónico (AA) que es el principal producto de la familia de los ácidos grasos n-6 y el ALA puede producir ácido eicosapentaenoico (EPA) y ácido docosahexaenoico (DHA) como principales productos de la familia de los ácidos grasos n-3[37].

1.2.1.1 Ácidos grasos omega-3

El ALA es el precursor de los ácidos grasos de la familia n-3 y la conversión de éste en sus derivados permite que se incorporen rápidamente en los fosfolípidos formando parte de las membranas[28]. Mediante la vía enzimática de elongación y desaturación, ALA produce EPA y DHA, (Fig.3); sin embargo, algunos estudios sugieren que ésta ruta tiene una capacidad de producción limitada, puesto que el 7% aproximadamente de ALA es convertido a EPA y solo el 0.013% a DHA[38]. Las fuentes dietéticas principales de ALA son los aceites de plantas y semillas; por otra parte, los ácidos grasos EPA y DHA abundan en los pescados, mariscos y aceites marinos[36].

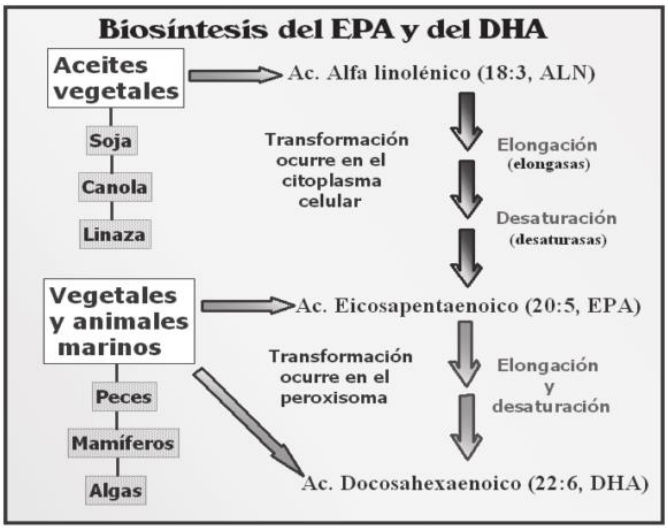

Fig. 3 Biosíntesis de EPA y DHA[56]

El EPA y DHA tienen efectos directos sobre la capacidad de adhesión de proteínas a las membranas y la expresión de moléculas de histocompatibilidad y de proteínas intracelulares básicas para el crecimiento y la división de las células[28].

También tienen efectos relacionados con el tejido adiposo y antiinflamatorios. Dentro de los efectos relacionados al tejido adiposo, el EPA y DHA activan la AMPK (cinasa activada por monofosfato de adenina), que dentro de sus funciones está promover la β-oxidación de los ácidos grasos en tejido adiposo[39] (Fig.4); también, aumentan el metabolismo energético debido a que promueven la biogénesis mitocondrial[40]. En el hígado, el EPA y DHA aumentan la oxidación de ácidos grasos. Debido a estos efectos lipooxidantes

y de disminución de lipogénesis, éstos ácidos grasos se consideran coadyuvantes en el tratamiento del sobrepeso y obesidad[41,42].

Dentro de los efectos antiinflamatorios, el EPA y el DHA disminuyen la resistencia a la insulina y mejoran el estado de inflamación tisular debido a un aumento de las adipocinas anti-inflamatorias como adiponectina y una disminución de citocinas pro-inflamatorias como el factor de necrosis tumoral alfa (TNFα), la interleucina-6 (Il-6), la proteína quimioatrayente de monocitos-1 (MCP-1) y el inhibidor del activador de plasminógeno-1 (PAI-1)[40].

Otros efectos anti-inflamatorios incluyen la inhibición del aumento de eicosanoides pro-inflamatorios mediados por el AA. Además, el EPA y el DHA tienen efectos en la activación de los PPARγ y los receptores acoplados a proteínas G (GRP-120); cuyo efecto en macrófagos y células madre de tejido adiposo es la inhibición de la vía de NF-kB (Fig. 4) [43,44]. Diversos estudios también han demostrado su efecto en el metabolismo de lipoproteínas, en la función plaquetaria, endotelial y vascular[45], en la función inflamatoria[46] y en la coagulación[47], por lo tanto disminuyen la incidencia de enfermedades cardiovasculares y constituyen precursores de eicosanoides como prostaglandinas y leucotrienos[36].

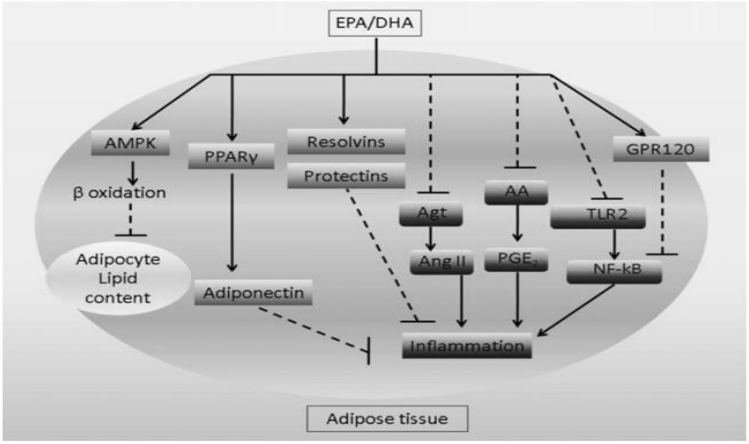

Fig.4. Mecanismos anti-inflamatorios de EPA y DHA[58]

1.3 EPA y DHA extraídos a partir de microalgas

Tradicionalmente, las fuentes más comunes de ácidos grasos omega-3 han sido los aceites marinos por su alto contenido de EPA y DHA (25 a 30%)[48] y la harina de pescado (8% omega-3)[49]. Sin embargo, una desventaja de éstos productos, son algunos efectos nutricionales y organolépticos no deseados, como la oxidación (debido a su alta poliinsaturación) y el olor característico del producto[50].

En los últimos años, las fuentes marinas se han visto disminuidas debido a que la captura de peces ha superado los niveles máximos sustentables[51]. Otra desventaja del uso de aceites marinos, es el riesgo de contaminación con metales pesados y pesticidas, cuya solución puede ser la refinación del aceite, pero éste proceso implica un costo mayor en su producción[52].

Una alternativa al consumo de fuentes marinas, es el aumento del consumo de alimentos ricos en ALA, sin embargo, es conocido que la conversión de ALA a EPA y DHA es muy limitada, por lo que siempre existe el riesgo de deficiencia de dichos ácidos grasos[48]. Las microalgas, son un grupo eucariota evolutivamente diverso de organismos fotosintéticos unicelulares y predominantemente acuáticos que han sido recientemente estudiados por su potencial para producir compuestos de alto valor biológico y benéficos para la salud como carotenoides, AGP y AGP de cadena muy larga.[53] Son productoras naturales primarias de ácidos grasos EPA y DHA debido a que poseen la maquinaria biosintética para secuencialmente alternar entre desaturación y elongación en las cadenas carbonadas [52]

Debido a que las microalgas se encuentran en la base de la cadena alimenticia, los peces son los principales consumidores de éstos ácidos grasos, los cuales son incorporados en los lípidos de las membranas y se acumulan en los aceites y carne de muchas especies marinas.[54] Una ventaja importante de ésta fuente, es que la mayoría de las especies de microalgas acumulan sus AGP en los fosfolípidos de las membranas, a diferencia de las fuentes animales que acumulan sus AGP en forma de triacilglicéridos. Este aspecto es sumamente relevante ya que la biodisponibilidad de los AGP omega-3 en el sistema digestivo es mucho mayor cuando se aportan en forma de fosfolípidos.[55]

Por todo esto y el creciente conocimiento de la composición y propiedades de las microalgas, así como de la posibilidad de realizar su cultivo de forma artificial; ha despertado un interés por éstos microorganismos como fuente renovable y sustentable de ácidos grasos EPA y DHA[56].

2. Planteamiento del problema

La diabetes mellitus (DM) es una enfermedad crónica no transmisible de etiopatogenia multifactorial que se caracteriza por un defecto en la secreción de insulina, en su acción o ambos. Esta condición desencadena un aumento de las concentraciones de glucosa en sangre (hiperglucemia) debido a su incapacidad para ser introducida en la célula y ser utilizada como fuente primaria de energía. Esta condición favorece la formación de productos finales de la glicación avanzada (AGES); considerados los principales causantes de las complicaciones de la diabetes. Dichas moléculas actúan uniéndose a sus receptores en la célula, desencadenando procesos celulares que incluyen el aumento de estrés oxidante y de la producción de citosinas proinflamatorias.

Una de las estrategias para combatir o minimizar los daños ocasionados por los estados de oxidación e inflamación en la diabetes, es el aumento en el consumo de ácidos grasos omega-3 específicamente EPA y DHA. Sus efectos biológicos incluyen beneficios en el metabolismo de lipoproteínas, en la función plaquetaria, endotelial y vascular, antioxidante y antiinflamatoria. Las fuentes dietéticas principales de EPA y DHA son de origen marino (vegetales: algas y microalgas, y animales: peces, crustáceos, mariscos y algunos mamíferos). Por lo tanto, la búsqueda de fuentes adecuadas, sustentables y de bajo costo de estos ácidos grasos representa un desafío en nutrición y en el desarrollo biotecnológico.

Recientemente se ha planteado el uso de microalgas como fuente sustentable y renovable de ácidos grasos omega 3 específicamente EPA y DHA, sin embargo, no existen estudios aún que aclaren sus efectos antioxidante y antiinflamatorio como los ya descritos para ácidos grasos de origen animal (pescado)

Por lo anterior surge la siguiente pregunta de investigación: ¿Cuál será el efecto de la suplementación con ácidos grasos EPA y DHA extraídos a partir de microalgas sobre los marcadores de estrés oxidante e inflamación en ratones db/db?

3. Justificación

El aumento de la prevalencia de enfermedades crónico no transmisibles como la diabetes mellitus, representa un desafío para los sistemas de salud e investigación. La búsqueda de estrategias que permitan la disminución de la prevalencia o la limitación de las complicaciones ha derivado en el uso de nuevas tecnologías para el desarrollo e innovación de productos alimenticios de alto valor biológico y que a su vez representen una opción renovable y sustentable.

El uso de microalgas nativas de la región recolectadas y aisladas en México, representan un panorama nuevo para el sector alimenticio y con alta competitividad mundial

Con el estudio de los efectos de ácidos grasos EPA y DHA extraídos a partir de microalgas sobre los marcadores de estrés oxidante e inflamación, se ampliarán los conocimientos sobre la efectividad de estos productos en el tratamiento y prevención de enfermedades crónicas como la diabetes.

4. Hipótesis

Hi: La suplementación con ácidos grasos EPA y DHA extraídos a partir de microalgas reducirá el estrés oxidante y los marcadores de inflamación en ratones db/db.

Ho: La suplementación con ácidos grasos EPA y DHA extraídos a partir de microalgas no reducirá el estrés oxidante ni los marcadores de inflamación en ratones db/db

5. Objetivos: Ggeneral y específicos

5.1 General

- Evaluar el efecto de la suplementación con ácidos grasos EPA y DHA extraídos a partir de microalgas sobre los marcadores de estrés oxidante e inflamación en ratones db/db

5.2 Específicos

- Determinar por medio de citometría de flujo las poblaciones linfocitarias productoras de IL-4, IL-5, IL-6, IL-10, TGF-β, IFN-γ, TNF-α, IL-12 y IL-17 en linfocitos de sangre total.
- Determinar las concentraciones de productos finales de lipoperoxidación mediante el ensayo de TBARS, así como la capacidad total antioxidante.

6. Diseño metodológico

6.1 Diseño de estudio

El presente estudio tiene un diseño analítico experimental aleatorizado

6.2 Muestra

Se utilizaron 60 ratones machos de 8 semanas de edad, de dos cepas diferentes: 30 ratones db/db (BKS.Cg-+Leprdb+LeprdbOlaHsd Harlan®) y 30 ratones CD1 (Crl: CD1 (ICR) Universidad Autónoma del Estado de México). Para ambas cepas se formó un grupo inicial (I) y 4 grupos de estudio (n=6): i) Control (CL); ii) Croqueta con 2.9% EPA+DHA (CR); iii) Liofilizado de ácidos grasos (LI) y iv) Grasa Saturada (GSAT). Los grupos de estudio (CL, CR, LI, GSAT) se trabajaron por duplicado. (Tabla 1)

Muestra: 60 ratones	30 ratones db/db BKS.Cg-+Lepr^{db}+Lepr^{db}OlaHsd Harlan®	Inicial (I) n=6 Control (CL) n=6 Croqueta (CR) n=6 Liofilizado (LI) n=6 Grasa Saturada (GSAT) n=6
	30 ratones CD1 Crl: CD1(ICR) Universidad Autónoma del Estado de México	Inicial (I) n=6 Control (CL) n=6 (x2) Croqueta (CR) n=6 (x2) Liofilizado (LI) n=6 (x2) Grasa Saturada (GSAT) n=6 (x2)

Tabla 1. Distribución de grupos de estudio

6.3 Características de la muestra

Los ratones db/db tienen una mutación en los genes del receptor de leptina que da lugar a un déficit de señalización de dicha hormona adipocitaria cuyas manifestaciones clínicas se presentan de manera bifásica, mostrando primero hiperinsulinemia y después hipoinsulinemia. Algunas características son la elevación de la insulina plasmática a partir

de los 10-14 días de nacidos, hiperglicemia a partir de las 8 semanas, obesidad a partir de la 5 semana con proteinuria y glucosuria, polifagia, poliuria y polidipsia. Los ratones CD1 son la cepa más utilizada en investigación para diversos propósitos, especialmente en estudios oncológicos, teratológicos, inmunológicos toxicológicos y de envejecimiento.

6.4 Cuidado y manejo de animales de experimentación

Los animales se albergaron en el bioterio del Laboratorio de Investigación en Nutrición y Salud en la Facultad de Medicina de la Universidad Autónoma del Estado de México, en jaulas cría ratón de 19 x 29 x 13 cm de acrílico, con ciclos de luz/oscuridad de 12/12 hrs; a temperatura controlada (21 ±1°C), agua y alimento *ad libitum* con registro semanal de ingestión.

6.5 Procedimientos

6.5.1 Suplementación

El grupo CR fue alimentado con una formulación de croquetas igual a las del consumo de los otros grupos de estudio a excepción en su contenido de EPA y DHA (2.0% vs. 0.2%). El grupo LI fue suplementado con liofilizado de EPA y DHA extraídos de microalgas a una dosis diaria de 1mg/g de peso diluido en agua destilada y administrado con pipeta por vía oral en un horario de 8 a 9 de la mañana. El grupo GSAT fue suplementado con aceite de coco como fuente de grasa saturada a una dosis diaria de 1 mg/g de peso. La suplementación se llevó a cabo por 8 semanas, es decir a partir de la 8ª semana de vida hasta la 16ª semana de vida.

6.5.2 Peso, longitud y glucemia

El peso de los ratones se obtuvo con una báscula pesa ratón Triple Beam 700/800 series marca Ohaus® y la longitud se midió desde la nariz hasta el ano utilizando una cinta de fibra de vidrio. Las mediciones se realizaron semanalmente a la misma hora y los resultados se reportaron en gramos (g) y centímetros (cm) respectivamente. La glucosa se determinó semanalmente con un glucómetro Contour TS de Bayer mediante punción en cola.

6.5.3 Obtención y procesamiento de las muestras

Al final de la 16ª semana de vida para los grupos CL, CR, LI, GSAT y 8ª semana de vida del grupo I, se procedió al sacrificio del animal mediante el uso de cámara de éter, considerando las especificaciones técnicas para la producción, cuidado y uso de animales de laboratorio de la Norma Oficial Mexicana NOM-062-ZOO-1999.

Posteriormente se obtuvieron:

1. Sangre total para citometría de linfocitos frescos para la fenotipificación y determinación de citocinas: IL-4, IL-5, IL-6, IL-10, TGF-β, IFN-γ, TNF-α, IL-12 y IL-17.
2. Plasma para la determinación de
    a. Triacilglicéridos, colesterol total, colesterol HDL, colesterol LDL mediante un método colorimétrico ultrasensible (Selectra II®)
    b. Marcadores de estrés oxidante (MDA y capacidad antioxidante) mediante kits comerciales de la casa Merck-Millipore.

6.6 Variables de estudio

Variables independientes: Suplementación

Variables dependientes: Glucemia, Perfil de lípidos, marcadores de estrés oxidante, marcadores de inflamación.

Intervinientes: peso, índice de masa corporal, consumo de alimento, consumo de agua.

Variable	Def. Concept	Def. Operativa	Tipo de variable	Escala de medición	Análisis estadísticos
Variable independiente					
Tratamiento	Conjunto de medidas que se aplican para aliviar o curar una enfermedad	Tipo de ácido graso y presentación al cual el grupo de estudio será sometido: Croqueta 2.0% EPA+DHA (CR) Liofilizado EPA+DHA (LI) Grasa Saturada /aceite de Coco (GSAT)	Categórica	g (CR) mg/g (LI y GSAT)	
Variables dependientes					
Glucemia	Medida de la concentración de glucosa libre en sangre, suero o plasma.	Concentraciones de glucosa en sangre	Cuantitativa	mg/dl	ANOVA de una via
Perfil de lípidos	Prueba que mide concentraciones de grasa en sangre.	Concentraciones de triacilglicéridos, colesterol total, colesterol-HDL, colesterol-LDL en sangre	Cuantitativa	mg/dl	ANOVA de una via
Marcadores de estrés oxidante	Sustancia presente en orina u otros líquidos corporales que puede servir para diagnosticar un estado de estrés oxidante	Proteínas carboniladas en sangre, bazo y Placas de Peyer Ensayo de malondialdehido (MDA) Capacidad antioxidante	Cuantitativa	mg/dl	ANOVA de una via
Marcadores de inflamación	Sustancia presente en orina u otros líquidos corporales que puede indicar un estado de inflamación.	IL-4, IL-5, IL-6, IL-10, TGF-β, IFN-γ, TNF-α, IL-12 y IL-17.	Cuantitativas	mg/dl	ANOVA de una via

6.7 Implicaciones bioéticas

El estudio se llevó a cabo con base en la Norma Oficial Mexicana NOM-062-ZOO-1999: Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio. Además, se contará con previa autorización del Comité de Ética en Investigación de la Facultad de Medicina de la Universidad Autónoma del Estado de México.

6.8 Recolección de datos

Los datos fueron recabados en el programa estadístico SPSS v.23 para Windows

6.9 Análisis estadístico

Se realizó un análisis de medidas de tendencia central y de dispersión.

Para la comparación entre grupos de estudio, se realizaron pruebas de ANOVA de una vía con ajuste de la p por el método de Bonferroni

7. Resultados

7.1 Artículo y/o capítulo de libro aceptado

7.1.1 Título del artículo y/o capítulo de libro aceptado

Effect of Supplementation with n-3 Fatty Acids Extracted from Microalgae on Inflammation Biomarkers from Two Different Strains of Mice

7.1.2 Página frontal del manuscrito

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Research Article

Effect of Supplementation with *n*-3 Fatty Acids Extracted from Microalgae on Inflammation Biomarkers from Two Different Strains of Mice

L. E. Gutiérrez-Pliego,[1] B. E. Martínez-Carrillo,[1] A. A. Reséndiz-Albor,[2] I. M. Arciniega-Martínez,[2] J. A. Escoto-Herrera,[1] C. A. Rosales-Gómez,[1] and R. Valdés-Ramos[1]

[1]*Laboratorio de Investigación en Nutrición, Facultad de Medicina, Universidad Autónoma del Estado de México, Paseo Tollocan y Venustiano Carranza s/n, Col. Universidad, 50180 Toluca, MEX, Mexico*
[2]*Laboratorio de Inmunidad de Mucosas, Sección de Investigación y Posgrado, Escuela Superior de Medicina, Instituto Politécnico Nacional, Av. Plan de San Luis S/N, Colonia Casco de Santo Tomas, Miguel Hidalgo, 11350 Ciudad de México, Mexico*

Correspondence should be addressed to R. Valdés-Ramos; rvaldesr@uaemex.mx

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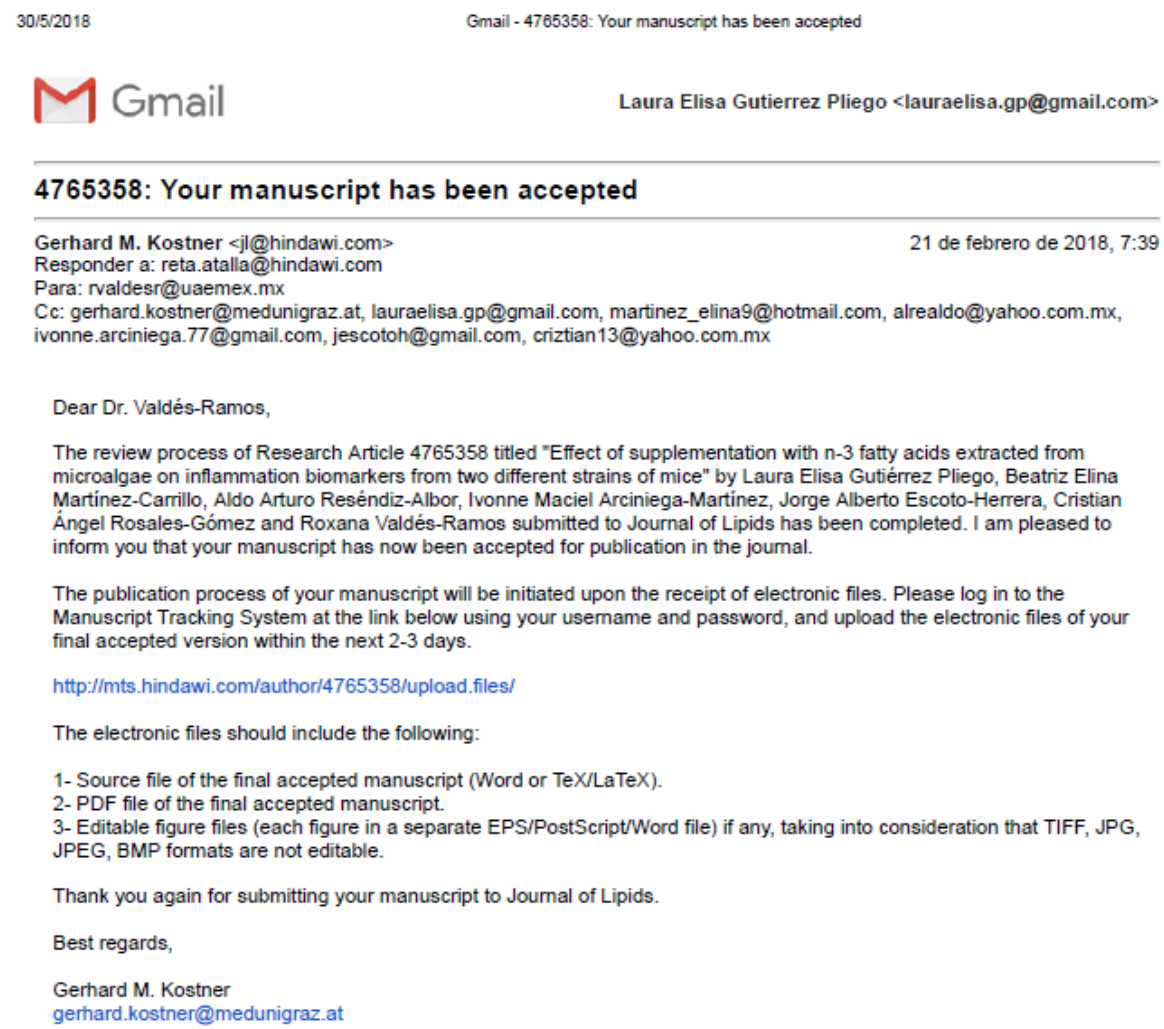
Background. Diabetes mellitus is considered a chronic noncommunicable disease in which inflammation plays a main role in the progression of the disease and it is known that *n*-3 fatty acids have anti-inflammatory properties. One of the most recent approaches is the study of the fatty acids of microalgae as a substitute for fish oil and a source rich in fatty acids EPA and DHA. *Objective.* To analyze the effect of supplementation with *n*-3 fatty acids extracted from microalgae on the inflammatory markers from two different strains of mice. *Methods.* Mice of two strains, db/db and CD1, were supplemented with *n*-3 fatty acids extracted from microalgae in lyophilized form and added to food; the experiment was carried out from week 8 to 16 of life. Flow cytometry was performed to determine the percentage of TCD4+ cells producing Th1 and Th2 cytokines. *Results.* Supplementation with microalgae fatty acids decreased the percentage of TCD4+ cells producing IFN-γ and TNF-α and increased the ones producing IL-17A and IL-12 in both strains; on the other hand, supplementation decreased percentage of TCD4+ cells producing IL-4 and increased the ones producing TGF-β. *Conclusions.* Microalgae *n*-3 fatty acids could be a useful tool in the treatment of diabetes as well as in the prevention of the appearance of health complications caused by inflammatory states.

1. Introduction

Diabetes mellitus is a multifactorial chronic noncommunicable disease, characterized by states of hyperglycemia resulting from defects in insulin secretion, its action, or both [1]. In Mexico, according to the National Health and Nutrition Survey Half Way 2016 (ENSANUT MC 2016 for its acronym in Spanish), 9.4% of the adult population has been diagnosed with diabetes [2]. It also represents the leading cause of negative health outcomes such as heart failure, blindness, kidney failure, amputations, and premature death [3]. The main cause of health complications in diabetes is chronic hyperglycemia, which is associated with changes in immunomodulation and inflammation [4].

The use of *n*-3 polyunsaturated fatty acids as a strategy to minimize damage caused by hyperglycemia has been deeply studied [5]. Its biological effects include benefits on the metabolism of lipoproteins [6], platelet, and endothelial and vascular function [7], as well as antioxidant and anti-inflammatory impact [8]. Evidence suggests that *n*-3 inhibits the proliferation of T lymphocytes in murine models and in humans [9, 10] and inhibits degranulation of cytotoxic T lymphocytes [11]. Thus, it suggests that polyunsaturated fatty acids have potentially immunosuppressive properties. Moreover, the supplementation with EPA (eicosapentaenoic acid) for 12 weeks has been shown to modify the fatty acids composition of the phospholipids of plasma, platelets, neutrophils, monocytes, and T and B lymphocytes [12].

7.1.3 Carta de aceptación



30/5/2018 Gmail - 4765358: Your manuscript has been accepted

Gmail Laura Elisa Gutierrez Pliego <lauraelisa.gp@gmail.com>

4765358: Your manuscript has been accepted

Gerhard M. Kostner <jl@hindawi.com> 21 de febrero de 2018, 7:39
Responder a: reta.atalla@hindawi.com
Para: rvaldesr@uaemex.mx
Cc: gerhard.kostner@medunigraz.at, lauraelisa.gp@gmail.com, martinez_elina9@hotmail.com, alrealdo@yahoo.com.mx, ivonne.arciniega.77@gmail.com, jescotoh@gmail.com, criztian13@yahoo.com.mx

Dear Dr. Valdés-Ramos,

The review process of Research Article 4765358 titled "Effect of supplementation with n-3 fatty acids extracted from microalgae on inflammation biomarkers from two different strains of mice" by Laura Elisa Gutiérrez Pliego, Beatriz Elina Martínez-Carrillo, Aldo Arturo Reséndiz-Albor, Ivonne Maciel Arciniega-Martínez, Jorge Alberto Escoto-Herrera, Cristian Ángel Rosales-Gómez and Roxana Valdés-Ramos submitted to Journal of Lipids has been completed. I am pleased to inform you that your manuscript has now been accepted for publication in the journal.

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1- Source file of the final accepted manuscript (Word or TeX/LaTeX).
2- PDF file of the final accepted manuscript.
3- Editable figure files (each figure in a separate EPS/PostScript/Word file) if any, taking into consideration that TIFF, JPG, JPEG, BMP formats are not editable.

Thank you again for submitting your manuscript to Journal of Lipids.

Best regards,

Gerhard M. Kostner
gerhard.kostner@medunigraz.at

7.1.4 Resumen

Background. Diabetes mellitus is considered a chronic noncommunicable disease in which inflammation plays a main role in the progression of the disease and it is known that n-3 fatty acids have anti-inflammatory properties.One of themost recent approaches is the study of the fatty acids of microalgae as a substitute for fish oil and a source rich in fatty acids EPA and DHA. Objective.To analyze the effect of supplementation with n-3 fatty acids extracted from microalgae on the inflammatory markers from two different strains of mice. Methods. Mice of two strains, db/db and CD1, were supplemented with n-3 fatty acids extracted from microalgae

in lyophilized form and added to food; the experiment was carried out from week 8 to 16 of life. Flow cytometry was performed to determine the percentage of TCD4+ cells producingTh1 andTh2cytokines. Results. Supplementationwith microalgae fatty acids decreased the percentage of TCD4+ cells producing IFN-γ and TNF-α and increased the ones producing IL-17A and IL-12 in both strains; on the other hand, supplementation decreased percentage of TCD4+ cells producing IL-4 and increased the ones producing TGF-β. Conclusions. Microalgae n-3 fatty acids could be a useful tool in the treatment of diabetes as well as in the prevention of the appearance of health complications caused by inflammatory states

7.1.5 Apartados del artículo

Introduction

Diabetes mellitus is a multifactorial chronic noncommunicable disease, characterized by states of hyperglycemia resulting from defects in insulin secretion, its action, or both [1]. In Mexico, according to the National Health and Nutrition Survey Half Way 2016 (ENSANUT MC 2016 for its acronym in Spanish), 9.4% of the adult population has been diagnosed with diabetes [2]. It also represents the leading cause of negative health outcomes such as heart failure, blindness, kidney failure, amputations and premature death [3]. The main cause of health complications in diabetes is chronic hyperglycemia which is associated to changes in immunomodulation and inflammation[4].

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Within their anti- and pro-inflammatory effects, it has been shown that in cell cultures, EPA and DHA (docosahexaenoic acid) have high anti-inflammatory and immunosuppressive effects [13–15]. Same findings have been shown on animal studies supplemented with fish oil [16–18]. It

has been proven that EPA and DHA supplementation decreases pro-inflammatory cytokines such as tumor necrosis factor Alpha (TNF-α), interleukin-6 (IL-6), monocyte-1 chemoattractant protein (MCP-1) and plasminogen activator-1 (PAI-1) inhibitor [19].

The main dietary sources of EPA and DHA fatty acids are fish, shellfish and marine oils [20]. However, some disadvantages of the use of these sources are undesirable nutritional and organoleptic effects, such as oxidation (due to their high polyunsaturation) and the characteristic odor of the product [21]. Another disadvantage of the use of marine oils is the risk of contamination with heavy metals and pesticides, the solution of which may be oil refining, but this process involves a higher cost of production [22]. In addition, in recent years, marine sources have been diminished because fish catch has exceeded the maximum sustainable levels [23].

Microalgae are an evolutionarily microscopic diverse eukaryotic group of unicellular and predominantly aquatic photosynthetic organisms that have recently been studied for their potential to produce compounds of high biological value and beneficial for health such as carotenoids, polyunsaturated fatty acids and very long chain polyunsaturated fatty acids [24]. They are the primary natural producers of EPA and DHA, because they have the biosynthetic machinery to sequentially alternate between desaturation and elongation in their carbon chains [25]. Because microalgae are at the beginning of the food chain, fish are the main consumers of these fatty acids, which is why they are incorporated into the lipids of membranes and accumulated in the fats and meat of many marine species [24]. However, there are less studies that describe their anti-inflammatory effects as those already described for fatty acids of animal origin [26–28].

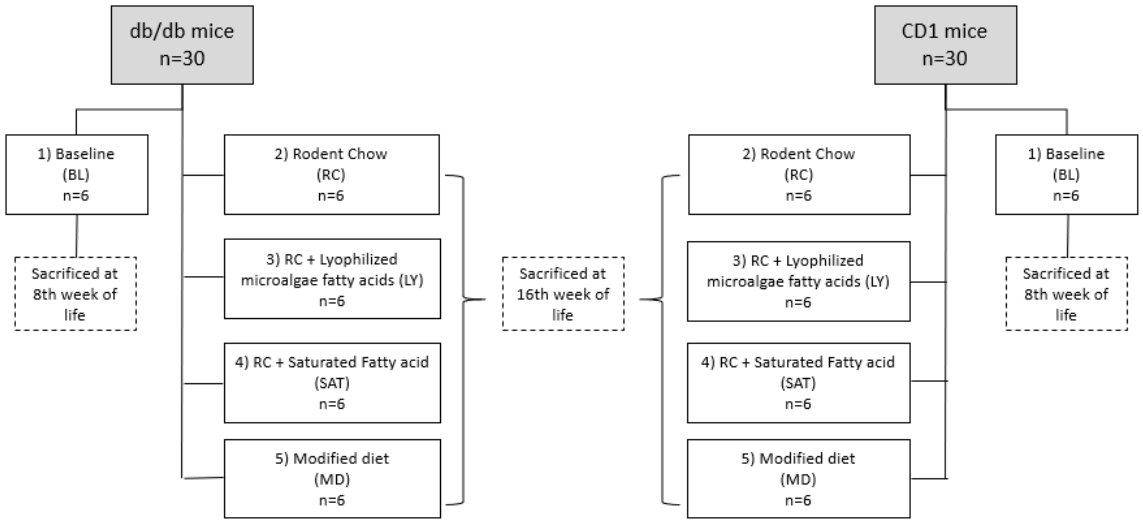
For all the above and the growing study of the composition and properties of microalgae, as well as the possibility of cultivating them in artificial form; interest has aroused in the study of these microorganisms as a renewable source of omega-3 fatty acids. The aim of this study was to analyze the effect of the consumption of omega-3 fatty acids extracted from microalgae (Chlorophyceae and Eustigmatophyceae families) provided either as a supplement or incorporated to diet, on the inflammatory markers from two different strains of mice: db/db mice as a model of obesity and diabetes mellitus in which an inflammation state is expected and CD1 mice as a model of optimal sate of health without inflammation.

Methods

1. Animals and study groups

The present experimental, prospective, controlled, and randomized study was conducted with sixty male mice 8-week old from two different strains: 30 db/db mice (BKS.Cg+Leprdb+ LeprdbOlaHsd Harlan®) and 30 CD1 mice (Crl: CD1 (ICR) belonging to the Faculty of Medicine from the Autonomous University of Mexico State). For each strain, five study groups were formed (n=6): 1) A baseline group (BL) to obtain baseline values; 2) A Rodent Chow group (RC); 3) A RC+Lyophilized microalgae Omega-3 fatty acids (LY); 4) A RC+Saturated fatty acid group (SAT); 5) Modified diet (MD) supplemented with microalgae omega-3 fatty acids. Supplementation was administered from 8th to 16th week of life (Fig 1). The animals were housed in acrylic cages of 19 x 29 x 13 cm, with light / dark cycles of 12/12 h with controlled temperature at 21 ± 1° C. Groups 2, 3, and 4 were fed a standard normal diet (Rodent Laboratory Chow 5001 from Purina [3.02 kcal/g]) and water *ad libitum*. Water consumption (mL/week) and food (g/week) were recorded weekly. Animal care and experimental procedures in rodents were carried out in accordance with the rules of the Internal Regulations for the Use of Laboratory Animals and the Committee of Ethics in Research of the UAEMex, as well as the guidelines of the Ministry of Health and Agriculture of Mexico for the Production and Care of Laboratory Animals (NOM-062- ZOO-1999), Mexico City, Mexico. This protocol was approved by the Ethical Research Committee from the Faculty of Medicine of the UAEMex.

Fig1. Experimental groups for both strains



2. Obtaining of omega-3 fatty acids (EPA and DHA) from microalgae

The microalgae used in this project were native, collected and isolated by BIOMEX SA. de CV. The strains used were from *Chlorophyceae* and *Eustigmatophyceae* families which have a high content of EPA and DHA. The use of these microalgae for such purpose is of recent interest. The process for obtaining the EPA and DHA includes the cultivation of microalgae, separation of biomass, extraction of total lipids, and finally chromatographic procedures for EPA and DHA content determination (25.7% EPA+DHA). EPA and DHA were provided as free fatty acids form.

3. Supplementation

a) LY group was fed with Rodent Chow and supplemented with lyophilizate powder containing EPA+DHA obtained from microalgae. The supplemental dose was 1mg/g of mouse weight, reconstituted in 100µl of distilled water and administered with micropipette by direct oral deposition every day at 8:00 am.

b) SAT group was fed with Rodent Chow and supplemented with coconut oil. The daily dose of coconut oil was 1mg/g of mouse weight administered with micropipette by oral deposition at 8:00am.

c) MD group was fed with a Rodent Chow added with microalgae EPA+DHA for a total content of 2.0% Omega-3 fatty acid which means 10x the original content; Chow was administered ad libitum. (Table 1)

Table 1: Nutrient composition of study groups' diet

RC group		LY group		SAT group		MD group	
Protein, %	23.9	Protein, %	23.9	Protein, %	23.9	Protein, %	23.9
Starch, %	31.9	Starch, %	31.9	Starch, %	31.9	Starch, %	31.9
Glucose, %	0.22	Glucose, %	0.22	Glucose, %	0.22	Glucose, %	0.22
Fiber (Crude), %	5.10	Fiber (Crude), %	5.10	Fiber (Crude), %	5.10	Fiber (Crude), %	5.10
Cholesterol, ppm	200	Cholesterol, ppm	200	Cholesterol, ppm	200	Cholesterol, ppm	200
EPA+DHA, %	**0.2**	**EPA+DHA, %**	**0.2**	**EPA+DHA, %**	**0.2**	**EPA+DHA*, %**	**2.0**
Metabolizable Energy: 3.02 kcal/g		**Metabolizable Energy: 3.02 kcal/g + 0.09 kcal/mg of Lyophilized fatty acids***		**Metabolizable Energy: 3.02 kcal/g + 0.09 kcal/mg of coconut oil**		**Metabolizable Energy: 3.07 kcal/g**	

*Microalgae source

4. Determination of body mass index (BMI) and blood glucose concentration

The BMI and blood glucose concentrations of animals were determined at the 8th and 16th week of life. The formula used for BMI determination was BMI= [weight (g)/ length (cm)2

*100]. Weight was determined using a mouse Triple Beam 700/800 series Ohaus® brand weighing scale and length was determined by measuring the animal from nose to anus. Blood glucose was determined with a Bayer Contour TS glucometer through tail puncture.

5. Collection of biological samples

The BL groups were sacrificed at the 8th week of life and the rest of groups were sacrificed at the 16th week of life. Animals were anesthetized by ether camera, bled by direct cardiac puncture (using a heparinized syringe, obtaining 1mL of blood), and then sacrificed by cervical dislocation. 500µl of the collected blood was used for lymphocyte isolation using

Ficoll-Hypaque Plus (GE Healthcare Bio-Sciences AB, Sweden); lymphocytes where stored with a PBS (Phosphate-buffered saline) solution to obtain a final volume of 1ml for further Flow cytometry.

6. Flow cytometry assays

Cell suspensions of peripheral blood mononuclear cell (PBMC) were adjusted to 1×106 cells/mL in PBS for the cytofluorometric analysis with briefly modifications [29]. i) Surface phenotype of T cells was detected by using fluorescent labeled monoclonal antibodies: anti-CD3 FITC (Cat. No.553063), anti-CD8α PE (Cat. No 553035), anti-CD4 PerCP (Cat. No. 553052) (all from BD Biosciences). Cells were incubated for 30 min at room temperature. Finally, the cells were then washed with PBS and fixed in 1% paraformaldehyde. ii) For the detection of intracellular cytokine production, lymphocytes were stimulated with a mixture containing phorbol myristate acetate, ionomycin and Brefeldin A (Leucocyte Activation Cocktail Kit, BD Pharmingen) and incubated for 4 h at 37°C and 5% CO2. Then, antibodies to cell surface markers, anti-CD4 PerCP were added and incubated as before. For intracellular staining of CD4+ T cells, fixation and permeabilization were performed using Cytofix/Cytoperm Kits (BD Pharmingen) according to the manufacturer's instructions. These cells were incubated with anti-IL-4 PE (Cat. No.554435), anti-IL-5 PE (Cat. No. 554395), anti-IL-6 APC (Cat. No. 561367), anti-IL-10 FITC (Cat. No. 554466), anti-IL-17A FITC (BioLegend Cat. No. 506907), anti-IFN-γ FITC

(Cat. No. 554411), and anti-TNF-α PE antibodies (Cat. No. 554419). For all samples, the expression of CD69 was measured as an activation control. The fluorescent signal intensity was recorded and analyzed by FACS Aria Flow Cytometer (Becton Dickinson). Events were collected from the lymphocyte gate on the FSC/SSC dot plot. 20,000 gated events were acquired from each sample using the CellQuest research software (Becton Dickinson). Data was analyzed using Summit software v4.3 (Dako, Colorado Inc.). Data from six mice per group are reported as the mean ± standard deviation (SD).

7. Statistical analysis

One-way ANOVA was performed for comparison between groups from each strain (BL, RC, LY, SAT and MD), Bonferroni *post hoc* was applied. Differences were considered significant at $p<0.05$. Software used to run statistical analysis was SPSS v.23 for Windows.

Results

BMI was higher in the MD group for diabetic mice and blood glucose was higher in all the db/db groups

In the diabetic db/db mice, the MD group showed a significantly higher BMI than the BL group; blood glucose concentrations were significantly higher in all groups compared to the BL group; the food intake was significantly lower in the MD group and higher in the LY group and finally the water consumption was significantly higher in the MD group, all of this compared with the BL group (Bonferroni *post hoc*, $p <0.001$). In the healthy mice (CD1), there were no significant differences in BMI and blood glucose. On the other hand, the consumption of food was significantly higher in the SAT group and the water consumption was significantly higher in the LY and SAT groups, all this compared with the BL group (Bonferroni *post hoc*, $p <0.001$). (Table 2)

Table 2. Effect of supplementation with EPA and DHA extracted from microalgae on Body Mass Index, blood glucose, food and water consumption in db/db and CD1 mice.

		8 weeks old	16 weeks old			
		BL	RC	MD	LY	SAT
		Mean±SD n=6	Mean±SD n=6	Mean±SD n=6	Mean±SD n=6	Mean±SD n=6
db/db	BMI *g/cm²*	57.3±4.9	61.2±2.5	65±1.7*	62.2±1.5	59.7±2.3
	Glucose *mg/dL*	293.8±131.0	551.8±83.7*	505±74*	525.5±51*	580.7±22*
	Food intake, *g/week*	34.3±2.1	32.9±0.3	27.6±1.0*	37.5±0.8*	36.3±2.4
	Water intake, *mL/week*	64.8±11.8	78.2±7.2	81.4±10.4*	79.6±1.8	67.2±8.3
CD1	BMI *g/cm²*	29.6±2.0	31.4±1.9	32.2±3.1	34.4±4.1	34.2±2.4
	Glucose *mg/dL*	126.2±18.6	119.3±13	127.5±18.7	109.7±13.3	106.8±12.4
	Food intake, *g/week*	54.6±1.7	58.3±5.6	42.7±5	61±1.9	73.3±20.8*
	Water intake, *mL/week*	63±13.1	67.3±3.2	53.9±1.3	89.8±6.1*	93.2±4.8*

Data are presented as means ± standard deviations. One-way ANOVA* for comparison of differences between BL group at 8 weeks vs. all the groups at 16 weeks. P value was significant at<0.05

Microalgae fatty acids modified lymphocyte populations in db/db mice by lowering CD3+ and CD8+ populations and in CD1 mice by lowering CD3+

In the db/db strain, the percentage of CD3+ lymphocytes were significantly higher in all the groups when compared to the BL group (Bonferroni *post hoc*, p <0.001); Regarding CD4+ lymphocytes, the MD group showed a significantly lower percentage and the SAT group a higher percentage Bonferroni *post hoc*, p <0.001). Finally, the CD8 + lymphocytes have a higher percentage in the MD, LY and SAT groups (Bonferroni *post hoc*, p <0.001).

For CD1 strain mice, the percentage of CD3+ lymphocytes were lower in the MD, LY and SAT groups and higher in the RC group, all compared with the BL group (Bonferroni *post hoc*, p <0.001). Regarding CD4 + lymphocytes, the percentage was significant in the MD and SAT groups (Bonferroni *post hoc*, p <0.001). Finally, the percentage of CD8+ lymphocytes was significantly higher in the RC and LY groups, but lower in the MD and SAT groups, all compared with the BL group (Bonferroni *post hoc*, p <0.001). (Table3)

Table 3. Effect of supplementation with EPA and DHA extracted from microalgae in lymphocytes populations in db/db and CD1 mice.

		8 weeks old	16 weeks old			
		BL	RC	MD	LY	SAT
		Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
		n=6	n=6	n=6	n=6	n=6
db/db	CD3+, %	71.8±0.5	70.4±0.8*	69.8±0.4*	70.7±0.4*	68.3±0.2*
	CD4+, %	62.0±0.2	62.1±0.5	60.6±0.3*	61.8±0.9	65±0.5*
	CD8+, %	30.0±1.0	29.3±1.0	24.9±0.3*	28.4±0.2*	23±0.1*
cd1	CD3+, %	74.7±0.02	77.8±0.04*	70.6±0.03*	72.4±0.01*	69.5±0.06*
	CD4+, %	67.4±0.2	67.5±0.05	70±0.04*	67.6±0.03	72.3±0.4*
	CD8+, %	21.4±0.05	24.8±0.01*	17.4±0.1*	24.2±0.03*	19.9±0.02*

Data are presented as means ± standard deviations. One-way ANOVA* for comparison of differences between BL group at 8 weeks vs. all the groups at 16 weeks. P value was significant at <0.05

Supplementation with Microalgae fatty acids increases IL-17A, IL-12, IL-4, IL-6, IL-10 and TGF-β but decreases IFN-γ, TNF- α and IL-5 in diabetic mice.

In the diabetic mice, the behavior of Th1 type cytokines was the same for all study groups; the proportion of TCD4+ cells producing IFN-γ and TNF-α were significantly lower in all groups

compared to the BL (Bonferroni *post hoc*, p <0.001). On the contrary, the percentage of TCD4+ cells producing IL-12 and IL-17A was significantly lower in all groups (Bonferroni *post hoc*, p <0.001). As for the Th2 type cytokines, the percentage of TCD4+ cells producing IL-4 was higher in the RC, MD and SAT groups and, on the other hand, it was lower in the LY group. The percentage of TCD4+ cells producing IL-5 was significantly lower in all groups compared to the baseline group (Bonferroni *post hoc*, p <0.001). For all groups, the percentage of TCD4+ cells producing IL-6 and IL-10 was significantly lower compared to the initial group (Bonferroni *post hoc*, p <0.001). Finally, the percentage of TCD4+ cells producing TGF-β was significantly lower in the RC group and higher in the MD, LY and SAT groups. (Table 4)

Table 4: Effect of supplementation with EPA and DHA fatty acids extracted from microalgae on Th1 and TH2 cytokines in db/db

db/db mice		8 weeks old BL Mean±SD n=6	16 weeks old RC Mean±SD n=6	MD Mean±SD n=6	LY Mean±SD n=6	SAT Mean±SD n=6
Th1 %TCD4+/	IFN-γ	22.5±0.4	11.5±0.4*	7.2±0.5*	2.1±0.4*	15.7±0.4*
	TNF-α	10.2±0.4	7.7±0.4*	8.8±0.4*	2.4±0.4*	1.4±0.4*
	IL-12	1.2±0.1	12.4±0.5*	10.2±0.5*	6.8±0.1*	3.6±0.5*
	IL-17A	1.4±0.1	4.2±0.3*	3.7±0.1*	11.4±0.3*	11.4±0.3*
Th2 %TCD4+/	IL-4	1.8±0.08	8.2±0.3*	6.6±0.3*	1.1±0.1*	10.3±0.2*
	IL-5	19.9±0.8	9.4±0.4*	7.5±0.6*	2.1±0.2*	6.5±0.2*
	IL-6	1.5±0.1	3.1±0.4*	4.5±0.2*	4.0±0.1*	14.8±0.2*
	IL-10	1.44±0.2	16.0±1.3*	7.3±0.2*	2.7±0.08*	14.5±0.2*
	TGF-β	3.2±0.4	2.1±0.05*	5.5±0.1*	4.9±0.09*	7.1±0.1*

Data are presented as means ± standard deviations of percentage of TCD4+ cells producing cytokines. One-way ANOVA* for comparison of differences between BL group at 8 weeks vs. all the groups at 16 weeks. P value was significant at <0.05

Supplementation with Microalgae fatty acids Lyophilized increases IL-17A but decreases IFN-γ, TNF- α, IL-12, IL-4 and IL-6 in healthy mice.

In CD1 mice, for Th1 type cytokines, the percentage of TCD4+ cells producing IFN-γ and TNF-α was significantly lower in the RC and LY groups and significantly higher in the SAT group (Bonferroni *post hoc*, p <0.001). The percentage of TCD4+ cells producing IL-12 was significantly lower in the RC, MD and LY groups and higher in the SAT group, all compared with the BL group (Bonferroni *post hoc*, p<0.001); finally, the percentage of TCD4+ producing

IL-17A were significantly lower in all groups compared to the BL group (Bonferroni *post hoc*, p <0.001). Regarding the Th2 type cytokines, the SAT group showed a significantly higher percentage of TCD4+ cells producing IL-4, IL-5, IL-6 and TGF-β compared to the BL group (Bonferroni *post hoc*, p<0.001), the MD group showed similar behavior for IL-5 and TGF-β. On the other hand, the percentage of TCD4+ cells producing IL-4 and IL-6 was significantly lower in the LY groups (Bonferroni *post hoc*, p <0.001). (Table 5)

Table 5: Effect of supplementation with EPA and DHA fatty acids extracted from microalgae on Th1 and TH2 cytokines in CD1

CD1 mice		8 weeks old	16 weeks old			
		BL	RC	MD	LY	SAT
		Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
		n=6	n=6	n=6	n=6	n=6
Th1	IFN-γ, %	1.8±0.18	1.2±0.09*	2.7±0.1*	1.0±0.1*	4.3±0.1*
%TCD4+/	TNF-α, %	2.5±0.13	1.5±0.1*	2.6±0.1	1.4±0.09*	4.6±0.07*
	IL-12, %	2.8±0.1	1.7±0.08*	2.4±0.09*	1.3±0.08*	4.4±0.08*
	IL-17A, %	1.6±0.08	1.8±0.08*	1.8±0.1*	4.1±0.08*	3.6±0.09*
Th2	IL-4, %	2.6±0.1	1.8±0.09*	2.4±0.1	1.6±0.1*	4.4±0.1*
%TCD4+/	IL-5, %	2.1±0.1	1.4±0.1*	2.6±0.1*	1.9±0.1	3.4±0.1*
	IL-6, %	1.8±0.1	1.7±0.08	1.7±0.1	1.5±0.09*	2.6±0.07*
	IL-10, %	1.6±0.08	1.5±0.1	2.2±0.1	1.2±0.1	2.9±1.6
	TGF-β, %	1.5±0.06	1.3±0.8*	2.3±0.07*	1.5±0.08	5.1±0.1*

Data are presented as means ± standard deviations of percentage of TCD4+ cells producing cytokines. One-way ANOVA* for comparison of differences between BL group at 8 weeks vs. all the groups at 16 weeks. P value was significant at <0.05

Discussion

The results provided in this study show evidence that supplementation with Omega-3 fatty acids obtained from microalgae improves the inflammatory profile in general by reducing the secretion of many cytokines. Therefore, these results suggest that microalgae extracts may be considered as an anti-inflammatory strategy against different diseases. These findings are summarized in Fig. 2

The BMI was significantly higher in the MD group from the diabetic mice. These results are different from those reported by Zhuang in which C57B1/6 mice were supplemented with fish oil and did not show changes in BMI [30]. There were no significant differences in plasma glucose attributable to treatment; no studies were found to match our findings; however, a study

with C57B / 6 mice supplemented with EPA suggests a protective effect of omega-3 fatty acids on glucose metabolism [19]. Further studies on the effect of microalgae fatty acids on glucose metabolism are needed.

In this study, food consumption was lower in the MD groups for the diabetic mice. Other studies with C57Bl/6 mice fed with normal chow enriched with EPA and DHA extracted from fish oil showed no changes in food consumption [31,32]. However, a study from Díaz- Reséndiz explains that mice regulate food intake according to the composition of the food or the presence of an extra source of energy [33].

Effect of Microalgae Omega-3 fatty acids on lymphocytes populations and their intracellular cytokines

The percentage of total T lymphocytes was lower in all study groups from both strains. In contrast to this study, Marano et.al. [34] suggest that consumption of omega-3 fatty acids increases CD3+ lymphocyte populations including CD4+. In agreement with our findings, several supplementation studies report that there were no changes in lymphocyte populations [35,36].

On the other hand, in both strains the SAT groups showed a significantly lower percentage of CD8+ cells. The CD4+ populations in the SAT groups increased significantly compared to their BL group. These results are consistent with those of Baccan [37] who showed that consumption of high-fat diets significantly increase lymphocyte populations.

The db/db strain is characterized by a chronic inflammatory state such as diabetes disease, which causes the pro and anti-inflammatory cytokines to be in higher concentrations compared to the CD1 strain, however, although the strains are very different between them, the decrease in cytokine concentrations occurred in a similar way.

In this study, supplementation with omega 3 fatty acids extracted from microalgae and administered either lyophilized or added to the feed, significantly decreased the percentage of TCD4+ cells producing IFN-γ and TNF-α. These cytokines play different roles in inflammatory states such as in diabetes; IFN-γ directs the differentiation of CD4+ lymphocytes into helper

lymphocytes type 1 (Th1), also intervenes in the activation of macrophages and induces a greater secretion of IL-12 [38]. However, in the diabetic mice, microalgae fatty acids showed to increase the percentage of TCD4+ cells producing IL-12. The main functions of IL-12 are the activation of Th1 lymphocytes and to stimulate the production of IFN-γ [39]. On the other hand, TNF-α is a cytokine involved in the acute and chronic phase as well as in the activation of the production of certain anti-inflammatory cytokines such as IL-4, IL-5 and IL-6 as a form of self-regulation of the inflammatory state [40].

A study by Vigerust et. al. made in transgenic TNF-α C57B/6 mice that were fed with diets enriched whether with fish oil or krill oil showed no modification in this cytokine [40]. Similarly, in a study in Wistar rats supplemented with fish or soybean oil, no significant differences were found in the concentrations of IFN-γ and TNF-α [41]. Although Navarro-Xavier [41] showed there are no changes in TNF-α after fish oil supplementation, there are also many studies that report a significant decrease in this cytokine [42–44]. A study made by Sierra [45] reports that in Balb/c mice fed with modified diet either with EPA or with DHA for 3 weeks, spleen lymphocytes decreased their production of TNF-α only in the diet with EPA, but not in the diet with DHA. When approaching the effect of microalgae fatty acids; a study carried out in cell lines of macrophages exposed to LPS and added with extracts of different microalgae, showed a significant decrease of the TNF-α compared against the control cultures. A study by Sierra et al. reported that only EPA enriched diet was able to decrease IL-12 concentrations [45].

In this study, the percentage of TCD4+ cells producing IL-17A showed a significant increase in both strains. IL-17A is known as an inflammatory cytokine whose main function is exerted on myeloid and mesenchymal cells by inducing the expression of granulocyte colony-stimulating factor (G-CSF), IL-6 and other chemokines, which increase granulopoiesis and recruit neutrophils into the site of infection [46]. Vigerust et al. also reported that after supplementation with fish oil and krill oil enriched diets, IL-17A showed to be increased only in the fish oil group [40], these results agree with our findings.

The supplementation with omega 3 fatty acids in lyophilized form and added in the food showed a significantly higher percentage of TCD4+ cells producing IL-10 in both strains. IL-4 is produced by type 2 T cells (Th2), basophils and mast cells. It has anti-inflammatory function by

blocking the synthesis of Il-1, TNF-α and IL-6; In addition, it promotes the proliferation and differentiation of B lymphocytes and is considered a potent inhibitor of apoptosis [47]. IL-10 is also known as cytokine synthesis inhibiting factor (CSIF) and can inhibit the synthesis of proinflammatory cytokines by T lymphocytes and macrophages. It also regulates the growth and differentiation of B lymphocytes, NK cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells [48]. A study in Wistar rats fed with a fish oil enriched diet was found to show lower concentrations of IL-4 and IL-5 at the alveolar level compared to control [41]. Also, Sierra [45] showed similar results in Balb/c mice. Our findings agree with Li [42] who demonstrated that fish oil supplementation decreased IL-10; however, Sierra reported that only EPA supplementation was able to increase IL-10 concentrations [45]. These results suggest that the consumption of microalgae fatty acids has attenuating effects of systemic inflammation even in chronic diseases and not only in acute states of inflammation.

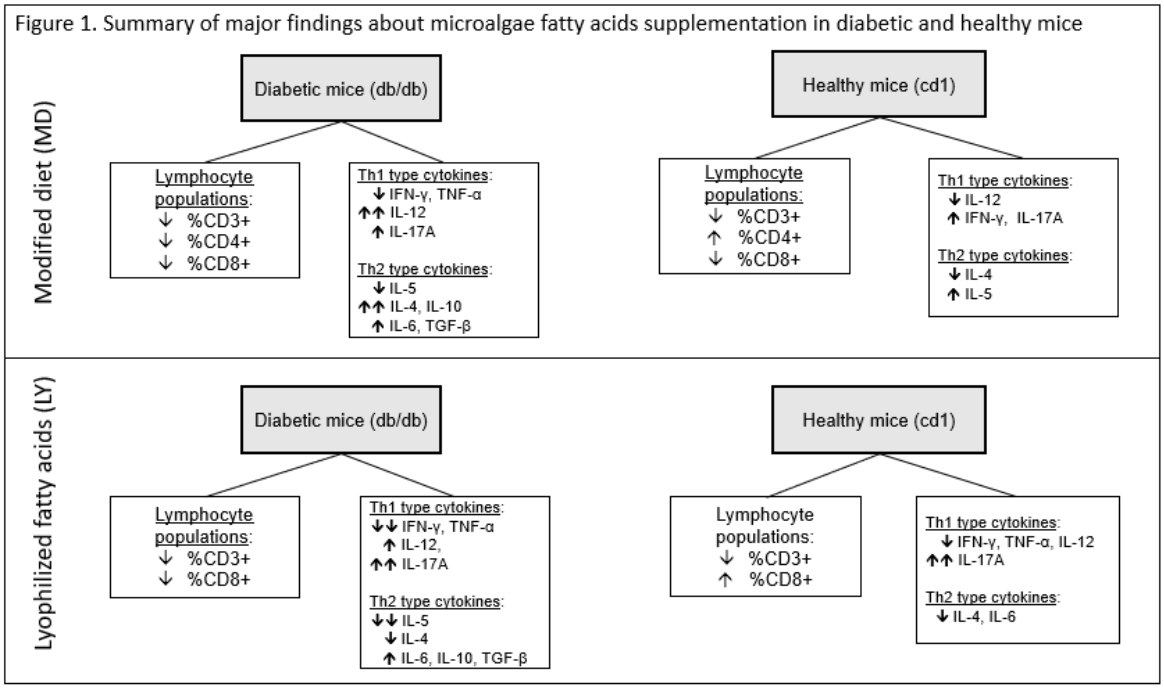
TGF-β is a cytokine with pleiotropic functions in hematopoiesis, angiogenesis, cell proliferation, differentiation, migration and apoptosis. Has a strong anti-inflammatory action but may increase some immune functions. Thus, in knock-out mice for TGF-β they show defects in regulatory T lymphocytes, which generates an extensive inflammation with abundant proliferation of T lymphocytes and differentiation of CD4 + in Th1 and Th2 lymphocytes [49]. In our study both strains showed higher percentage of TCD4+ cells producing TGF-β in the LY group when compared with BL, however, they were much lower than those for the SAT group so we could agree that microalgae fatty acids have a positive effect on TGF-β ex. A study with apoE-deficient mice infused with angiotensin and treated with EPA and DHA orally for 3 weeks showed that the TGF-β gene expression was significantly decreased in the EPA group and DHA compared to untreated mice [50]. In contrast, a study in Wistar rats [51] exposed to LPS during gestation and whose offspring were supplemented with fish oil showed that TGF-β concentrations were increased as compared to controls [52].

IL-6 release is induced by IL-1 and TNF-a. It is involved in the production of immunoglobulins, in the differentiation of active B lymphocytes, plasma cells, modulates hematopoiesis and is responsible, together with IL-1, for the synthesis of acute phase liver proteins like fibrinogen [53]. Percentage of TCD4+ cells producing IL-6 showed to be significantly increased in LY group from both strains, however SAT group reported the highest IL-6 concentrations; we

suggest that microalgae fatty acids could have a protective effect against IL-6 expression. Two studies report decreasing concentrations of IL-6 when using fish oil, one was conducted in mice [42] and the other in overweight pregnant women [43]. Additionally, a study by Robertson [54] in macrophage cell line cultures treated with microalgae extracts showed a significant decrease in IL-6.

Regarding the effects of the consumption of coconut oil as a source of saturated fat; there is controversy about the properties of coconut oil, this is because despite being a source of saturated fat, several studies have shown that it has anti-inflammatory properties [55,56], in this study, the consumption of coconut oil showed a high percentage of TCD4+ cells producing Th1 and Th2 type cytokines which is consistent with other studies [57].

Figure 1. Summary of major findings about microalgae fatty acids supplementation in diabetic and healthy mice



Conclusion

The results provided in this study show evidence that supplementation with Omega-3 fatty acids obtained from microalgae improves the inflammatory profile in general by reducing the

secretion of many cytokines. Therefore, these results suggest that microalgae extracts may be considered as an anti-inflammatory strategy against different chronic diseases.

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7.2 Segundo artículo aceptado

7.2.1 Título del Artículo y/o capítulo de libro aceptado

Effect on adipose tissue of diabetic mice supplemented with n-3 fatty acids extracted from microalgae

7.2.2 Página frontal del manuscrito

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RESEARCH ARTICLE

Effect on Adipose Tissue of Diabetic Mice Supplemented with *n-3* Fatty Acids Extracted from Microalgae

Laura E. Gutiérrez-Pliego[1], Beatriz E. Martínez-Carrillo[1], Aldo A. Reséndiz-Albor[2] and Roxana Valdés-Ramos[1,*]

[1]*School of Medicine, Universidad Autónoma del Estado de México, Paseo Tollocan y Venustiano Carranza s/n, Col. Moderna de la Cruz, 50180 Toluca, State of Mexico, Mexico;* [2]*Laboratory of Mucosal Immunity, Research and Graduate Section, Superior School of Medicine, Instituto Politécnico Nacional. Av. Plan de San Luis s/n, Col. Casco de Santo Tomás, Miguel Hidalgo 11350, Mexico City, Mexico*

Abstract: ***Background:*** Type 2 Diabetes Mellitus (T2DM) is considered a chronic noncommunicable disease in which oxidative stress is expected as a result of hyperglycaemia. One of the most recent approaches is the study of microalgae fatty acids and their possible antioxidant effect.

Objective: This study aimed to analyse the effect of supplementation with *n-3* fatty acids extracted from microalgae on the total antioxidant capacity (TAC) and lipid peroxidation of adipose tissue and plasma from diabetic (db/db) and healthy (CD1) mice.

Methods: Mice were supplemented with lyophilized *n-3* fatty acids extracted from microalgae or added to the diet, from week 8 to 16. TAC assay and Thiobarbituric Acid Reactive Substances assay (TBARS) were performed on adipose tissue and plasma samples.

Results: The supplementation of lyophilized *n-3* fatty acids from microalgae increased the total antioxidant capacity in adipose tissue of diabetic mice (615.67µM Trolox equivalents vs 405.02µM Trolox equivalents from control mice, $p<0.01$) and in the plasma of healthy mice (1132.97±85.75µM Trolox equivalents vs 930.64±32µM Trolox equivalents from modified diet mice, $p<0.01$). There was no significant effect on lipid peroxidation on both strains.

Conclusion: The use of *n-3* fatty acids extracted from microalgae could be a useful strategy to improve total antioxidant capacity in T2DM.

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1. INTRODUCTION

Diabetes mellitus is considered a noncommunicable chronic disease and the leading cause of many health-related complications and premature deaths [1, 2]. According to the World Health Organization, in 2014, 422 million adults worldwide had diabetes [3]. In Mexico, the prevalence of diabetes mellitus amounts to 9.4% in the adult population according to data from the Mexico National Survey of Health and Nutrition Mid-way 2016 (ENSANUT 2016 MC for its acronym in Spanish) [4]. This disease is characterized by a state of chronic oxidative stress due to the continuous condition of hyperglycaemia [5-7].

Oxidative reactions are essential in most cellular metabolic processes [8]. Oxidative stress is the excessive formation or insufficient elimination of highly reactive molecules called reactive oxygen species (ROS) and reactive nitrogen species (RNS) [9]. In diabetes, the accumulation of advanced glycation end products (AGES), the activation of the sorbitol and hexosamine pathways, as well as various pathways mediated by protein kinases C, result in increased oxidative stress [10-12]. This oxidative stress imbalance may lead to cellular damage on several macromolecules such as lipids, proteins and DNA [13, 14]. Lipids are the main target of free radicals, causing lipid peroxidation; this occurs when free radicals attack fatty acids that contain carbon double bonds, especially polyunsaturated fatty acids (PUFAs) [13, 15]. The damage lies in the modification of the physical and chemical properties of the cell membrane, resulting in the alteration of function, oedema and cell death [14, 16, 17]. The most studied sub-product of lipid peroxidation is Malondialdehyde (MDA) [18, 19]. However, on a regular basis, enzymatic and non-enzymatic antioxidant mechanisms are capable of minimizing the damage caused by oxidative stress [20, 21].

N-3 fatty acids are known because of their wide range of biological effects, among which are benefits to lipoprotein metabolism, platelet, endothelial and vascular function, as well as their anti-inflammatory and antioxidant potential [22-25]. Therefore, the consumption of *n-3* fatty acids, especially EPA

*Address correspondence to this author at the Faculty of Medicine, Universidad Autónoma del Estado de México, Paseo Tollocan esq. Jesús Carranza, Col. Moderna de la Cruz, Toluca, Edo. Mex, 50180, Mexico; Tel/Fax: +52-722-217-4831; E-mails: rvaldesr@uaemex.mx

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7.2.3 Carta de aceptación

Gmail

Laura Elisa Gutierrez Pliego <lauraelisa.gp@gmail.com>

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Endocrine, Metabolic & Immune Disorders - Drug Targets EMIDDT <emiddt@benthamscience.net> 12 de enero de 2020, 9:06
Para: Roxana Valdes Ramos <rvaldesr@uaemex.mx>
Cc: alrealdo@yahoo.com.mx, martinez_elina9@hotmail.com, lauraelisa.gp@gmail.com

Dear Dr. Valdés-Ramos,

I am pleased to inform you that your submission entitled **"Effect on Adipose Tissue of Diabetic Mice Supplemented with n-3 Fatty Acids Extracted from Microalgae"** has been accepted for publication in the journal **"**Endocrine, Metabolic & Immune Disorders-Drug Targets**"**. You will be notified about the official acceptance soon.

Before we proceed further, we need the following details:

1. The department names, along with the complete affiliation details translated in **English**.
2. A running/short title.
3. If abbreviations are used in the text they should be provided in **"List of Abbreviations".**
4. Please check the **"Reference section"** in the attached Word file named "Composer References" if it is correctly formatted as it has been formatted by a software so please check for any potential errors.
5. The information regarding **"Ethics Approval and Consent to Participate"** should be given in the following manner:

This study was approved by the Ethics Committee/Institutional Review Board (Ethics Committee/ Institutional Review Board Name???) (provide City, Country name??) (Provide Protocol/Reference Number????).

6. For research involving animals, the authors should indicate whether the procedures followed were in accordance with the standards set forth in the eighth edition of the "Guide for the Care and Use of Laboratory Animals" (grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals_prepub.pdf published by the National Academy of Sciences, The National Academies Press, Washington, D.C.).

Research work on animals should be carried out in accordance with the NC3Rs ARRIVE Guidelines. For In Vivo Experiments, please visit https://www.nc3rs.org.uk/arrive-guidelines

Authors should clearly state the name of the approval committee, highlighting that legal and ethical approvals were obtained prior to initiation of the research work carried out on animals, and that the experiments were performed in accordance with the relevant guidelines and regulations stated below.

7.2.4 Resumen

Background: Type 2 Diabetes Mellitus (T2DM) is considered a chronic noncommunicable disease in which oxidative stress is expected as a result of hyperglycaemia. One of the most recent approaches is the study of microalgae fatty acids and their possible antioxidant effect. Objective: This study aimed to analyse the effect of supplementation with n-3 fatty acids extracted from microalgae on the total antioxidant capacity (TAC) and lipid peroxidation of adipose tissue and plasma from diabetic (db/db) and healthy (CD1) mice. Methods: Mice were supplemented with lyophilized n-3 fatty acids extracted from microalgae or added to the diet, from week 8 to 16. TAC assay and Thiobarbituric Acid Reactive Substances assay (TBARS) were performed on adipose tissue and plasma samples. Results: The supplementation of lyophilized n-3 fatty acids from microalgae increased the total antioxidant capacity in adipose tissue of diabetic mice (615.67μM Trolox equivalents vs 405.02μM Trolox equivalents from control mice, $p<0.01$) and in the plasma of healthy mice (1132.97±85.75μM Trolox equivalents vs 930.64±32μM Trolox equivalents from modified diet mice, $p<0.01$). There was no significant effect on lipid peroxidation on both strains. Conclusion: The use of n-3 fatty acids extracted from microalgae could be a useful strategy to improve total antioxidant capacity in T2DM.

7.2.5 Apartados del artículo

Introduction

Diabetes mellitus is considered a noncommunicable chronic disease and the leading cause of many health-related complications and premature deaths [1, 2]. According to the World Health Organization, in 2014, 422 million adults worldwide had diabetes [3]. In Mexico, the prevalence of diabetes mellitus amounts to 9.4% in the adult population according to data from the Mexico National Survey of Health and Nutrition Mid-way 2016 (ENSANUT 2016 MC for its acronym in Spanish) [4]. This disease is characterized by a state of chronic oxidative stress due to the continuous condition of hyperglycaemia [5-7]. Oxidative reactions are essential in most cellular metabolic processes [8]. Oxidative stress is the excessive formation or insufficient elimination of highly reactive molecules called reactive oxygen species (ROS) and reactive nitrogen species (RNS) [9]. In diabetes, the accumulation of advanced glycation end products (AGES), the activation of the sorbitol and hexosamine pathways, as well as various pathways mediated by

protein kinases C, result in increased oxidative stress [10-12]. This oxidative stress imbalance may lead to cellular damage on several macromolecules such as lipids, proteins and DNA [13, 14]. Lipids are the main target of free radicals, causing lipid peroxidation; this occurs when free radicals attack fatty acids that contain carbon double bonds, especially polyunsaturated fatty acids (PUFAs) [13, 15]. The damage lies in the modification of the physical and chemical properties of the cell membrane, resulting in the alteration of function, oedema and cell death [14, 16, 17]. The most studied sub-product of lipid peroxidation is Malondialdehyde (MDA) [18, 19]. However, on a regular basis, enzymatic and non-enzymatic antioxidant mechanisms are capable of minimizing the damage caused by oxidative stress [20, 21]. N-3 fatty acids are known because of their wide range of biological effects, among which are benefits to lipoprotein metabolism, platelet, endothelial and vascular function, as well as their anti-inflammatory and antioxidant potential [22-25]. Therefore, the consumption of n-3 fatty acids, especially EPA and DHA, has been shown to have beneficial effects against chronic diseases such as diabetes mellitus in which oxidative stress is present [26]. Traditionally, the main source of these fatty acids has been fish oil [27]; however, among the disadvantages of its use, we find that exploitation in the fishing industry has reached the sustainable limits. In addition, the contamination by heavy metals and pesticides has become a major problem [28, 29]. Other disadvantages of the use of these sources are undesirable nutritional and organoleptic effects, such as oxidation (due to their high polyunsaturation) and the characteristic odor of the product [30]. A new focus on biotechnological advances is the use of microalgae as an alternative source of polyunsaturated fatty acids. Microalgae are an evolutionarily microscopic diverse eukaryotic group of unicellular and predominantly aquatic photosynthetic organisms [31]. They are the primary natural producers of EPA and DHA, because they have the biosynthetic machinery to sequentially alternate between desaturation and elongation in their carbon chains [32]. However, there are fewer studies that describe their antioxidant effects as those already described for fatty acids of animal origin [31, 33, 34]. The aim of this study was to analyse the effect of the consumption of n-3 fatty acids extracted from two families of microalgae (Chlorophyceae and Eustigamatophyceae) provided either as a supplement or incorporated to the diet, on the total antioxidant capacity and lipid peroxidation in adipose tissue of diabetic mice.

Methods

Animals

The present study is an experimental, prospective, controlled and randomized study. Forty-eight 8-week old male mice from two different strains were used: 24 diabetic mice (db/db mice BKS.Cg-+Leprdb+LeprdbOlaHsd Harlan®) and 24 healthy mice (CD1 mice. Crl: CD1 [ICR] Universidad Autónoma del Estado de México, UAEMex). Animal care and experimental procedures were carried out in accordance with the standards of the guidelines of the Mexican Ministry of Health for the Production and Care of Laboratory Animals (NOM-062-ZOO-1999 Ministry of Agriculture, Mexico City, Mexico) and the Internal Regulations for the Use of Laboratory Animals and the Committee of Ethics in Research of the UAEMex. Animals were housed in acrylic cages, three mice per cage. food and water were offered ad libitum during the entire experiment and all animals were maintained on a 12/12 h light/dark cycle.

Experimental groups

For both strains, mice were randomly assigned to one of the five study groups: 1) A Rodent Chow group (RC); 2) a group supplemented with lyophilized microalgae n-3 fatty acids (LY); 3) a group supplemented with coconut oil group (CO); 4) a modified diet group with n-3 fatty acids from microalgae incorporated in the chow (MD). Groups 1, 2, and 3 were fed a standard normal diet (Rodent Laboratory Chow 5001 from Purina® [12.63 kJ/g]). The experimental study was conducted from the 8th to the 16th week of life.

Supplementation

a) The LY group was fed with a standard rodent diet and supplemented with lyophilized powder containing EPA+DHA extracted from microalgae (Chlorophyceae and Eustigamatophyceae families) at a dosage of 1mg/g of mouse weight; the powder was reconstituted in 10uL of distilled water and administered by direct oral gavage with a micropipette at 8:00 am every day. b) The CO group was also fed with a standard rodent diet and supplemented with coconut oil (C1758-500 Sigma Aldrich®) at a dosage of 1mg/g of mouse weight administered by direct oral gavage with a micropipette at 8:00 am every day. c) The MD group was fed with a rodent chow enriched with microalgae EPA+DHA for a total content of 2.0% n-3 fatty acid meaning 10x of

the original content. Chow was administered ad libitum. The EPA and DHA contents of the diet are shown in Supplementary Table 1.

Collection of biological samples

The sacrifice of animals was conducted in the 16th week of life of all treatment groups. Anaesthesia was given using an ether chamber; afterward, animals were bled by direct cardiac puncture to obtain 1mL of blood and then sacrificed by cervical dislocation. Blood was centrifuged at 2500rpm for 15min, plasma was collected and stored in 1.5mL microtubes at -80°C until used. 200mg of the mesenteric adipose tissue was removed and stored into 1.5mL microtubes at -80° C until used.

Sample preparation

Plasma samples were assayed directly. The adipose tissue samples were homogenized with 1xPBS at 12,000 rpm for 1 minute per sample. The homogenization equipment was a rotary Dragon Lab brand(R) homogenizer used for the preparation of samples. Afterward, samples were centrifuged at 14,000rpm for 10 minutes to form pellets and remove any debris. Finally, the supernatant was used for the determination of antioxidant capacity and lipid peroxidation.

Total Antioxidant Capacity Determination (TAC)

One common way to calculate the total antioxidant capacity (TAC) of an organism is through assays based on the reduction of metals such as copper and iron. For this study, a quantitative colorimetric determination of total antioxidant capacity assay kit was used (QuantiChrom™ Antioxidant Assay Kit), the procedure was performed following manufacturer´s instructions and samples were read in a spectrophotometer at an optical density (OD) of 570nm.

Lipid peroxidation determination

A common method for the detection of MDA is the thiobarbituric acid reactive substances fluorometric assay (TBARS) in which MDA reacts with thiobarbituric acid to give colorful products that can be read in a colorimetric plate reader. For this purpose, a commercial kit (QuantiChrom™ TBARS Assay Kit) was used and the manufacturer's specifications were

followed according to the colorimetric test instructions. Finally, the samples were read in a spectrophotometer at an OD of 535nm.

Blood glucose, Body Mass Index, Food and Water Intake

Blood glucose was measured with a Bayer Contour TS glucometer through tail puncture at the beginning and end of the study. The body mass index (BMI) was calculated weekly, using the weight and length of each mouse. Mice were weighed weekly until sacrifice, on a Triple Beam 700/800 Series mouse scale (Ohaus Cat. No. 2,729,439). The length was measured under anaesthesia (0.1 mL of sodium pentobarbital at 1%) with a tape from the nose to the anus. BMI was calculated with the following formula: BMI = Weight (g)/length (cm2) [35]. Food and water intake was recorded at the beginning and end of the study.

Statistical analysis

For parametric values, One-way ANOVA was performed for comparison between the groups from each strain (MD, LY, SAT and CO), Bonferroni post hoc was applied. For non-parametric values, the Kruskal-Wallis H test was performed. Two-way ANOVA was run to compare mice and treatment groups. Differences were considered significant at $p<0.05$. The software used to run statistical analysis was SPSS v.23 for Windows.

Results

Food and water intake, BMI, and glucose values

With respect to food intake, all Db/db mice consumed less than the CD1 groups. The MD was the lowest food consuming group and the LY was the highest ($p<0.05$) in the Db/db mice. Food intake in CD1 mice was only found to be increased in the CO group. Water intake was higher in the MD Db/db and in the LY and CO CD1 groups.

In the Db/db mice, the MD group had the highest BMI, their blood glucose levels were all higher than the CD1 mice with the highest value in the CO group. BMI in the CD1 mice was found to be significantly ($p<0.05$) increased in the LY and CO groups. Blood glucose was lower in the CO group, with no significant differences between treatment groups (Table 1).

Table 1. Food and water intake, BMI and glucose of Db/db and CD1 mice during the study.

	RC	MD	LY	CO	F (p)	Bonferroni (p<0.05)
	n=6	n=6	n=6	n=6		
Db/db mice						
Food intake, *g/week*	32.92±0.34	27.68±0.97	37.50±0.77	36.30±2.41	31.166 (**0.000**)	RC vs MD, LY vs MD, CO vs MD
Water intake, *mL/week*	78.25±7.18	81.39±10.40	79.61±1.81	67.23±3.41	1.305 (0.285)	NS
BMI, g/cm^2	61.27±2.46	65.05±1.67	62.22±1.52	59.66±2.34	0.768 (0.518)	NS
Glucose, *mg/dL*	551.83±83.66	505.00±73.95	525.50±50.93	580.67±22.01*	0.122 (0.947)	NS
CD1 mice						
Food intake, *g/week*	58.34±5.60	42.75±4.98	60.96±1.91	73.3±20.81	7.415 (**0.000**)	LY vs MD, CO vs MD
Water intake, *mL/week*	67.28±3.23	53.91±1.32	89.78±6.10	93.25±4.77	9.367 (**0.000**)	CO vs MD, LY vs MD
BMI, g/cm^2	31.40±1.95	32.18±3.09	34.44±4.11	34.27±2.41	0.728 (0.541)	NS
Glucose, *mg/dL*	119.33±12.97	127.50±18.68	109.67±13.31	106.83±12.40	0.891 (0.891)	NS

RC: Rodent Chow; MD: RC+EPA & DHA in chow; LY: RC + lyophilized EPA & DHA.
Data are presented as means ± SD. One-way ANOVA for comparison of differences between BL and all groups.
*p value was significant at <0.05. NS: Non-signficant.

When analyzing both CD1 and Db/db groups by two-way ANOVA, we found differences between treatment groups in BMI (F1=.028, p=0.384, Bonferroni=NS); blood glucose (F=0.179, p=0.910, Bonferroni=NS); food intake (F=0.3003, p=0.035, Bonferroni: CL vs MD= 0.007, CL vs CO= 0.031, LY vs MD=0.000, CO vs MD= 0.000), and water intake (F=5.944, p=0.001, Bonferroni: MD vs LY= 0.024, MD vs CO= 0.027).

Total Antioxidant Capacity in Adipose Tissue and Plasma of Diabetic Mice

In adipose tissue, the LY group showed a higher antioxidant capacity than the RC and CO group. On the other hand, the CO group had a significantly lower antioxidant capacity compared to LY and RC (post hoc p <0.05). In plasma, LY group showed higher antioxidant capacity than the CO group. Finally, the CO group showed a lower antioxidant capacity than the RC group (post hoc p <0.05) (Table 2).

Table 2. Effect of consumption of EPA and DHA fatty acids extracted from microalgae on the Antioxidant capacity and lipoperoxidation.

-	-	RC	MD	LY	CO	F (p)	Bonferroni (p<0.05)
	-	n=6	n=6	n=6	n=6	-	-
Db/db mice							
Antioxidant capacity	Adipose Tissue+	405.02	473.59	615.67	332.84	--- (**0.001**+)	RC vs LY, CO vs LY
µM Trolox equivalents	Plasma++	706.90± 22.14	639.20± 18.80	730.35± 41.00	379.27± 35.60	9.341 (**0.000**)	RC vs CO, MD vs CO, LY vs CO
Lipid peroxidation *µM MDA equivalents*	Adipose Tissue++	0.43± 0.07	0.44± 0.03	0.45± 0.08	0.60± 0.04	2.624 (0.062)	NS
	Plasma	0.08± 0.01	0.08± 0.01	0.08± 0.01	0.10± 0.01	1.437 (0.245)	NS
CD1 mice							
Antioxidant capacity, *µM Trolox equivalents*	Adipose Tissue++	487.31± 64.43	424.32± 65.67	545.48± 81.35	378.81± 53.84	0.883 (0.457)	NS
	Plasma++	1103.49± 79.78	930.64± 32.23	1132.97± 85.75	804.12± 26.29	8.679 (**0.000**)	RC vs CO, LY vs CO
Lipid peroxidation, *µM MDA Equivalents*	Adipose Tissue++	0.07± 0.01	0.08± 0.01	0.06± 0.01	0.07± 0.01	0.171 (0.915)	NS
	Plasma++	0.42± 0.11	0.46± 0.07	0.44± 0.02	0.46± 0.11	1.046 (0-382)	NS

+ Data are presented as median (p50); Kruskal-Wallis H Test as data were not normally distributed.
++ Data are presented as means ± SD; One-way ANOVA for comparison of differences between all groups.
NS: Non-significant.

Lipid Peroxidation in Adipose Tissue and Plasma of Diabetic Mice

Lipid peroxidation in both tissues we studied showed no significant differences between treatments in the Db/db mice.

Total Antioxidant Capacity in Adipose Tissue and Plasma of Healthy Mice

In adipose tissue, the CO group had the lowest antioxidant capacity, while the LY showed the highest values, although these differences were not significant. Plasma total antioxidant capacity showed a similar but statistically significant pattern, with the highest values in the LY group (Table 2).

Lipid Peroxidation in Adipose Tissue and Plasma of Healthy Mice

Although lipid peroxidation in adipose tissue and plasma in the MD group showed a tendency to be higher than the other groups, no significant differences were found between treatment groups (Table 2). When comparing Db/db and CD1 mice by a two-way ANOVA analysis, we found significant differences in the antioxidant capacity in adipose tissue (F=0.808, p=0.493, Bonferroni: LY vs CO= 0.002); antioxidant capacity in plasma (F= 0.642, p=0.590, Bonferroni: CL vs CO= 0.000, MD vs LY= 0.038, MD vs CO= 0.002, CO vs LY= 0.000); lipid peroxidation in adipose tissue (F= 1.044, p=0.377, Bonferroni=NS), and lipid peroxidation in plasma (F= 0.735, p= 0.534, Bonferroni=NS).

Discussion

The results of this study show that the consumption of n3 fatty acids from microalgae administered in the form of a supplement has beneficial effects on the total antioxidant capacity in adipose tissue of diabetic mice and in the plasma of healthy mice, as these values did not decrease significantly in comparison with the group consuming saturated fat. Our results on BMI are different from those reported in C57B1/6 mice, where fish-oil supplementation caused no changes [36]. With respect to blood glucose, Kalupahana et al., found that EPA supplementation protects glucose metabolism in C57B1/6 mice [37]. The lower intake of food in our Db/db mice may be explained by the fact that n-3 supplementation may induce a modification in adiponectin production thus regulating food intake [38]. Additionally, Shklyaev et al., [39] showed that an increase in adiponectin in Sprague-Dawley rats promoted a reduction in food intake and body weight, however, the effect on our db/db mouse model may have been different particularly with respect to weight gain due to the metabolic characteristics of these animals. Another explanation for the differences in food intake may be that the MD diet may have had a more satiating effect than the rodent chow alone or with the addition of LY or CO by oral gavage. Several studies have previously shown the close relationship between obesity, hyperglycaemia states and the generation of reactive oxygen species [40-43] as well as the association with low levels of antioxidants [44, 45] and increased lipid peroxidation [46, 47]. The determination of total antioxidant capacity in tissue and plasma is of great importance, since

it gives a more complete measure of the antioxidant mechanisms present in the organism [45]. TBARS assay for lipid peroxidation applied on different types of samples has also been very useful [48-52]. However, to date, there are few studies that focused on the analysis of the antioxidant effect of the consumption of a specific nutrient [53]. The effect of supplementation of n-3 fatty acids from fish oil on oxidative stress has shown inconclusive results. A study on patients with Alzheimer's disease, who were supplemented with n-3 fish oil for 6 months, showed that it had no effect on the modulation of the formation of free radicals [54]. However, a study on patients with a high risk of psychosis showed that the antioxidant effect of n-3 fatty acids only occurred significantly when they were supplemented with vitamin E together with n-3 fatty acids [55]. Also, a study conducted by Di Nunzio et al., showed that supplementation with polyunsaturated fatty acids except for DHA, increased cellular susceptibility to oxidative stress [56]. On the other hand, a study carried out on Wistar rats, showed that the consumption of EPA and DHA in different proportions had a positive effect on inflammatory markers and oxidative stress [57]. Regarding the use of microalgae as sources of n-3 fatty acids and their antioxidant effect, currently, the evidence is limited, and most studies have focused on the first analysis of the content and their possible antioxidant capacity [58, 59]. A study conducted by Maadane et al., demonstrated the antioxidant capacity of the microalgae Nannochloropsis gaditana and Chlorella sp. using the DPPH radical scavenger assay [60]. It is important to mention that these species belong to the same family of microalgae used in this study. As for studies conducted on animal models for determining the antioxidant effect of n-3 fatty acids from microalgae; Haimeur et al., demonstrated that the supplementation with fatty acids of microalgae improved oxidative state by decreasing the production of MDA, increasing the concentrations of glutathione peroxidase in the liver and platelets [61].

Our study did not show a significant effect in the group fed with modified diet (MD); however, a study conducted in broilers fed with corn and soybean enriched with microalgae biomass (Nannochloropsis oceanica, belonging to the Eustigamatophiceae family) at several doses, showed that the liver, plasma, breast and thighs of chickens were high in EPA and DHA, suggesting that microalgae can be used to produce chicken meat enriched with n-3 fatty acids [62]. In our study, lipid peroxidation was not affected by supplementation or consumption of n-3 from microalgae, however, the group supplemented with coconut oil (CO) showed

significantly higher concentrations of MDA in diabetic mice. The above findings do not agree with the studies by Narayanankutty et al., and Alves et al., in which they show that supplementation with coconut oil decreases the serum MDA concentrations in rats fed with a high-fructose diet and hypertensive Wistar rats [63, 64]. We think that this difference in results is explained by the time of exposure and the dose administered to the experimental animals; while we administered an established dose of pure coconut oil orally for 8 weeks, the other studies used coconut oil added into modified diets, which may lead to variable intake of coconut oil and for a period of 4 weeks.

Conclusion

The present study concludes that the use of n-3 fatty acids extracted from microalgae could be a useful strategy to combat oxidative stress in chronic diseases such as diabetes mellitus by increasing the total antioxidant capacity, however, more studies are necessary.

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8. Discusión y conclusiones generales

La suplementación con ácidos grasos EPA y DHA extraídos de microalgas, no tuvo efecto significativo sobre el metabolismo de la glucosa en los ratones diabéticos, sin embargo, es importante profundizar con más estudios. El consumo de alimento, la ganancia de peso y el Índice de Masa Corporal no mostraron modificaciones algunas atribuibles a la suplementación.

Aunque las poblaciones linfocitarias se vieron disminuidas en todos los grupos de tratamiento con ácidos grasos EPA y DHA, la disminución puede también explicarse en función del estado de inflamación crónico presente en los ratones y la progresión de la enfermedad. Dentro de los hallazgos principales referente a la producción de citocinas inflamatorias, se encontró que la suplementación de ácidos grasos EPA y DHA en forma de liofilizado, disminuyo la producción de células CD4+ productoras de IFN-γ de manera importante. El IFN-γ es una proteína involucrada en varios procesos biológicos importantes como la inducción de la secreción de TNF-a y la estimulación de la liberación de especies reactivas de oxigeno por parte de los macrófagos. Por otra parte, el porcentaje de células productoras de IL-17a se vio aumentado significativamente, se sabe que la IL-17a participa en procesos inflamatorios especialmente en las células mieloides y mesenquimales gracias a la inducción de la secreción de sustancias como la IL-6.

El uso de ácidos grasos EPA y DHA extraídos a partir de microalgas son una estrategia útil contra el estrés oxidante gracias a un aumento de la capacidad antioxidante en tejido adiposo y en plasma. Sin embargo, más estudios podrían esclarecer con mayor profundidad estos hallazgos.

Los resultados de este proyecto de investigación concluyen que el uso de ácidos grasos n-3 extraídos a partir de microalgas, podrían ser una estrategia favorable para emplearse contra los daños causados por estrés oxidante e inflamación en enfermedades crónico-degenerativas, como lo es la diabetes mellitus tipo 2.

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