UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO

INSTITUTO DE INVESTIGACIONES QUÍMICO-BIOLÓGICAS

Programa Institucional de Doctorado en Ciencias Biológicas

Opción en Biología Experimental

Efecto del aceite de aguacate en el desarrollo de la enfermedad hepática no alcohólica y su influencia en la dinámica y función mitocondrial en ratas

Tesis para obtener el grado de

Doctor en Ciencias en Biología Experimental

Presenta:

M.C. Claudia Isabel García Berumen

Directores de tesis:

D.C. Christian Cortés Rojo

D.C Ricardo Mejía Zepeda

(Facultad de Estudios Superiores –Iztacala, UNAM

Morelia, Michoacán, México, Mayo 2019

INSTITUTO DE INVESTIGACIONES QUÍMICO-BIOLÓGICAS UMSNH

Agradecimientos

A mis padres y hermanos por apoyarme siempre.

A mi esposo e hijo que son mi mayor motivación.

Al D.C Christian Cortés Rojo y al D.C Ricardo Mejía Zepeda por recibirme como su estudiante y apoyarme en la elaboración de este proyecto.

A mis sinodales D.C Alfredo Saavedra Molina, D.C Alain Rodríguez Orozco, D.C Homero Reyes de la Cruz por su tiempo y concejos para la realización del trabajo.

A mis compañeros del laboratorio de bioquímica por su apoyo, concejos y por hacer del laboratorio un lugar ameno de trabajo.

A CONACyT por la beca otorgada.

ÍNDICE

Resumen

La enfermedad hepática (EHNA), es una de las principales causas de enfermedad hepática en todo el mundo, la incidencia aumenta año tras año de modo paralelo a la obesidad y la diabetes, debido a los cambios en la dieta y en la baja actividad física. La EHNA se caracteriza por una acumulación excesiva de grasa, inflamación y disfunción de hepatocitos en el hígado en ausencia de consumo de alcohol. La patogenia es poco conocida y los mecanismos moleculares que causan la progresión de la esteatosis simple a la esteatohepatitis (EHPTNA) no han sido bien esclarecidos. Alteraciones en procesos mitocondriales (tales como la dinámica mitocondrial, la oxidación β de ácidos grasos, el transporte de electrones y la fosforilación oxidativa, están estrechamente relacionadas con la patogénesis, sugiriendo que la EHPTNA es una enfermedad mitocondrial. Se ha reportado que la ingesta de aceite de aguacate tiene efectos benéficos contra la disfunción mitocondrial durante la diabetes y la hipertensión, al aumentar la resistencia de la cadena respiratoria al estrés oxidativo, disminuir la producción de ERO y mejorar el estado redox del glutatión. Con base en lo anterior, el objetivo de este trabajo fue determinar si el aceite de aguacate protege contra las alteraciones inducidas por la EHNA en la función mitocondrial, el estrés oxidativo, la inflamación y dinámica mitocondrial en mitocondrias de hígado de rata. Se probaron dietas con alto contenido de carbohidratos, alto contenido de grasa y su combinación. A partir de este análisis, los efectos del aceite de aguacate se analizaron en ratas en donde la EHNA fue inducida por una dieta con alto contenido de grasa, fructosa y sacarosa, administrada durante 6 o 18 semanas. El aceite de aguacate fue suministrado 12 semanas posterior a las 6 semanas de administración de dicha dieta, o en las últimas 12 semanas cuando la dieta se administró por 18 semanas. Las histologías de ratas con EHNA mostraron esteatosis, inflamación e hinchamiento de hepatocitos. La EHNA inhibió la fosforilación oxidativa y la actividad del complejo III, lo cual fue atribuido a una disminución en el flujo de electrones en los citocromos *b* y $c + c_1$ de dicho complejo. Además, se observó un aumento en la producción de ERO, en la peroxidación lipídica y de la expresión de TNF-α e IL-6. La expresión de las proteínas de fisión mitocondrial DRP1 y Fis1 aumentó y disminuyó la de las proteínas de fusión Mfn1/2 y de OPA1. Todos estos efectos se revirtieron con la suplementación con

aceite de aguacate. Estos datos sugieren que el aceite de aguacate podría ser benéfico para disminuir la progresión de EHNA al mejorar la función mitocondrial.
PALABRAS CLAVE:ESTRES OXIDATIVO, MITOCONDRIA, ANTIOXIDANTE, EHNA, BLANCO TERAPEUTICO.

Abstract

Nonalcoholic Fatty Liver Disease (NAFLD) is a major cause of liver disease worldwide, its incidence increases parallel with the obesity pandemic, due to changes in diet and in low physical activity. NAFLD is characterized by excessive fat accumulation, inflammation, and hepatocyte dysfunction in liver in the absence of alcohol consumption. The pathogenesis is poorly understood and the molecular mechanisms that cause progression from the simple steatosis to steatohepatitis (NASH) are still undefined. Mitochondrial alterations in processes like mitochondrial dynamics, fatty acid β-oxidation and oxidative phosphorylation are closely related to NASH pathogenesis, suggesting that NASH is a mitochondrial disease. It has been reported that avocado oil intake has protective effects against mitochondrial dysfunction during diabetes and hypertension by increasing the resistance of the respiratory chain to oxidative stress, decreasing ROS production and improving the glutathione redox state. On this basis, we tested whether avocado oil protects from the alterations elicited by NAFLD on mitochondrial function, oxidative stress, inflammation and mitochondrial dynamics in liver mitochondria from rats with NAFLD. Diets with high carbohydrates content or high fat content or a combination of these diets was first tested. From this analysis, the effects of avocado oil were tested in rats where NAFLD was induced by a diet with high fat, high fructose and sucrose supplied by six or 18 weeks. Avocado oil was supplemented during 12 weeks after 6 weeks of the administration of the above diet or in the latter 12 weeks when the NAFLD diet was given for 18 week. Histologic analyses shown steatosis, inflammation and hepatocyte ballooning in the rats with NAFLD. Liver mitochondrial from rats with NAFLD exhibited inhibition of oxidative phosphorylation, impaired complex III activity and decreased electron transfer at cytochromes b and $c+c_1$ in this complex. Furthermore, it was observed increasing in ROS production, in lipid peroxidation and in the expression to TNF-α and IL-6 were increased. Increased expression of the proteins of mitochondrial fission DRP1 and Fis1 was detected, while the expression of proteins of fusion Mfn1/2 and OPA1 was decreased. All these effects were counteracted when avocado oil was supplemented after the establishment of NAFLD. These data suggest that avocado oil could be beneficial to decrease NAFLD progression by improving mitochondrial function.

1. Introducción

1.1 Enfermedad Hepática no Alcohólica

La enferedad hepática no alcohólica (EHNA) es la enfermedad del hígado más común a nivel mundial, su prevalencia está ligada íntimamente a enfermedades relacionadas con el síndrome metabólico como son: la resistencia a la insulina, la diabetes, la obesidad, la hipertensión y la dislipidemia. Actualmente, es considerada como la manifestación hepática del síndrome metabólico (Marchesini et al, 2003), aunque también se presenta en la población en general; 7 de cada 10 adultos la presentan, en adolescentes 4 de cada 10 y en niños 3 de cada 10, estas cifras se incrementan en personas con obesidad y diabetes (ENSANUT 2016). Los mecanismos mediante los cuales se ha tratado de explicar la progresión de la EHNA se enmarcan en la teoría de los hits (Fig. 1). El primer hit es la acumulación de triglicéridos en el hígado, o esteatosis, lo cual incrementa la susceptibilidad del hígado al daño mediado por el segundo hit, como es el desbalance en la expresión de citocinas y adiponectinas (factor de necrosis tumoral α (TNF-α), interleucina 6 (IL6), adiponectina, leptina), la disfunción mitocondrial, el estrés oxidativo y la peroxidación de lípidos, lo cual conduce al desarrollo de esteatohepatitis no alcohólica (EHPTNA) (Day y James, 1998). En sujetos obesos, ocurre un flujo mayor de ácidos grasos libres (AGL) hacia el hígado, lo cuales son oxidados mediante la β-oxidación o bien son esterificados con glicerol en forma de triacilglicéridos, lo que causa acumulación de grasa en el hígado y genera lipotoxicidad (Feldstein et al, 2004). Las alteraciones mitocondriales están estrechamente relacionadas con la patogénesis de la EHPTNA, lo que sugiere que la EHPTNA es una enfermedad mitocondrial (Pessayre y Fromenty, 2005).

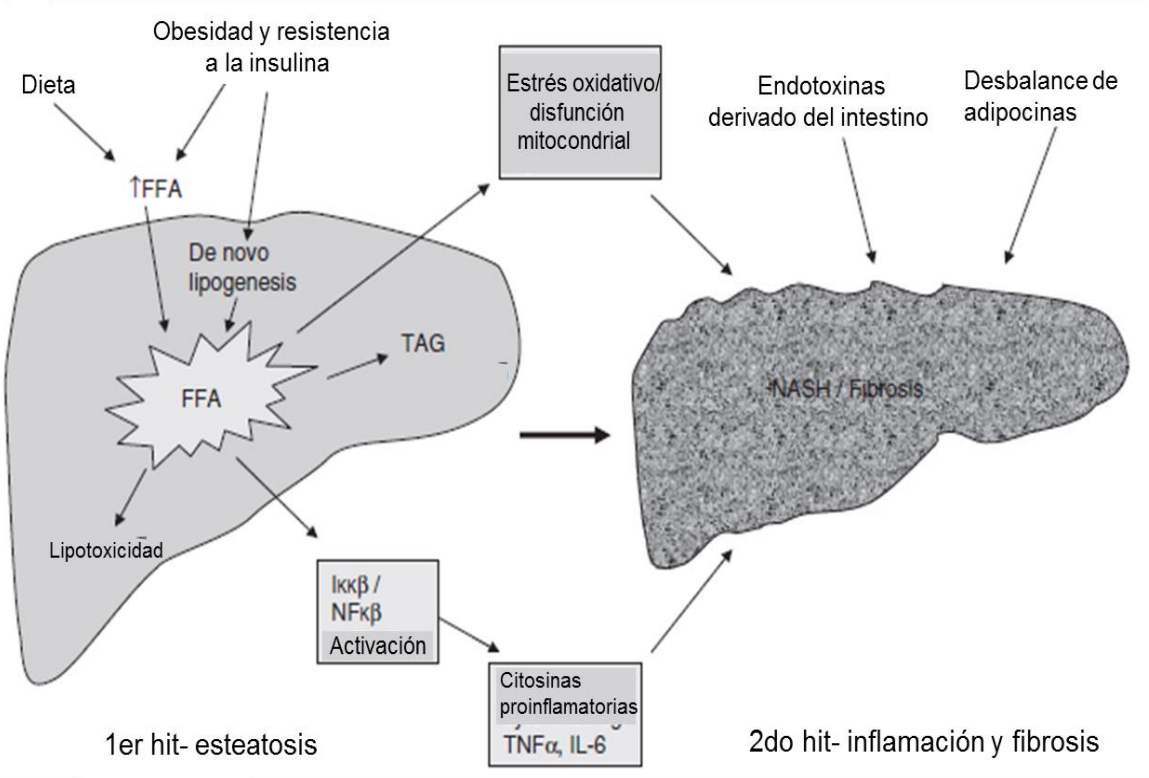

Figura 1. La teoría de los 2 hits: la esteatosis representa el "primer hit", lo cual sensibiliza al hígado para sufrir lesiones mediadas por un "segundo hit", consistente en un aumento de citocinas pro-inflamatorias, de adipocinas, del estrés oxidativo y el desarrollo de disfunción mitocondrial, lo que produce esteatohepatitis y fibrosis. La presencia de altos niveles de estrés oxidativo reduce la capacidad proliferativa de los hepatocitos maduros, lo que resulta en una reducción en la reparación del hígado (Modificado de Dowman et al, 2009).

1.2 Mitocondria

Las mitocondrias son orgánulos intracelulares presentes en todas las células eucariotas. Son las encargadas de suministrar la mayor parte de la energía necesaria para la actividad celular, sintetizan ATP a expensas de la glucosa, los ácidos grasos y los aminoácidos por medio de la fosforilación oxidativa (Alberts et al., 2002). Durante las reacciones enzimáticas involucradas en la glucólisis, en la oxidación de ácidos grasos y en el ciclo de Krebs, los equivalentes reductores nicotinamida adenindinucleótido reducido (NADH) y el dinucleótido de flavina y adenina reducido ($FADH_2$), generados por la oxidación de moléculas en dichas vías metabólicas, proveen de electrones a la fosforilación

oxidativa. La cadena respiratoria transfiere los electrones del NADH o el $FADH_2$, hasta el O_2 (Lehninger et al., 2000).

El NADH se oxida en el complejo I de la cadena respiratoria para reducir una molecula de ubiquinona (UQ) asociada a la membrana. El $FADH_2$ se oxida en el complejo II para reducir una molécula de UQ. El ubiquinol reducido (UQH_2) formado en los complejos I y II es oxidado en el complejo III, que a su vez reduce al citocromo *c*. El citocromo *c* reduce al complejo IV, el cual es la oxidasa terminal, al transferir cuatro electrones al oxígeno molecular para formar moléculas de H_2O. Los complejos I, III y IV translocan protones desde la matriz hacia el espacio intermembranal en contra de su gradiente de concentración, aprovechando la energía de las reacciones exergónicas que ocurren en el transporte de electrones. Esto genera un potencial electroquímico de protones a través de la membrana interna que dirige la síntesis del ATP por la F_1F_0 ATP sintasa.

Aunque el oxígeno molecular se reduce a agua en el complejo IV mediante una transferencia secuencial de cuatro electrones, una proporción menor de oxígeno puede reducirse de manera monovalente a radical superóxido ($O_2^{\bullet-}$), lo que ocurre predominantemente en el complejo III (Sugioka et al, 1988) pero también en el complejo I (Herrero y Baria 1997). La ruta de transferencia de electrones cíclica dentro del complejo III implica un sitio cerca de la cara citoplásmica de la membrana donde el UQH_2 transfiere un solo electrón a la subunidad citocromo *c1* a través de la subunidad hierro-azufre de Rieske, lo que genera un radical ubisemiquinona ($UQ^{\bullet}$) altamente reactivo. La pérdida del segundo electrón y la generación de UQ dependen de la transferencia del electrón a través de dos grupos hemo *b* de la subunidad citocromo *b* que se encuentran orientados de modo secuencial en lados opuestos de la membrana interna (Zhang et al, 1998). Esta transferencia se opone al potencial de membrana, de modo que un alto potencial de membrana disminuye la ocupación del sitio de unión a la $UQ^{\bullet}$ en el citocromo *b* por lo que aumenta la probabilidad de que este segundo electrón pueda ser transferido al oxígeno molecular, generando el anión $O_2^{\bullet}$ (Troy et al, 1996).

La mitocondria es fuente de generación de especies reactivas de oxígeno (ERO) y es al mismo tiempo blanco del daño por las mismas, es por ello que el desarrollo de diversas enfermedades se relacionan con el estrés oxidativo mitocondrial, siendo una de ellas la EHNA.

1.3 EHNA y mitocondria

Las mitocondrias, además de ser el compartimento donde ocurren numerosas reacciones bioquímicas esenciales en la homeostasis energética, tienen un papel clave en la muerte y el envejecimiento celular (Scheffler, 2001). Este orgánulo se encuentra constituyendo una red compleja, interconectada y altamente dinámica, mantenida por eventos opuestos y balanceados de fusión y fisión mitocondrial (Westermann, 2002). A este proceso se le ha denominado "dinámica mitocondrial". La fusión mitocondrial está regulada por las proteínas mitofusinas 1 y 2 (Mfn1 y Mfn2) que se localizan en la membrana externa mitocondrial (Chen et al, 2003) y la proteína de la atrofia óptica (OPA1), que se localiza en el espacio intermembranal asociada a la membrana interna mitocondrial, y que participa en el remodelado de las crestas mitocondriales y la fusión de la membrana interna (Olichon et al, 2002). La fisión mitocondrial es dirigida por proteínas como la dinamina 1 (Drp-1), que se localiza principalmente en el citoplasma, pero con una fracción que se localiza en la membrana externa mitocondrial y cuya localización representa futuros puntos de fisión. Se ha sugerido que Drp1 actúa como mecanoenzima que participa activamente en el corte de membranas por constricción (Bossy-Wetzel et al, 2003). Drp1 carece de secuencia destino, por lo que es reclutada hacia la membrana por la proteína Fis1, la cual se encentra inserta en la membrana externa y en conjunto con Drp1 formarán un anillo de escisión para de esta manera poder fisionar las mitocondrias fusionadas (Liesa et al, 2009) (Fig. 2).

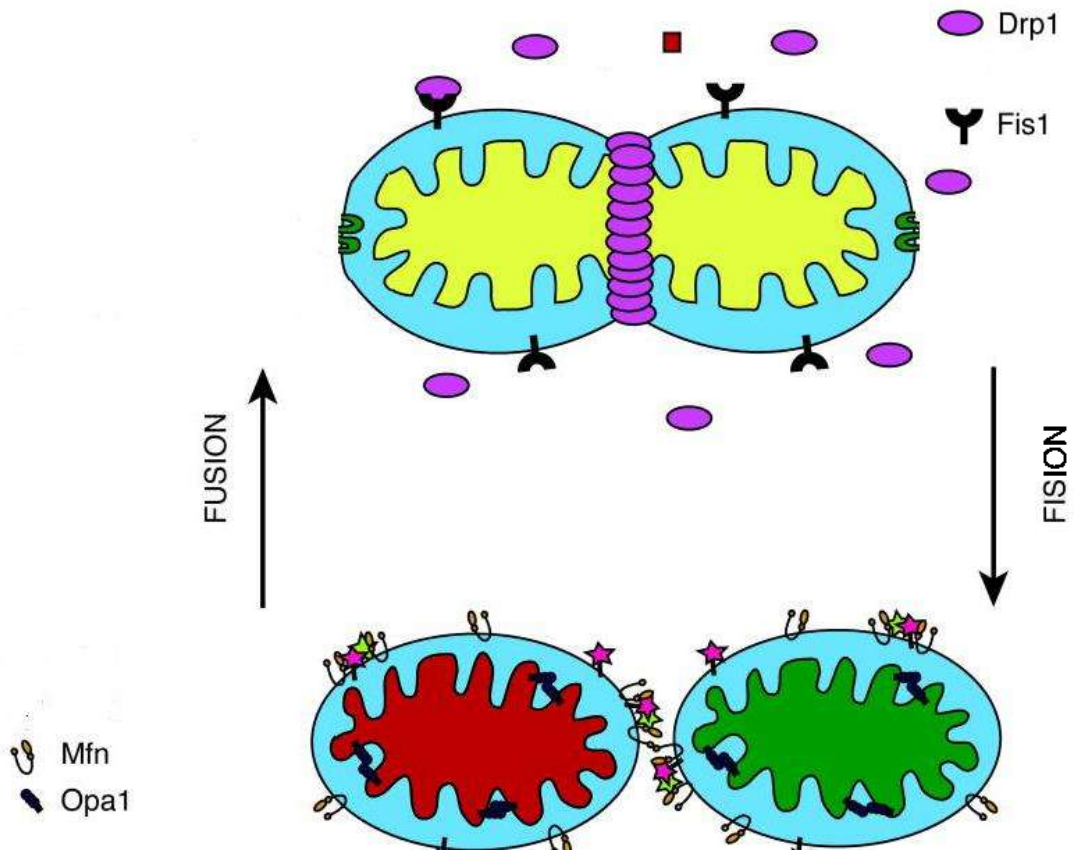

Figura 2. Maquinaria de fusión y fisión mitocondrial. En el esquema se ilustra la localización de las proteínas involucradas en la dinámica mitocondrial. La fisión mitocondrial divide una mitocondria en dos unidades hijas por las acciones coordinadas de Drp1 y Fis1. La unión de dos mitocondrias da como resultado la fusión mitocondrial de las membranas mediadas por Mfn y Opa1 (Modificado de Thomas y Cookson 2009).

La fusión mitocondrial regula directamente el metabolismo mitocondrial, de tal modo que la disminución de la concentración de la proteína OPA1 o de cualquiera de las dos Mfn mediante ARN de interferencia conduce a la formación de mitocondrias fragmentadas con menor consumo de oxígeno y menor potencial de membrana (Chen et al, 2005). Aunque se conoce la función de dichas proteínas en la fusión y el remodelado mitocondrial, su relación con la maquinaria metabólica se desconoce, así como por qué la pérdida de algunas de estas proteínas interfiere directamente con la respiración celular. Por el contrario, la sobrexpresión de Mfn2 incrementa directamente la actividad de los complejos respiratorios, la oxidación mitocondrial y la utilización de glucosa (Pich et al, 2005). La expresión de la Mfn2 está disminuida en el músculo esquelético de ratas Zucker obesas y en pacientes obesos y diabéticos, lo que destaca la importancia patológica de las

alteraciones en la dinámica y la morfología mitocondrial y evidencia que la plasticidad mitocondrial es crítica en el mantenimiento de la función de este organelo (Bach, 2003).

1.4 La dieta como un factor importante en el desarrollo de EHNA

Los hepatocitos desempeñan un papel fundamental en el metabolismo de los carbohidratos, lípidos y proteínas. Los metabolitos derivados del catabolismo de los lípidos y de la glucosa son utilizados por las mitocondrias para generar ATP (Lehninger, 1965). Cada hepatocito contiene aproximadamente 800 mitocondrias (~ 18% del volumen celular total), que desempeñan funciones cruciales en la oxidación de los ácidos grasos y la fosforilación oxidativa (Mitchell, 1961).

La demanda de energía y la ingesta calórica se deben encontrar balanceadas en el organismo. La obesidad es una de las principales anomalías de este equilibrio. La obesidad y sus consecuencias, como la resistencia a la insulina y el síndrome metabólico, son una amenaza creciente para la salud de la población mundial (Keaney, 2003). Una dieta basada en proporciones altas de colesterol, grasas saturadas y fructosa (comida rápida) recapitula las características del síndrome metabólico y la EHPTNA con fibrosis progresiva. La fructosa se ha usado cada vez más como edulcorante desde la introducción de los jarabes de maíz con alto contenido de fructosa en la década de 1960 (Tetri et al, 2008) y ahora es una fuente abundante de carbohidratos en la dieta. El consumo de fructosa en México se ha incrementado considerablemente debido a que se encuentra en los refrescos, los néctares y los jugos son ahora elaborados con mieles fructosadas, siendo estos productos ahora las principales fuentes “hidratantes” en el día a día. El aumento del consumo de estos productos a su vez se ha relacionado con el aumento de la prevalencia de obesidad, diabetes tipo 2 e hígado graso, posicionando a México en segundo lugar en obesidad (OCDE, 2017).

La fructosa fue aceptada previamente como un componente dietético beneficioso porque no estimula la secreción de insulina. Sin embargo, dado que la señalización por insulina juega un papel importante en los mecanismos centrales de desarrollo de la EHNA, esta propiedad de la fructosa puede ser indeseable (Teff et al, 2004). La fructosa es metabolizada principalmente en el hígado, a una tasa excepcionalmente alta debido a una

cantidad extensa de fructocinasa en este órgano, la cual fosforila la fructosa a fructosa 1-fosfato, y la subsecuente conversión de la fructosa 1-fosfato a triosa fosfato. A diferencia de lo que ocurre con la glucolisis, esto sucede a una alta tasa independientemente de la presencia de altos niveles de ATP o de NADH porque el metabolismo de la fructosa evita el control de flujo ejercido por la fosfofructocinasa, la cual es el primer paso de la glucólisis (James et al, 2004). Estudios previos comparando el metabolismo de la fructosa y la glucosa en post-absorción en intervalos cortos han demostrado que la fructosa se metaboliza más rápido que la glucosa. El consumo de fructosa en grandes cantidades puede provocar estrés hepático al disminuir la energía hepática por el alto consumo de ATP durante la fosforilación de la fructosa y al generar altos niveles de ácido úrico debido a la alta acumulación de AMP (van den Berghe et al, 1977).

1.5 Alternativas para el tratamiento de la EHNA

Para el tratamiento de esta enfermedad la aproximación principal es generar cambios en el estilo de vida del paciente, lo que incluye modificaciones en la dieta e incluir al ejercicio físico de manera cotidiana. La mejor evidencia de pérdida de peso como medio para mejorar la histología hepática en pacientes con EHPTNA proviene de un ensayo que asignó al azar a 31 personas obesas con EHPTNA con cambios intensos en el estilo de vida (modificación de la dieta y 200 minutos semanales de actividad física moderada durante 48 semanas). Con estos cambios se obtuvo una pérdida de peso del 9,3% y condujo a una mejoría en la esteatosis, en la necrosis y en la inflamación lobular, pero no en la fibrosis (Promrrat et al, 2010).

Se han sugerido algunas alternativas farmacológicas para el tratamiento de la EHNA tales como el uso de la metformina, donde se ha observado una disminución de la resistencia a la insulina y de los niveles séricos de las aminotransferasas, pero ninguna mejora significativa en la histología hepática de pacientes con EHPTNA (Uygun et al, 2004).

También se ha estudiado el efecto de tiazolidinedionas como la pioglitazona y rosiglitazona sobre las aminotransferasas y la histología hepática en adultos con EHPTNA, donde se ha observado que el consumo de estos fármacos mejora la esteatosis hepática y los niveles de aminotransferasas, pero no la inflamación y la necrosis. La pioglitazona se puede usar para tratar la EHPTNA diagnosticada por biopsia. Sin embargo, se debe tener en cuenta que la mayoría de los pacientes que participaron en ensayos clínicos que investigaron pioglitazona para tratar EHPTNA no eran diabéticos y que la seguridad y eficacia a largo plazo de pioglitazona en pacientes con EHPTNA no está establecida (Ratziu et al, 2008). En general, no existe ningún fármaco aprobado por la Food and Drug Administration (FDA) en los Estados Unidos de América para el tratamiento de la EHNA.

El estrés oxidativo se considera un mecanismo clave de la lesión hepatocelular y la progresión de la enfermedad a EHPTNA. La vitamina E es un antioxida
investigado su uso en el tratamiento de la EHNA. En los diferentes ensayos clínicos con vitamina E se han utilizado diversos criterios para el ingreso al estudio, diferentes dosis y formulaciones de vitamina E poco claras de vitamina E que podrían afectar su biodisponibilidad, el uso adicional de otros antioxidantes u otros fármacos y existen datos histológicos limitados para evaluar los resultados. A pesar de estas limitaciones, se puede resumir que (1) el uso de vitamina E se asocia con una disminución de las aminotransferasas en sujetos con EHPTNA; (2) los estudios en los que se evaluaron los puntos finales histológicos indican que la vitamina E causa mejoría en la esteatosis, la inflamación y el ballooning hepatocelular; (3) la vitamina E no tiene ningún efecto sobre la fibrosis hepática (Dufour et al, 2006; Yakaryilamz et al, 2007).

1.6 Características y propiedades del aceite de aguacate

El aceite de aguacate (*Persea americana Mill*) posee una alta proporción de ácidos grasos monoinsaturados, siendo su componente principal el ácido oleico (C18:1), al cual se le han atribuido algunos efectos benéficos tales como descenso del colesterol total, del LDL y de triacilglicéridos, con aumento de HDL y mejoría del índice aterogénico en pacientes

con colesterol normal, con hiperlipidemia, con hipertrigliceridemia y con diabetes mellitus tipo 2 (Alvizouri et al., 2009).

Además del alto contenido de ácidos grasos monoinsaturados, se ha reportado que el aceite de aguacate también posee una amplia variedad de antioxidantes tales como la luteína, los α y β-carotenos, la anteraxantina, la neoxantina, la zeaxantina, la violaxantina y la β-criptoxantina, así como tocoferoles (alfa y gamma) y las clorofilas a y b. La más abundante de estas moléculas antioxidantes es la luteína (Ashton et al., 2006).

Respecto a la disfunción mitocondrial durante la diabetes y al consumo de aceite de aguacate, en estudios realizados en un modelo de diabetes tipo I en ratas a las cuales se les administro durante 90 días este aceite, se observó un efecto protector en la funcionalidad de mitocondrias de riñón y de hígado, ya que la administración del aceite protegió contra el daño inducido por la diabetes en la actividad de los complejos I y III, disminuyó el daño sobre los citocromos c+c_1, del complejo III, disminuyó la formación de ERO y la peroxidación de lípidos y aumentó la resistencia al estrés oxidativo, además de que, de manera importante, no se observó desarrollo de esteatosis hepática por la administración del aceite (Ortiz-Avila et al., 2013; 2015). Por lo tanto, es factible proponer que la administración del aceite de aguacate puede disminuir el daño hepático en la diabetes al mejorar la función y la dinámica mitocondrial al prevenir la peroxidación de lípidos, prevenir la fisión mitocondrial excesiva, disminuir la formación de ERO y atenuar la expresión de citosinas pro-inflamatorias que son inducidas por los productos de peroxidación de lípidos y las ERO y que están involucradas en la progresión de la enfermedad hepática no alcohólica.

2. Justificación

La EHNA está vinculada con el desarrollo de diabetes, obesidad y enfermedades cardiovasculares, en las cuales se sabe que la producción mitocondrial de ERO y la peroxidación de lípidos juegan un papel importante en su progresión debido a alteraciones en la dinámica mitocondrial. Se ha observado que el aceite de aguacate disminuye los niveles de ERO y de peroxidación de lípidos, y que mejora el funcionamiento de la cadena respiratoria en mitocondrias de hígado de ratas con diabetes tipo 1. Por lo anterior, en el presente trabajo se propone que el aceite de aguacate puede retrasar el desarrollo de la EHNA, al disminuir el estrés oxidativo en la mitocondria, mejorar el funcionamiento de la cadena respiratoria, y disminuir la producción de ERO y la peroxidación de lípidos, lo cual podría estar asociado con una mejora de la dinámica mitocondrial y disminución de la expresión de citosinas pro-inflamatorias que son activadas por ERO y productos de la peroxidación de lípidos.

3. Hipótesis

El aceite de aguacate revierte el desarrollo de la enfermedad hepática no alcohólica al disminuir las alteraciones en la dinámica y la función mitocondrial asociadas a la peroxidación de lípidos.

4. OBJETIVOS

4.1 Objetivo general

Determinar el efecto del aceite de aguacate en la función y dinámica mitocondrial y su relación con la peroxidación de lípidos en ratas con EHNA.

4.2 Objetivos específicos

1. Evaluar el efecto del aceite de aguacate en la progresión de la EHNA en ratas.

2. Determinar el efecto del aceite de aguacate en la función mitocondrial y en el estrés oxidativo en hígado de ratas con EHNA.

3. Determinar el efecto del aceite de aguacate en el desarrollo de inflamación inducida por la EHNA.

4. Analizar el efecto del aceite de aguacate en la dinámica mitocondrial en hígado de ratas con EHNA.

5. Resultados

Los resultados generados durante la realización del presente proyecto se presentan en los siguientes capítulos:

5.1.Capítulo I

García-Berumen, C. I -Avila, O., Vargas-Vargas, M. A., del Rosario-Tamayo, B. A., Guajardo-López, C., Saavedra-Molina, A., Rodríguez-Orozco, A. R., Cortés-Rojo, C. (2019). The severity of rat liver injury by fructose and high fat depends on the degree of respiratory dysfunction and oxidative stress induced in mitochondria. *Lipids in Health and Disease*, *18*(1), 78.

5.2. Capítulo II

Avocado oil consumption protects mitochondrial function and dynamics trough control of ROS accumulation and inflammatory cytokines expression in liver of rats with Non-Alcoholic Fatty Liver Disease. Escrito no publicado.

García-Berumen et al. Lipids in Health and Disease (2019) 18:78
https://doi.org/10.1186/s12944-019-1024-5

Lipids in Health and Disease

RESEARCH Open Access

The severity of rat liver injury by fructose and high fat depends on the degree of respiratory dysfunction and oxidative stress induced in mitochondria

Claudia Isabel García-Berumen[1], Omar Ortiz-Avila[1], Manuel Alejandro Vargas-Vargas[1], Bricia A. del Rosario-Tamayo[2], Clotilde Guajardo-López[4], Alfredo Saavedra-Molina[1], Alain Raimundo Rodríguez-Orozco[3] and Christian Cortés-Rojo[1*]

Abstract

Background: High fat or fructose induces non-alcoholic fatty liver disease (NAFLD) accompanied of mitochondrial dysfunction and oxidative stress. Controversy remains about whether fructose or fat is more deleterious for NAFLD development. To get more insights about this issue and to determine if the severity of liver disease induced by fructose or fat is related to degree of mitochondrial dysfunction, we compared the effects of diets containing high fat (HF), fructose (Fr) or high fat plus fructose (HF + Fr) on NAFLD development, mitochondrial function, ROS production and lipid peroxidation.

Methods: Wistar rats were assigned to four groups: Control, fed with standard rodent chow; High fat (HF), supplemented with lard and hydrogenated vegetable oil; Fructose (Fr), supplemented with 25% fructose in the drinking water; High fat plus fructose group (HF + Fr), fed with both HF and Fr diets. Rats were sacrificed after 6 weeks of diets consumption and the liver was excised for histopathological analysis by hematoxylin and eosin staining and for mitochondria isolation. Mitochondrial function was evaluated by measuring both mitochondrial respiration and complex I activity. Lipid peroxidation and ROS production were evaluated in mitochondria by the thiobarbituric acid method and with the fluorescent ROS probe 2,4-H_2DCFDA, respectively.

Results: Fr group underwent the lower degree of both liver damage and mitochondrial dysfunction that manifested like less than 20% of hepatocytes with microvesicular steatosis and partial decrease in state 3 respiration, respectively. HF group displayed an intermediate degree of damage as it showed 40% of hepatocytes with microvesicular steatosis and diminution of both state 3 respiration and complex I activity. HF + Fr group displayed more severe damage as showed microvesicular steatosis in 60% of hepatocytes and inflammation, while mitochondria exhibited fully inhibited state 3 respiration, impaired complex I activity and increased ROS generation. Exacerbation of mitochondrial lipid peroxidation was observed in both the Fr and HF + Fr groups.

Conclusion: Severity of liver injury induced by fructose or fat was related to the degree of dysfunction and oxidative damage in mitochondria. Attention should be paid on the serious effects observed in the HF + Fr group as the typical Western diet is rich in both fat and carbohydrates.

Keywords: NAFLD, liver steatosis, Mitochondria, Respiratory chain, Complex I, lipid peroxidation

* Correspondence: christiancortesrojo@gmail.com
[1]Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Edificio B-3, Ciudad Universitaria, 58030 Morelia, Michoacán, Mexico
Full list of author information is available at the end of the article

Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined as excessive hepatic lipid accumulation in individuals whose alcohol intake is not significant and without any other hepatic disease. NAFLD encompasses a spectrum of liver alterations ranging from non-alcoholic fatty liver (NAFL), when steatosis is observed in absence of hepatocyte ballooning, non-alcoholic steatohepatitis (NASH), when steatosis is accompanied of ballooning and inflammation, to cirrhosis [1]. NAFLD is one of the most prevalent liver diseases in the world [2], affecting one third of the population in developed countries and increasing overall liver-related morbidity and mortality [3]. High-fat diets were initially believed to be the primary driver of the obesity epidemic [4], which was associated with increasing NAFLD prevalence [5]. Consequently, fat was substituted by fructose because this carbohydrate, in contrast with glucose, does not stimulate insulin secretion. However, fructose leads to liver stress due to excessive phosphorylation of this carbohydrate at the expense of ATP, causing phosphate deficiency, AMP accumulation, and increased synthesis of both uric acid and triglycerides [6, 7].

Diets with excessive amounts of fructose or fat induce hepatic mitochondrial dysfunction. Fructose increases the supply of electrons to the electron transport chain (ETC) by upregulating the tricarboxylic acid cycle [8], this along with impaired complex IV activity, increased mitochondrial levels of ROS and lipid peroxidation [9]. On the other hand, excessive fat intake impairs mitochondrial respiration in the phosphorylating state (i.e. mitochondrial state 3 respiration), inhibits complex IV activity, increases lipid peroxidation and augments ROS generation [10, 11].

The central role of mitochondrial dysfunction in NAFLD progression has been revealed by its alleviation with mitochondria-targeted strategies. For example, the deletion of the lysocardiolipin acyltransferase ALCAT-1 ameliorated NAFLD by inhibiting cardiolipin remodeling and improving both mitochondrial respiration and oxidative stress [11]. Furthermore, the alleviation of NAFLD in rodents by mitochondria-targeted antioxidants has been attributed to decreased lipid peroxidation, attenuation of oxidative stress and the inhibition of apoptosis [12].

On the other hand, it has been shown that diets containing high fat plus fructose induce more damage in both liver and mitochondria than a diet containing only high fat [13]. However, the effects of fructose alone were not compared with the effects of high fat or high fat plus fructose. This is an important issue to address, because the role of fructose in the development of metabolic syndrome has been put in doubt, thus there is not yet a definitive consensus about whether fat or fructose is more detrimental for NAFLD progression [14, 15]. Furthermore, it is unknown whether the severity of liver disease is related to the degree of mitochondrial dysfunction caused by fructose or fat. To address these issues, we have compared the effects of diets enriched in fructose (Fr), high fat (HF) and high fat plus fructose (HF + Fr) on NAFLD development, dyslipidemia, mitochondrial function, ROS levels and lipid peroxidation.

Materials and methods

Animals and experimental groups

Male Wistar rats weighing 250–350 g were used in this study. Each rat was housed in individual cages and maintained at room temperature with day/night cycles of 12 h/12 h, with free access to diets and water or fructose. Animals were randomly assigned to four groups (Table 1): 1) control group: fed only with standard rodent chow; 2) fructose group (Fr): fed with standard rodent chow plus 25% fructose in the drinking water; 3) high-fat group (HF): fed with the HF diet; 4) high fat plus fructose group (HF + Fr): fed with the HF diet plus 25% fructose in the drinking water. Diets were provided for 6 weeks. Food intake was limited to 20 g daily per rat to avoid the rancidity of not immediately ingesting food in the groups whose diets contained high fat. 250 mL of water or fructose solution was given daily to each rat, except that fructose was given to the HF + Fr group every other day at the beginning of the 4th week, as its consumption decreased significantly at that time in this group. All the procedures with animals were performed according to the Federal Regulations for the Use and Care of Animals (NOM-062-ZOO-1999) issued by the Mexican Ministry of Agriculture.

High-fat diet (HF) was prepared with 47.5% standard rodent chow (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO, USA), 10.1% lard, 40.6% hydrogenated vegetable oil, 1.3% sodium cholate, 0.3% choline chloride and 0.2% thiouracil (Table 1). Standard rodent chow contained 23.9% protein (28.5% of total calories), 5.0% fat

Table 1 Experimental groups and preparation of diets

Diet components	Experimental groups			
	Control	HF	Fr	HF + Fr
Standard rodent chow[b], %	100	47.5	100	47.5
Lard[c], %		10.1		10.1
Hydrogenated vegetable oil[d], %		40.6		40.6
Sodium cholate[e], %		1.3		1.3
Choline chloride[e], %		0.3		0.3
Thiouracil[e], %		0.2		0.2
Fructose[f], %*w/v*			25[a]	25[a]

Diets were provided during 6 weeks to Wistar rats with an initial weight of 250-350 g. [a]Fructose was given in the drinking water. [b]Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO, USA. The composition of this diet can be consulted in [49]. [c]JC Fortes, Empacadora San Benito, México. [d]Manteca Inca, ACH Foods México, S. de R.L. de C.V. [e]Sigma-Aldrich, St. Louis, MO, USA. [f]Grupo Químico Contreras, S.A. de C.V. México

(13.4% of total calories) and 48.7% carbohydrate (58.1% of total calories). HF diet contained 14.7% protein (8.8% of total calories), 53.8% fat (73.2% of total calories) and 29.8% carbohydrate (18.0% of total calories). Laboratory Rodent Diet 5001, which was used as the starting material to prepare the diets, exceeding several-fold the recommendations in vitamins and minerals made by the committee on AIN-93 purified diets for laboratory rodents [16], except for chromium, that is contained in similar concentrations in both diets, and for vitamin E, that is 1.78-fold higher in the AIN-93 diet than in the Laboratory Rodent Diet 5001.

Isolation of mitochondria

At the end of the treatments, rats were fasted 14 h and sacrificed by decapitation. The liver was excised and placed into ice-cold isolation solution (Medium 1) containing 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, and 2 mM MOPS (pH 7.4). The liver was cut, washed, and homogenized with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 314 x g. Subsequently, the supernatant was decanted and centrifuged at 4410 x g. The resulting pellet was washed with a solution (Medium 2) containing 220 mM mannitol, 70 mM sucrose, and 2 mM MOPS (pH 7.4), and centrifuged at 6350 x g. Finally, the pellet was re-suspended in 500 μl of medium 2. Each centrifugation was performed during 10 min at 4 °C [17]. Mitochondrial protein concentration was measured by the Biuret method.

Determination of biochemical parameters in serum and weight gain

At the end of the six-weeks of treatments with diets, rats were fasted for 14 h and blood was recollected after the sacrifice for obtaining serum. Glucose, total cholesterol (TC), and triglyceride (TG) levels were measured by enzymatic methods with kits from VITROS Chemistry Products (Ortho Clinical Diagnostics Inc. Rochester, NY, USA), according to the manufacturer's instructions. Weight gain was determined by subtracting the weight of the animals after the six-weeks of treatments, measured right before sacrifice, minus the weight at the beginning of the treatments.

Histological analyses of livers

Small sections of the livers, obtained at the sacrifice of animals, were fixed in 10% formalin, embedded in paraffin blocks, sectioned (5 μm thick) and stained with hematoxylin and eosin. Light microscopy was used for evaluation of steatosis, inflammation and hepatocyte ballooning. Microvesicular steatosis was evaluated like cytosolic accumulation of little lipid droplets not perturbing the central location of the nucleus. Macrovesicular steatosis was evaluated like cytosolic presence of large lipid drops that move the nucleus from its central position into the cell periphery [18].

Evaluation of mitochondrial respiration

Mitochondrial respiration was measured in basal, oligomycin-induced, state 4 (state 4_O) and phosphorylating state (state 3) by determining the oxygen consumption rate of freshly isolated mitochondria using a Clark-type electrode coupled to a YSI 5300A biological oxygen monitor and connected to a computer for data acquisition. 1.25 mg of mitochondrial protein was placed into a sealed glass chamber containing respiratory buffer with 100 mM KCl, 10 mM HEPES, 3 mM KH_2PO_4 and 3 mM $MgCl_2$ (pH 7.4). The final volume was adjusted to 2.5 mL. Respiration traces were started after adding mitochondria and 10 mM glutamate/malate as respiratory substrate. State 3 was stimulated with 0.2 mM ADP. State 4_O was induced by adding 1.4 μg/mL oligomycin. Respiratory control ratio (RCR) was calculated by dividing the respiration rate in state 3 vs the respiration rate in state 4_O.

Determination of complex I activity

0.1 mg/mL of mitochondrial protein were resuspended in a buffer with 50 mM KH_2PO_4 (pH 6.9) and incubated by 5 min with 1 μg antimycin A and 1 mM KCN in a final volume of 1 mL. Then, 5 mM $K_3Fe(CN)_6$ was added as electron acceptor and absorbance was registered at 340 nm in a Shimadzu UV2550 spectrophotometer. After 1 min, NADH was added and its oxidation was monitored during 4 min. The rate of NADH oxidation was calculated using the molar extinction coefficient of $16.3\ mM^{-1}\ cm^{-1}$ for NADH and the slopes of the time-traces of NADH oxidation.

Measurement of ROS levels

ROS levels were determined by measuring the oxidation of the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). 0.5 mg/mL of intact mitochondria and 1.25 mM H_2DCFDA were incubated in a solution with 100 mM KCl, 10 mM HEPES, 3 mM $MgCl_2$ and 3 mM KH_2PO_4 (pH 7.4) during 20 min at 4 °C under constant stirring. Then, mitochondrial suspension was placed into a quartz cuvette and basal fluorescence was recorded over time. After 1 min, 10 mM glutamate/malate was added as substrate for the ETC and the changes in H_2DCFDA fluorescence were further monitored by 15 min. Fluorescence changes were detected in a RF-5301PC spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan) (λ_{ex} 491 nm; λ_{em} 518 nm). ROS levels were calculated by subtracting the fluorescence (ΔF) detected after 15 min of substrate addition minus the fluorescence detected when substrate was added. ΔF was divided then by the milligrams of mitochondrial protein used in the assay.

Lipid peroxidation assay

Lipid peroxidation was evaluated with the thiobarbituric acid (TBA) method [19]. Mitochondrial pellets (0.1 mg/mL protein) were washed twice and resuspended with 50 mM KH_2PO_4 buffer (pH 7.6) immediately before TBA assay to avoid false positive results due to the interaction of thiobarbituric acid with the carbohydrates present in mitochondria isolation buffers. Lipid peroxidation levels were reported as thiobarbituric acid reactive substances (TBARS) per milligram of mitochondrial protein.

Data analysis

Data are expressed as mean ± standard error of the mean. Statistical differences of data were determined with two-way analysis of variance (ANOVA), followed by multiple comparisons analysis performed with post hoc Tukey test. Statistical significance was set at $P < 0.05$. Analysis were done with Sigma Plot 11.0 software (Systat Software, Inc., San Jose, CA, USA).

Results

Effects of fructose and fat on physiological parameters

Control group showed a weight gain at the end of the study of 59.6 g (Fig. 1a). Higher weight gains of 82.7 and 80.7 g were observed in both Fr and HF groups, respectively. In contrast, there were no differences in weight gains between control and HF + Fr groups. Regarding food consumption, there were no differences in this parameter among all the groups, since the animals fully consumed the amount (20 g) of food that was provided daily. No differences in water or fructose intake were detected within the groups, as all the rats drank the 250 mL that were given daily, except for the HF + Fr group, which started to drink ~ 110 mL fructose at the beginning of the 4th week of treatment.

Serum glucose concentration of the control group was 110 mg/dL (Fig. 1b) and lower levels were detected in the Fr, HF and HF + Fr groups, with values of 90.4, 91.6, and 68.5 mg/dL, respectively. Serum triglycerides levels are shown in the Fig. 1c. In comparison to control group, triglycerides levels were 2.7- and 1.7-fold higher in both Fr and HF + Fr groups, respectively, while no significant changes were observed in the HF group. Serum cholesterol levels (Fig. 1d) augmented moderately in the Fr group when compared to the control group. In contrast, cholesterol levels incremented up to 1.9 - and 1.7 - fold in the HF and HF + Fr groups, respectively.

Effects of fructose and fat on liver histology

Liver histological examination (Fig. 2 and Table 2) shows that livers from the Fr group displayed a lower percentage of hepatocytes with macrovesicular (10–20%, black lines) and microvesicular (15%, dotted arrows) steatosis, followed by the HF group with 40% of hepatocytes with

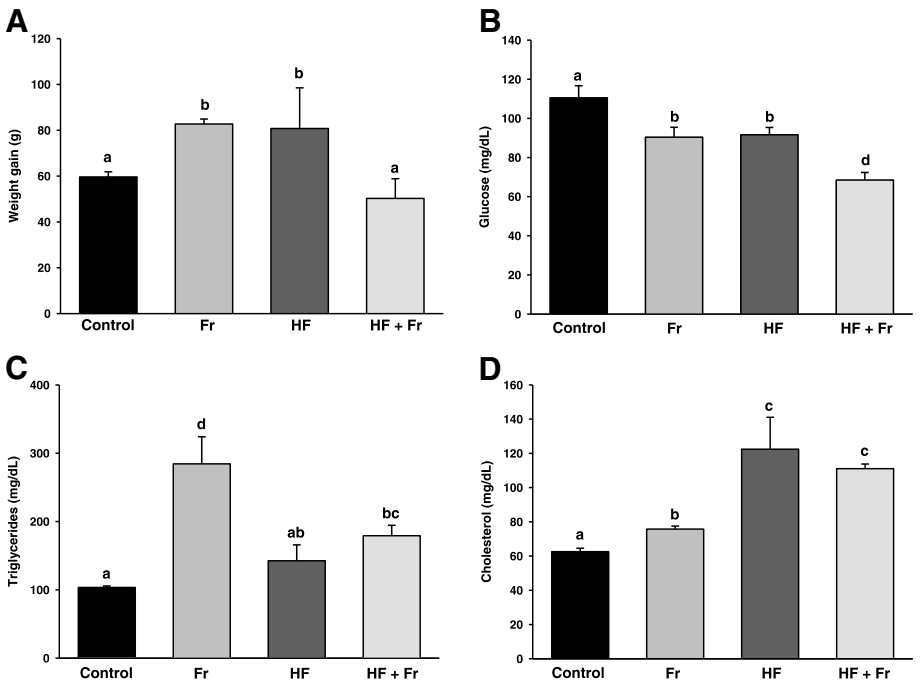

Fig. 1 Weight gain (**a**) fasting serum glucose (**b**), fasting serum triglycerides (**c**) and fasting serum cholesterol (**d**) in rats that were fed for 6 weeks with diets containing normal rodent chow (Control), fructose (Fr), high fat (HF), and high fat plus fructose (HF + Fr). Weight gain was determined by subtracting the weight of the animals after six-weeks treatments with the diets, determined right before sacrifice, minus the weight at the beginning of the treatments. The results are presented as the mean ± S.E. of $n \geq 4$. Different letters indicate statistically significant differences at $P < 0.05$

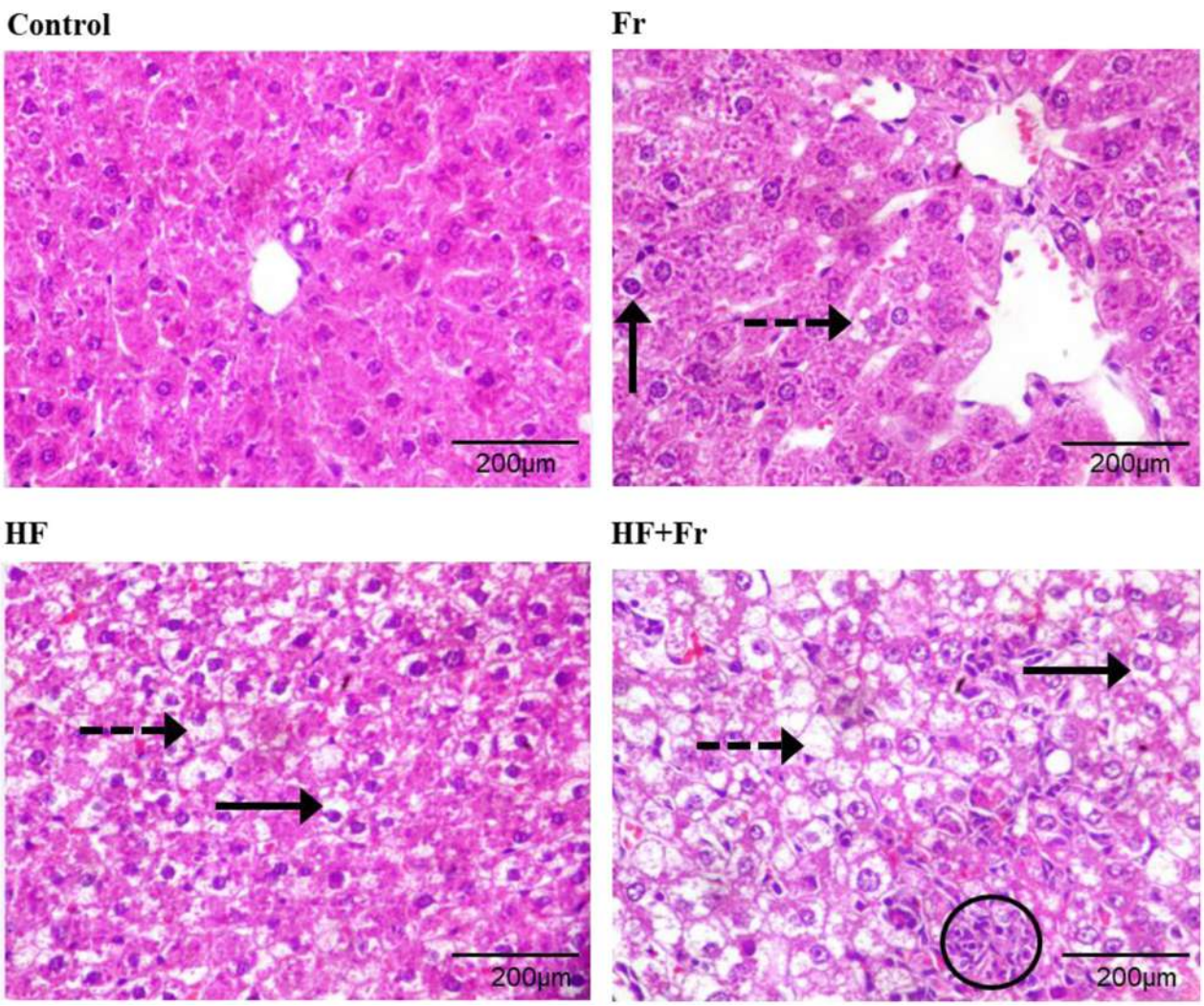

Fig. 2 Histological examination of livers from rats that were fed for 6 weeks with diets containing normal rodent chow (Control), fructose (Fr), high fat (HF) and high fat plus fructose (HF + Fr). Black arrows indicate the presence of macrovesicular steatosis; black dotted arrows indicate the presence of microvesicular steatosis; circle indicates inflammatory infiltrate. Liver sections were stained with hematoxylin-eosin and pictures were taken at 40X magnification

microvesicular steatosis and 60% with macrovesicular steatosis. The highest degree of steatosis was observed in the HF + Fr group, with 60% of hepatocytes with microvesicular steatosis and 80% with macrovesicular steatosis (in some cases, hepatocytes displayed both forms of steatosis). Furthermore, the percentage of ballooned cells was 60% for both Fr and HF groups and 70% for the HF + Fr group. (Fig. 2). In addition, the HF + Fr group also showed chronic inflammatory infiltrate (circle).

Table 2 Quantification of histological alterations induced in the livers of fructose (Fr), high fat (HF) and high fat plus fructose (HF + Fr) groups

Liver alterations	Experimental groups			
	Control	Fr	HF	HF + Fr
Microvesicular steatosis	1%	10–20%	40%	60%
Macrovesicular steatosis	N.D.	15%	60%	80%
Ballooning	N.D.	60%	60%	70%

Data are expressed as the percentage of hepatocytes showing each type of alteration

Effects of fructose and fat on mitochondrial function

In mitochondria from the control group, the respiration rate was ~ 7.7-fold higher in state 3 than in state 4_O, thus resulting in a respiratory control ratio (RCR) of 7.7 (Fig. 3a). Respiration rate in state 3 decreased in the Fr and HF groups 1.6- and 1.4- fold, respectively, in comparison to the control group. This resulted in RCR values of 2.5 and 4.1, respectively. A 1.8-fold increase in state 4 respiration also contributed to the lower RCR observed in the Fr group. Rate of respiration in state 3 decreased 3.9-fold in the HF + Fr group when compared

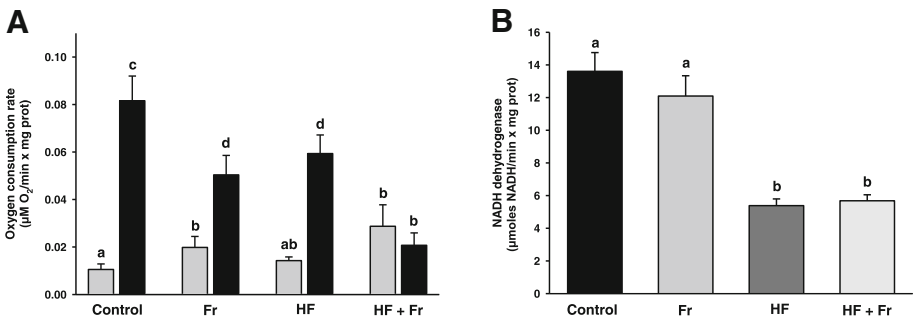

Fig. 3 Rate of respiration (**a**) and complex I activity (**b**) of liver mitochondria from rats that were fed for six weeks with diets containing normal rodent chow (Control), fructose (Fr), high fat (HF) and high fat plus fructose (HF + Fr). Mitochondria were fueled with glutamate – malate. Respiration was measured in state 3 (black bars) and in oligomycin-induced state 4 (gray bars). The results are presented as the mean ± S.E. of $n \geq 3$. Different letters indicate statistically significant differences at $P < 0.05$

to the control group, while state 4 respiration increased 2.7-fold, which resulted in a RCR of 0.7. On the other hand, complex I activity decreased ~ 2.3-fold in mitochondria for both the HF and the HF + Fr groups (Fig. 3b). In contrast, the activity in the Fr group remained unaltered.

Effects of fructose and fat on mitochondrial ROS generation and lipid peroxidation

Mitochondrial ROS levels doubled in the HF + Fr group in comparison to the control group (Fig. 4a). In contrast, no changes in the ROS levels were found in mitochondria for both the Fr and the HF groups. The levels of mitochondrial lipid peroxidation are presented in the Fig. 4b. In comparison to mitochondria of the control group, the levels of lipid peroxidation increased ~ 8 times in mitochondria from both the Fr and the HF + Fr groups, while no changes were detected in the HF group.

Discussion

Inclusion of fructose and/or fat in the diet induced a variable degree of hepatic damage. The HF + Fr group exhibited the most negative outcome, as evidenced by the higher percentage of hepatocytes with microvesicular and macrovesicular steatosis and inflammation. The HF group showed an intermediate presence of steatosis; while the lowest percentage of hepatocytes with both types of steatosis was observed in the Fr group (Fig. 2 and Table 2).

As shown in Fig. 2, deleterious effects of fat were aggravated by fructose, since the HF group exhibited a lower extent of steatosis than the HF + Fr group (Fig. 2 and Table 2), besides the latter group also exhibited inflammation. In this regard, it has been hypothesized that the severity of liver damage correlates with the degree of oxidative stress in hepatocytes [20]. Accordingly, it was observed that the consumption of fat did not increase lipid peroxidation per se, as can be seen in the HF group (Fig. 4b). In contrast, fructose alone or combined with fat increased several-fold the levels of lipid peroxidation. Lipid peroxidation has been identified as an elicitor of NASH by triggering signaling cascades that mediates inflammation via an augmentation in the levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), the end-products of lipid peroxidation [21, 22]. This suggest that lipid peroxidation induced by fructose might be triggering inflammation only when a higher degree of

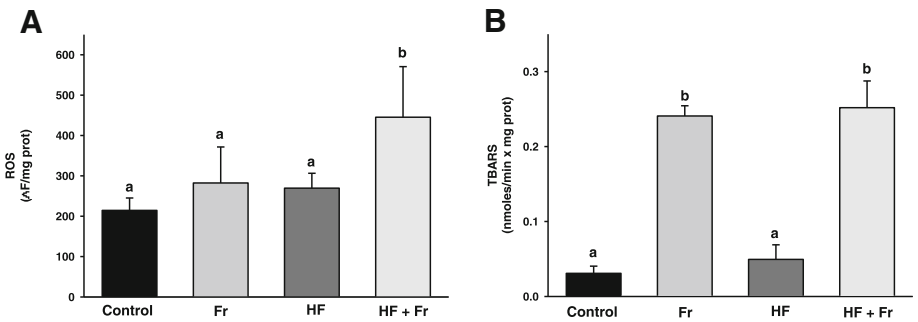

Fig. 4 Levels of ROS (**a**) and lipid peroxidation (**b**) of liver mitochondria from rats that were fed for six weeks with diets containing normal rodent chow (control), fructose (Fr), high fat (HF) and high fat plus fructose (HF + Fr). The results are presented as the mean ± S.E. of n ≥ 3. Different letters indicate statistically significant differences at $P < 0.05$

steatosis is established by high fat intake. Nevertheless, it cannot be ruled out that an increase of lipid peroxidation and inflammation might occurs by the consumption of a HF diet over a longer period time. Despite the Fr group exhibited high levels of mitochondrial lipid peroxidation, it also showed a low level of steatosis without changes in ROS production, which suggest that mitochondrial lipid peroxidation per se does not cause severe liver damage in the absence of other factors such as significant accumulation of fat or increased mitochondrial ROS production as was only observed in the HF + Fr group (Figs. 1 and 4a).

Another factor that may explain the presence of inflammation in the HF + Fr group is the pronounced mitochondrial dysfunction resulting from a sharp decrease in state 3 respiration yielding a RCR of 0.7 (Fig. 3a), which can be interpreted as a full impairment of oxidative phosphorylation. ATP depletion due to both augmented fructose metabolism [7] and impaired oxidative phosphorylation (Fig. 3a) might lead to cell death by necrosis in livers of the HF + Fr group, as ATP depletion is a hallmark of necrosis [23]. In turn, liver necrosis drives the release of intracellular content and activation of macrophages and neutrophils [24], which would be in concordance with the presence of inflammatory infiltrate seen in the HF + Fr group (Fig. 2).

A relationship was not found between the levels of lipid peroxidation and ROS, as lipid peroxidation increases in both the Fr and the HF + Fr groups, but ROS levels increases only in the latter one (Fig. 4). The absence of an apparent correlation among ROS and lipid peroxidation suggest that other factors different from ROS generation were responsible for increased lipid peroxidation by fructose. In this regard, induction of lipid peroxidation in liver by fructose has been associated to depletion of antioxidant defenses [25]. Lipid peroxidation is counteracted in mitochondria by phospholipid hydroperoxide glutathione peroxidase 4 (GPx4) using reduced glutathione (GSH) as an electron donor [26]. One catalytic round of GPx4 produces a molecule of oxidized glutathione (GSSG) that it is reduced back to two molecules of GSH by glutathione reductase (GR). It has been found that methylglyoxal (MGO), a reactive dicarbonyl involved in the production of advanced glycation end-products [27], inhibits the activities of GPx and GR [28]. Thus, a possible explanation for the several-fold increase of lipid peroxidation observed exclusively in the groups consuming fructose is that GPx4 and GR becomes inactivated by MGO produced during the hepatic metabolism of fructose.

Higher levels of ROS observed in mitochondria from the HF + Fr group (Fig. 4a) correlates well with null stimulation of respiration by ADP that yielded an RCR of 0.7 (Fig. 3a). Decreased respiration due to inhibition of ATP production by F_1F_0-ATP synthase leads to high rates of ROS production [29]. The lack of response to ADP addition can be interpreted like an impairment of F_1F_0-ATP synthase to produce ATP, which leads to decreased electron transfer and increased ROS generation. Furthermore, the partial decline of state 3 respiration observed in both the HF and the Fr groups, and therefore, their RCR of 4.1 and 2.5, respectively, is in concordance with their lower levels of ROS with respect to the HF + Fr group. Overall, these data suggest that the main factor increasing ROS production was the full impairment of state 3 respiration, although it cannot be discarded that severe impairment in the activity of antioxidants systems (e.g. glutathione system) may also account for the higher ROS levels observed in the HF + Fr group.

Low rates of mitochondrial respiration in the liver increase the $NADH/NAD^+$ ratio [30], which leads to defective fatty acid β-oxidation and hepatic accumulation of triglycerides [31, 32]. This could explain exacerbated steatosis in the HF + Fr group (Fig. 2) as mitochondria from this group displayed the lower rate of state 3 respiration (Fig. 3a). Conversely, the lower severity of steatosis observed in the HF and Fr groups agree with their higher rates of state 3 respiration. On the other hand, complex I activity was lower in the HF group than in the Fr group (Fig. 3b); this might be involved in the higher degree of steatosis observed in the HF group by virtue of the role of complex I in NADH re-oxidation (i.e., the lower the complex I activity, the higher the NADH levels) and the dependence of fatty acid catabolism on low $NADH/NAD^+$ ratios. On this basis, it can be hypothesized that higher accumulation of fat in the livers of HF group was due to a low rate of NADH re-oxidation caused by a highly decreased complex I activity, while the Fr group had a low hepatic fat accumulation thanks to a fully functional complex I. In summary, there is an inverse relationship between complex I activity, the rate of state 3 respiration, and steatosis severity, which might be related to modulation of fatty acid β-oxidation rate exerted by $NADH/NAD^+$ ratio and with the role of complex I activity on NADH redox turnover.

It has been considered that macrovesicular steatosis has a good prognosis when presented alone, with rare progression to NASH or cirrhosis. Microvesicular steatosis, by the contrary, is a less benign entity than macrovesicular steatosis because it has a serious prognosis and it is associated to impaired β-oxidation [33]. Taking into account the association between impaired β-oxidation and the presence of microvesicular steatosis, it can be postulated that excessive fat deposition in the HF + Fr group might be largely due to the inability of mitochondria to re-oxidize NADH molecules produced during the β-oxidation, because low complex I activity in conjunction with fully inhibited oxidative phosphorylation. In

the case of the HF group, intermediate fat deposition would be the result of low complex I activity along with partially functional oxidative phosphorylation, which may allow to re-oxidize NADH at intermediate levels. Finally, the relatively low deposition of fat in the Fr group would be attributed to higher capacity for NADH oxidation than in the other groups due to unaffected complex I activity and partially functional oxidative phosphorylation.

The pronounced deleterious effects of the HF + Fr diet are not trivial since the typical Western diet is abundant in high fat and high carbohydrates [34]. Besides, our results are consistent with another study showing that a high fat diet was significantly less deleterious than a high fat plus fructose diet, as the former induced only steatosis while the latter caused NASH. Furthermore, it was concluded that fructose was responsible for NASH development as the knockdown of the fructokinase gene prevented inflammation and fibrosis [35], which is also in line with our suggestion that fructose worsens the effects of fat by inducing necrotic cell death and inflammation. In contrast, it was shown in another report, that in comparison to our results, a high fat plus fructose diet had discrete effects on both respiration and lipid peroxidation in rat liver mitochondria [13], besides there were no differences between the effects produced by a high fat diet and the high fat plus fructose diet on these parameters. However, it must be stressed that in that work, diets were supplied for a shorter time (2 weeks) in comparison to this study (6 weeks), which may explain these different outcomes.

Several studies have shown that fructose does not affect weight gain [36–38], while others agree with our finding of increased weight by fructose [39–41]. Tillman et al. [38] have proposed that differences in weight response to fructose might be attributed to the way fructose was provided to animals (i.e. liquid or solid). For example, fructose was administered like aqueous solutions with high fructose corn syrup (HFCS) [39] or sucrose [41] in studies where fructose induced positive weight gain. In contrast, pelleted diet containing 60% fructose was given in studies showing no alteration on body mass [36–38]. Nevertheless, in one of these studies where weight gain was observed, sucrose was given in solid form [40]. Thus, there is no clear relation between the physical form in which fructose is administered and its effects on weight gain. Another possibility might be the different effects that fructose-containing carbohydrates may have in anorexigenic hormones like leptin. Fructose promotes weight gain by decreasing leptin blood levels and dysregulating its actions in energy balance [42]. On the contrary, it would be expected that leptin actions on body weight were not so altered with the HFCS or sucrose-containing diets, since these carbohydrates also contains glucose, which may counteract the effects of fructose on leptin secretion by inducing insulin secretion. However, this explanation does not fit with the outcomes of the studies referred above, since sucrose or HFCS was given in the studies where increased weight gain occurred [39–41]. In contrast, fructose was administered in the studies where no differences in weight gain were observed [36–38], which conflicts with our finding about weigh gain with fructose (Fig. 1a). Thus, the possibility remains that Wistar rats, the strain used in this study, present a different phenotypical response to fructose ingestion with respect to body mass, which guarantee further research to compare the effects of HFCS, sucrose or fructose, given in aqueous solution or pelleted, on the body mass of rat strains with different genetic backgrounds.

It was unexpected that there were no differences in weight gain between the HF + Fr and the control groups, and that even weight gain of the HF + Fr group was lower than that observed in both the HF and Fr groups (Fig. 1a). The most likely explanation for this finding is that fructose intake decreased more than 50% in the HF + Fr group at the beginning of the 4th week of treatment, which would be limiting calorie intake, and hence body weight gain. On the other hand, it can be hypothesized that lower weight gain in the HF + Fr group with respect to both the HF and the Fr groups may be due to combined result of exacerbated liver damage, highly impaired mitochondrial function, and a probable hyperinsulinemic status in rats of the HF + Fr group. Fructose is known to induce hyperinsulinemia [42]. Hyperinsulinemia in turn stimulates lipolysis in adipocytes and the release of free fatty acids into the bloodstream. Once in the liver, fatty acids are incorporated into triglycerides for their exportation in VLDL particles. Nevertheless, hyperinsulinemia also inhibits VLDL exportation to adipocytes [43], which may contribute in conjunction with increased lipolysis in adipocytes to decrease peripheric adiposity, as well to reduce fasting triglyceride levels as was observed in the HF + Fr group with respect to the Fr group (Fig. 1c). Probable hyperinsulinemic status might promote de novo triglyceride synthesis [44]; however, the deep impairment of oxidative phosphorylation in the HF + Fr group (Fig. 3a), and hence, the consequent failure in ATP synthesis, may counteract lipogenesis as this process is highly dependent on ATP [45], in this way leading to low availability of triglycerides to be exported to adipose tissue via VLDL. Probable inhibition of triglyceride synthesis also agrees with the severe degree of liver damage and higher levels of ROS observed in the HF + Fr group (Figs. 2 and 4a, respectively), as hepatic accumulation of free fatty acids due to inhibited lipogenesis produces oxidative stress and liver damage [44]. It may be argued against this hypothesis that the Fr group would have also exhibited a similar phenotype of

decreased weight gain due to fructose-induced hyperinsulinemia. However, the different outcomes observed among the HF + Fr and the Fr groups for the levels of glucose and triglycerides, and in weight gain suggest important differences between them in hormonal metabolic regulation. The idea that the HF + Fr diet induces lower gain weight associated to hyperinsulinemia is supported by another study where reduction of weight gain, hyperinsulinemia and severe liver damage was found in rats fed with a HF + Fr + ethanol diet [46], although this comparison must be taken with caution, as the degree at which ethanol contributes to this phenotype has not been elucidated.

Regarding the impact of diets on blood lipids, the more prominent effect was the increase in triglycerides in the Fr group (Fig. 1c), which agrees with the hyperlipidemic effect of this carbohydrate due to stimulation of de novo lipogenesis [47]. Unexpectedly, serum triglycerides in the HF + Fr group were 1.6-fold lower than in the Fr group. A possible explanation for this observation is that the inhibition of oxidative phosphorylation in the HF + Fr group (Fig. 3a) might be decreasing the rate of the de novo lipogenesis since the latter process is highly dependent on ATP produced by mitochondrial respiration [45]. On the other hand, the hypercholesterolemic effect of the diets containing HF (Fig. 1d) was not surprising due to the high content of cholesterol in the lard used for diets preparation.

A limitation of this study is that we did not determine if the severity of NAFLD induced by high fat or fructose is dependent on differential expression of pro-inflammatory cytokines like IL-6 or TNFα, which have been involved in NAFLD pathogenesis [48]. Likewise, we did not analyze the impact of the mitochondrial alterations observed with each diet on cytokine expression profile. Another limitation is that we did not examine the impact over NAFLD progression of inhibiting lipid peroxidation, ROS generation, or of counteract the impairment of oxidative phosphorylation. All that information would be useful to establish a causal link between the alterations induced by high fat or fructose in mitochondrial function and the progression of NAFLD. Another limitation is the lack of data about the $NADH/NAD^+$ ratio, the hepatic triglyceride content, and the rate of mitochondrial β-oxidation, which would allow to verify the negative influence of fructose or fat in the mitochondrial utilization of fatty acids and its relationship with the severity of steatosis. Finally, we did not analyze the effects of fructose and high fat for longer time periods.

Conclusions

Despite producing the higher levels of lipid peroxidation, fructose provoked the less deleterious effects on both mitochondrial function and NAFLD since this carbohydrate partially decreased oxidative phosphorylation and induced the lower percentage of microvesicular steatosis. High fat exerted intermediate effects that manifested as decreasing in both oxidative phosphorylation and complex I activity in mitochondria, and intermediate levels of steatosis. The combination of high fat plus fructose produced the more deleterious effects in mitochondria including lipid peroxidation, enhanced ROS production, decreased complex I activity, and the full inhibition of oxidative phosphorylation, which fits well with the more severe liver damage induced by this diet that manifested as high levels of steatosis and inflammation. The latter might have implications for development of innovative strategies against NAFLD as the Western diet involves the simultaneous intake of excessive fructose and high fat. Hence, therapeutic approaches should be focusing in counteracting lipid peroxidation, excessive ROS production, and enhancing both oxidative phosphorylation and complex I activity.

Abbreviations

2,4-H_2DCFDA: 2′,7′-dichlorodihydrofluorescein diacetate; 4-HNE: 4-hydroxynonenal; ALCAT-1: acyl-CoA:lysocardiolipin acyltransferase 1; ANOVA: analysis of variance; EGTA: ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; ETC: electron transport chain; Fr: fructose; GPx4: phospholipid hydroperoxide glutathione peroxidase 4; GR: glutathione reductase; GSH: reduced glutathione; GSSG: oxidized glutathione; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HF + Fr: high fat plus fructose; HF: high fat; MDA: malondialdehyde; MGO: methylglyoxal; MOPS: 3-(N-Morpholino)propanesulfonic acid; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; RCR: respiratory control ratio; ROS: reactive oxygen species; TBA: thiobarbituric acid; TBARS: thiobarbituric acid reactive substances; TC: total cholesterol; TG: triglycerides

Acknowledgements

English language assistance was provided by Fidelmar Cortés Pérez.

Funding

This work was funded by a grant from Programa de Investigación 2018–2019 de la Coordinación de la Investigación Científica, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México (3757633 to CC-R).

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CC-R and ARR-O conceived and designed the study. CIG-B, MAV-V and OO-A prepared and administered the diets, prepared the livers for histological analyses, performed the experiments in mitochondria and measured blood biochemical parameters. BAR-T and CG-L analyzed and interpreted the histological preparations. CC-R, AS-M and ARR-O analyzed and interpreted the data, CC-R and ARR-O drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval

All the procedures with animals were performed according to the Federal Regulations for the Use and Care of Animals (NOM-062-ZOO-1999) issued by the Mexican Ministry of Agriculture. The experimental protocol was also approved by the Institutional Committee for Use of Animals of the Universidad Michoacana de San Nicolás de Hidalgo.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Author details

[1]Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Edificio B-3, Ciudad Universitaria, 58030 Morelia, Michoacán, Mexico. [2]Hospital General Regional 36, Instituto Mexicano del Seguro Social – IMSS, 72090 Puebla, Puebla, Mexico. [3]Facultad de Ciencias Médicas y Biológicas "Dr. Ignacio Chávez", Universidad Michoacana de San Nicolás de Hidalgo, 58020 Morelia, Michoacán, Mexico. [4]Hospital Regional de Alta Especialidad del Instituto de Seguridad y Servicios Sociales de los Trabajadores del Estado – ISSSTE, Carr. Morelia-Atapaneo Km 6, Atapaneo, 58300 Morelia, Michoacán, Mexico.

Received: 27 December 2018 Accepted: 21 March 2019
Published online: 30 March 2019

References

1. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M, Sanyal AJ. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. Gastroenterology. 2012;142:1592–609.
2. Loomba R, Sanyal AJ. The global NAFLD epidemic. Nat Rev Gastroenterol Hepatol. 2013;10:686–90.
3. Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. Science. 2011;332:1519–23.
4. Chanmugam P, Guthrie JF, Cecilio S, Morton JF, Basiotis PP, Anand R. Did fat intake in the United States really decline between 1989–1991 and 1994–1996? J Am Diet Assoc. 2003;103:867–72.
5. Said A, Ghufran A. Epidemic of non-alcoholic fatty liver disease and hepatocellular carcinoma. World J Clin Oncol. 2017;8:429–36.
6. Abdelmalek MF, Suzuki A, Guy C, Unalp-Arida A, Colvin R, Johnson RJ, Diehl AM. Increased fructose consumption is associated with fibrosis severity in patients with nonalcoholic fatty liver disease. Hepatology. 2010;51:1961–71.
7. Lanaspa MA, Sanchez-Lozada LG, Choi YJ, Cicerchi C, Kanbay M, Roncal-Jimenez CA, Ishimoto T, Li N, Marek G, Duranay M, Schreiner G, Rodriguez-Iturbe B, Nakagawa T, Kang DH, Sautin YY, Johnson RJ. Uric acid induces hepatic steatosis by generation of mitochondrial oxidative stress: potential role in fructose-dependent and -independent fatty liver. J Biol Chem. 2012; 287:40732–44.
8. Satapati S, Sunny NE, Kucejova B, Fu X, He TT, Méndez-Lucas A, Shelton JM, Perales JC, Browning JD, Burgess SC. Elevated TCA cycle function in the pathology of diet-induced hepatic insulin resistance and fatty liver. J Lipid Res. 2012;53:1080–92.
9. Sil R, Chakraborti AS. Oxidative inactivation of liver mitochondria in high fructose diet-induced metabolic syndrome in rats: effect of glycyrrhizin treatment. Phytother Res. 2016;30:1503–12.
10. Mantena SK, Vaughn DP, Andringa KK, Eccleston HB, King AL, Abrams GA, Doeller JE, Kraus DW, Darley-Usmar VM, Bailey SM. High fat diet induces dysregulation of hepatic oxygen gradients and mitochondrial function in vivo. Biochem J. 2009;417:183–93.
11. Wang L, Liu X, Nie J, Zhang J, Kimball SR, Zhang H, Zhang WJ, Jefferson LS, Cheng Z, Ji Q, Shi Y. ALCAT1 controls mitochondrial etiology of fatty liver diseases, linking defective mitophagy to steatosis. Hepatology. 2015;61:486–96.
12. Ajith TA. Role of mitochondria and mitochondria-targeted agents in non-alcoholic fatty liver disease. Clin Exp Pharmacol Physiol. 2018;45:413–21.
13. Crescenzo R, Bianco F, Coppola P, Mazzoli A, Tussellino M, Carotenuto R, Liverini G, Iossa S. Fructose supplementation worsens the deleterious effects of short-term high-fat feeding on hepatic steatosis and lipid metabolism in adult rats. Exp Physiol. 2014;99:1203–13.
14. Zelman K. The great fat debate: a closer look at the controversy-questioning the validity of age-old dietary guidance. J Am Diet Assoc. 2011;111:655–8.
15. Stanhope KL. Sugar consumption, metabolic disease and obesity: the state of the controversy. Crit Rev Clin Lab Sci. 2016;5:52–67.
16. Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr. 1993; 123:1939–51.
17. Saavedra-Molina A, Devlin TM. Effect of extra-and intra-mitochondrial calcium on citrulline synthesis. Amino Acids. 1997;12:293–8.
18. Le TT, Ziemba A, Urasaki Y, Brotman S, Pizzorno G. Label-free evaluation of hepatic microvesicular steatosis with multimodal coherent anti-stokes Raman scattering microscopy. PLoS One. 2012;7:e51092.
19. Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol. 1978;52:302–10.
20. Seki S, Kitada T, Yamada T, Sakaguchi H, Nakatani K, Wakasa K. In situ detection of lipid peroxidation and oxidative DNA damage in non-alcoholic fatty liver diseases. J Hepatol. 2002;37:56–62.
21. Alwahsh SM, Xu M, Seyhan HA, Ahmad S, Mihm S, Ramadori G, Schultze FC. Diet high in fructose leads to an overexpression of lipocalin-2 in rat fatty liver. World J Gastroenterol. 2014;20:1807–21.
22. George J, Pera N, Phung N, Leclercq I, Yun Hou J, Farrell G. Lipid peroxidation, stellate cell activation and hepatic fibrogenesis in a rat model of chronic steatohepatitis. J Hepatol. 2003;39:756–64.
23. Skulachev VP. Bioenergetic aspects of apoptosis, necrosis and mitoptosis. Apoptosis. 2006;11:473–85.
24. Farrell GC, van Rooyen D, Gan L, Chitturi S. NASH is an inflammatory disorder: pathogenic, prognostic and therapeutic implications. Gut Liver. 2012;6:149–71.
25. Armutcu F, Kanter M, Gurel A, Unalacak M. Excessive dietary fructose is responsible for lipid peroxidation and steatosis in the rat liver tissues. Turkiye Klinikleri J Med Sci. 2007;27:164–9.
26. Cozza G, Rossetto M, Bosello-Travain V, Maiorino M, Roveri A, Toppo S, Zaccarin M, Zennaro L, Ursini F. Glutathione peroxidase 4-catalyzed reduction of lipid hydroperoxides in membranes: the polar head of membrane phospholipids binds the enzyme and addresses the fatty acid hydroperoxide group toward the redox center. Free Radic Biol Med. 2017; 112:1–11.
27. Allaman I, Bélanger M, Magistretti PJ. Methylglyoxal, the dark side of glycolysis. Front Neurosci. 2015;9:23.
28. Park YS, Koh YH, Takahashi M, Miyamoto Y, Suzuki K, Dohmae N, Takio K, Honke K, Taniguchi N. Identification of the binding site of methylglyoxal on glutathione peroxidase: methylglyoxal inhibits glutathione peroxidase activity via binding to glutathione binding sites Arg 184 and 185. Free Radic Res. 2003;37:205–11.
29. Murphy MP. How mitochondria produce reactive oxygen species. Biochem J. 2009;417:1–13.
30. Kushnareva Y, Murphy AN, Andreyev A. Complex I-mediated reactive oxygen species generation: modulation by cytochrome *c* and $NAD(P)^+$ oxidation-reduction state. Biochem J. 2002;368:545–53.
31. Latipää PM, Kärki TT, Hiltunen JK, Hassinen IE. Regulation of palmitoylcarnitine oxidation in isolated rat liver mitochondria. Role of the redox state of NAD(H). Biochim Biophys Acta. 1986;875:293–300.
32. Saheki T, Kobayashi K, Iijima M, Horiuchi M, Begum L, Jalil MA, Li MX, Lu YB, Ushikai M, Tabata A, Moriyama M, Hsiao KJ, Yang Y. Adult-onset type II citrullinemia and idiopathic neonatal hepatitis caused by citrin deficiency: involvement of the aspartate glutamate carrier for urea synthesis and maintenance of the urea cycle. Mol Genet Metab. 2004;81(Suppl 1):20–6.
33. Tandra S, Yeh MM, Brunt EM, Vuppalanchi R, Cummings OW, Ünalp-Arida A, Wilson LA, Chalasani N. Presence and significance of microvesicular steatosis in nonalcoholic fatty liver disease. J Hepatol. 2011;55(3):654–9.
34. Nolan CJ, Damm P, Prentki M. Type 2 diabetes across generations: from pathophysiology to prevention and management. Lancet. 2011;378:169–81.
35. Ishimoto T, Lanaspa MA, Rivard CJ, Roncal-Jimenez CA, Orlicky DJ, Cicerchi C, McMahan RH, Abdelmalek MF, Rosen HR, Jackman MR, MacLean PS, Diggle CP, Asipu A, Inaba S, Kosugi T, Sato W, Maruyama S, Sánchez-Lozada LG, Sautin YY, Hill JO, Bonthron DT, Johnson RJ. High-fat and high-sucrose (western) diet induces steatohepatitis that is dependent on fructokinase. Hepatology. 2013;58:1632–43.
36. Huang BW, Chiang MT, Yao HT, Chiang W. The effect of high-fat and high-fructose diets on glucose tolerance and plasma lipid and leptin levels in rats. Diabetes Obes Metab. 2004;6:120–6.
37. D'Angelo G, Elmarakby AA, Pollock DM, Stepp DW. Fructose feeding increases insulin resistance but not blood pressure in Sprague-Dawley rats. Hypertension. 2005;46:806–11.
38. Tillman EJ, Morgan DA, Rahmouni K, Swoap SJ. Three months of high-fructose feeding fails to induce excessive weight gain or leptin resistance in mice. PLoS One. 2014;9:e107206.
39. Bocarsly ME, Powell ES, Avena NM, Hoebel BG. High-fructose corn syrup causes characteristics of obesity in rats: increased body weight, body fat and triglyceride levels. Pharmacol Biochem Behav. 2010;97:101–6.

40. Michaelis OE 4th, Ellwood KC, Judge JM, Schoene NW, Hansen CT. Effect of dietary sucrose on the SHR/N-corpulent rat: a new model for insulin-independent diabetes. Am J Clin Nutr. 1984;39(4):612–8.
41. Schemmel RA, Teague RJ, Bray GA. Obesity in Osborne-Mendel and S 5B/Pl rats: effects of sucrose solutions, castration, and treatment with estradiol or insulin. Am J Phys. 1982;243(3):R347–53.
42. Elliott SS, Keim NL, Stern JS, Teff K, Havel PJ. Fructose, weight gain, and the insulin resistance syndrome. Am J Clin Nutr. 2002;76:911–22.
43. Blasiole DA, Davis RA, Attie AD. The physiological and molecular regulation of lipoprotein assembly and secretion. Mol BioSyst. 2007;3:608–19.
44. Choi SS, Diehl AM. Hepatic triglyceride synthesis and nonalcoholic fatty liver disease. Curr Opin Lipidol. 2008;19:295.
45. Crescenzo R, Bianco F, Falcone I, Coppola P, Liverini G, Iossa S. Increased hepatic de novo lipogenesis and mitochondrial efficiency in a model of obesity induced by diets rich in fructose. Eur J Nutr. 2013;52:537–45.
46. Alwahsh SM, Dwyer BJ, Forbes S, Thiel DH, Lewis PJ, Ramadori G. Insulin production and resistance in different models of diet-induced obesity and metabolic syndrome. Int J Mol Sci. 2017;18:E285.
47. Vos MB, Lavine JE. Dietary fructose in nonalcoholic fatty liver disease. Hepatology. 2013;57:2525–31.
48. Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. Hepatology. 2010;52:1836–46.
49. https://www.labdiet.com/cs/groups/lolweb/@labdiet/documents/web_content/mdrf/mdi4/~edisp/ducm04_028021.pdf. Accessed 11 Mar 2019.

Capítulo II

Avocado oil consumption protects mitochondrial function and dynamics trough control of ROS accumulation and inflammatory cytokines expression in liver of rats with Non-Alcoholic Fatty Liver Disease

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common chronic liver disease, characterized by excessive accumulation of fat in absence of significant alcohol consumption (8,9). NAFLD comprises simple steatosis, steatohepatitis and cirrhosis. NAFLD is associated to the diseases involving the metabolic syndrome such as obesity, hypertension, glucose intolerance and dyslipidemia (10). The progression of steatosis to non-alcoholic steatohepatitis and cirrhosis it is hypothesized in the 'two-hit' theory (12). The first "hit" is hepatic triglyceride accumulation, or steatosis. During obesity and insulin resistance, there is an increased influx of free fatty acids (FFA) to the liver, these FFA undergo β-oxidation or are esterified with glycerol to form triglycerides, leading to hepatic fat accumulation, which increases the susceptibility of the liver to injury mediated by "second hits", which in the liver promotes dysfunction of the electron transport chain (ETC), increased reactive oxygen species (ROS) generation, augmented lipid peroxidation and release of inflammatory cytokines. The latter leads directly to the progression of steatosis to nonalcoholic steatohepatitis and/or fibrosis (7,13). Mitochondrial alterations are closely related to the pathogenesis of NAFLD, in such way that NAFLD has been considered as a mitochondrial disease (11). Therefore, mitochondria has been considered as a therapeutic target for the treatment of NAFLD.

Currently, there is no an effective pharmacological therapy for NAFLD

(16,17). Hypoglycemic drugs like pioglitazone and metformin, or vitamins E and D have been tested for the treatment of NAFLD with poor results as only a fraction of the individuals recruited in these studies showed improvement of some histological features of NAFLD (18, 19, 20). This guarantee the search for new strategies for

NAFLD treatment involving the improvement of mitochondrial function and oxidative stress.

The beneficial effects of avocado oil against the mitochondrial alterations elicited by diabetes and hypertension have been investigated, as avocado oil contains a myriad of bioactive compounds including lipophilic antioxidants, chlorophylls, phytosterols and monounsaturated fatty acids (MUFA), being oleic acid (C18:1) the main fatty acid (14). Avocado oil treatment improved the redox state of the mitochondria pool of glutathione in mitochondria from brain, liver and kidney from diabetic rats, ameliorated the impairment in the activity of the ETC, improved the oxidative phosphorylation and decreased ROS production of mitochondria from kidney mitochondria of either diabetic or hypertensive rats, and inhibited mitochondrial lipid peroxidation in liver mitochondria from diabetic rats (5, 30, 47, 48).

Besides NAFLD alters the activity of the ETC, increases mitochondrial ROS production and enhances lipid peroxidation, NAFLD is also accompanied by disrupted mitochondrial networks in hepatocytes by disbalancing mitochondrial dynamics (35), which consist in events of fission/fusion of individual mitochondria. Mitochondrial fusion is associated with improved ATP generation, decreased ROS generation and lower damage to biomolecules. Conversely, mitochondrial fission lead to impaired oxidative phosphorylation, augmented ROS production and, ultimately, to cell death (49). Mitochondrial dynamics is controlled by proteins mediating fusion/fission events. Mitochondrial fusion requires membrane phospholipids for the anchoring of proteins involved in this process. Thus, it is feasible to propose that decreased lipid peroxidation might improve mitochondrial dynamics by facilitating the function of proteins modulating mitochondrial fusion.

Given the role of mitochondrial oxidative stress, ETC dysfunction, enhanced lipid peroxidation and impaired mitochondrial dynamics in the pathogenesis of NAFLD, and the beneficial effects of avocado oil on mitochondria during diabetes and hypertension (i.e. two diseases involving mitochondrial dysfunction and oxidative stress), the aim of this work was to evaluate the effects of avocado oil on NAFLD development and its relationship with the effects of avocado oil on features of

mitochondrial function like respiration, activity of the ETC complexes, cytochromes status, levels of lipid peroxidation and ROS, as well as the effects of avocado oil on the expression of inflammatory cytokines and proteins involved in mitochondrial dynamics.

2. Materials and methods

Animals

Male Wistar rats weighing ≈ 330 g were used and kept in a bioterium with controlled temperature and light/dark cycles of 12h/12h. The animals were managed according to the recommendations from Mexican Federal Regulations for the Use and Care of Animals (NOM-062-ZOO-1999) by the Ministry of Agriculture, Mexico. This research was approved by the Institutional Committee for Use of Animals of the Universidad Michoacana de San Nicolás de Hidalgo.

NAFLD was induced with an experimental diet prepared by mixing equal quantities of standard rodent chow (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO, USA) and a dough prepared with 5% lard, 20% sucrose, 20% lactose, 20% hydrogenated vegetable oil, 2% sodium cholate, 0.4% choline chloride and 0.15% thiouracil. 25% fructose was included in the drinking water. This diet was denominated the HFHC+Fr diet.

Thirty rats were randomly divided into the following six groups of five rats each: 1) control group (CTL), fed with standard rodent chow; 2) H1 group, fed during 6 weeks with the HFHC+Fr diet; 3) H1+AvoD group: fed during 6 weeks with the HFHC+Fr diet and then, changed to the standard rodent chow plus avocado oil for 12 weeks; 4) H1+D group, fed during 6 weeks with the HFHC+Fr diet and then, changed to the standard rodent chow for 12 weeks; 5) H4 group, fed during 18 weeks with the HFHC+Fr diet; 6) H4+Avo group, fed during 6 weeks with the HFHC+Fr diet and then, added with avocado oil with the HFHC+Fr diet for 12 weeks. Avocado oil was orally administered daily at a dose of 1 mL/250 g weight, using a bottled, commercial presentation of avocado oil (Ahuacatlan, DIRI COM, S.A. de C.V., México), purchased from a local grocery.

Isolation of mitochondria

At the end of the treatments, the rats were sacrificed by decapitation, and the liver was excised and placed into ice-cold isolation medium containing 220mM mannitol, 70mM sucrose, 1mM EGTA, and 2mM MOPS (pH 7.4). The liver was cut, washed, and homogenized with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 314g. Subsequently, the supernatant was decanted and centrifuged at 4410g. The resulting pellet was washed with a solution (Medium 2) containing 220mM mannitol, 70mM sucrose and 2mM MOPS (pH 7.4) and centrifuged at 6350g. Finally, the pellet was re-suspended in 500 µl of medium 2. Each centrifugation was performed during 10 min at 4°C [1]. Mitochondrial protein concentration was determined by the Biuret method.

Histological analyses of liver

Liver sections were fixed in 10 % formalin, embedded in paraffin blocks, sectioned 5µM thick and stained with hematoxylin & eosin (H&E). Histology was read by a single independent pathologist, blinded to experimental design and treatment groups. Light microscopy was used for evaluation of steatosis, lobular inflammation and hepatocyte ballooning, following the criteria of the nonalcoholic fatty liver disease activity score (NAS) described by Kleiner et al. (2005) [2].

Measurement of blood TG and Total Cholesterol levels

Rats were fasted by 12 h before to sacrifice, blood was recollected, and serum was obtained. Triglycerides (TG) and total cholesterol (TC) levels were determined by enzymatic methods using kits from VITROS Chemistry Products (Rochester, USA), according to the manufacturer’s instructions.

Determination of mitochondrial respiration

Oxygen consumption in states 4 (i.e basal state) and 3 (i.e. phosphorylating state) was measured using a Clark-type oxygen electrode coupled to a YSI 5300 oxygen monitor connected to a computer for data acquisition. Mitochondrial protein (0.5mg/ml) was deposited in a sealed glass chamber containing respiratory buffer consisting of 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 0.05 mM EDTA (pH 7.4) to a final volume of 2500µL. Respiration traces were started by adding 10 mM glutamate/malate as respiratory substrate for complex I. State 3 was induced by adding 0.2 mM ADP. Three minutes later, 1.4µg/mL oligomycin was added to induce the state 4. Finally, KCN was adding to inhibit ETC activity and discriminate the rate of unspecific oxygen consumption.

Determination of the activities of the ETC complexes

To evaluate the activities of the ETC complexes, mitochondria were solubilized with Triton X-100 (50) before each assay in order to enhance the accessibility of substrates and inhibitors to the redox sites of the ETC complexes, except by complex I, where mitochondria were frozen and thawed twice. Complex I activity was assayed by a modification of the technique reported by Chomova et al. (2012) [13]. Briefly, 0.1 mg/mL mitochondria were resuspended in 2 mL of 50mM KH_2PO_4 buffer and incubated with 1µg antimycin A plus 1mM KCN. After 5 min, 5mM $K_3Fe(CN)_6$ was added and absorbance was followed during 1 min at 340 nm in a Shimadzu UV2550 spectrophotometer. Then, NADH was added and its oxidation was measured during 4 min. The rate of NADH oxidation was calculated using a molar extinction coefficient of 6.2 $mM^{-1}cm^{-1}$ for NADH. The activities of succinate-DCIP oxidoreductase (complex II) and antimycin A-sensitive succinate-cytochrome c oxidoreductase (complex III), were determined as described previously (Ortiz-Avila et al. 2013), while cytochrome *c* oxidase (complex IV) activity was evaluated by the protocol described by Cortés-Rojo et al (4).

Measurement of ROS levels

ROS generation was determined by measuring the oxidation of 2´,7´-dichlorodihydrofluorescein diacetate (H_2DCFDA). 0.5mg/mL intact mitochondria and 1.25mM H_2DCFDA were incubated in a buffer containing 10mM HEPES, 100mM KCl, 3mM $MgCl_2$, and 3mM KH_2PO_4 (pH 7.4) during 20 min at 4◦C under constant shaking. Later, mitochondrial suspension was placed in a quartz cuvette and basal fluorescence was recorded. After 1min, 10mM glutamate/malate was added and the changes in H_2DCFDA fluorescence were further followed by 15min. Fluorescence changes were detected in a Shimadzu RF-5301PC spectrofluorophotometer (λ_{ex} 491 nm; λ_{em} 518 nm).

Lipid peroxidation assay

This assay was carried out in 0.3mg/mL of mitochondrial protein by measuring the levels of thiobarbituric acid reactive substances (TBARS), according to the protocol of Buege and Aust (3). To avoid false positive, mitochondrial pellets were washed twice and resuspended with PBS buffer (pH 7.4), immediately before TBARS assays. In experiments where the sensitivity to in vitro lipid peroxidation was tested, mitochondria were incubated before the assay during 30 min with 50μM Fe^{2+} in 50 mM KH_2PO_4 buffer. Absorbance was measured at 532nm with a Shimadzu UV-2550 UV-VIS spectrophotometer. Data were expressed as μmoles of TBARS/mg of protein.

Measurement of cytochromes

Cytochromes *c*+c_1 and *b* were analyzed by obtaining the reduced minus oxidized spectra with a Shimadzu UV2550 double beam spectrophotometer. First, 1.0 mg/mL intact mitochondria were placed in both reference and sample cuvettes and a baseline was recorded. 5 min before spectra recording, mitochondria from sample cuvette were incubated with 0.75 mM KCN for 5 min and then 10 mM

succinate was added and absorbance was recorded. Spectra were scanned between 500 and 580 nm.

Western botting analysis

Total protein was extracted from liver homogenate and sonicated mitochondria. Total protein levels were determined by the Lowry method (6). Equal amounts of protein samples were separated by 10% and 12% polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked with 5% skimmed milk for 3h at 4°C. The membranes were then incubated with one of the following primary antibodies: β-actin (sc-1615), TNFα (sc-12744), TNFβ (sc-28345), DRP1 (ac-271583), Fis1 (sc-376447), Mfn1 (ac-166644), Mfn2 (sc-515647), Opa1(sc-393296), TOM20 (sc-17764), all acquired from Santa Cruz Biotechnology-Antibodies were diluted at 1:2000 with 3% TBS-T milk. IL-6 (ab6672) antibody was acquired from Abcam and diluted 1:4000. After three washes with TBS T, the membranes were incubated with 1:2000 donkey anti-rabbit secondary antibody (sc-2305) and m-Ig GK BP HRP (sc-516102) (Santa Cruz Biotechnology), incubated at 4°C for 3h. Finally, images were obtained from multifunctional gel imaging system (Bio-Rad, USA). Densitometry analyses of protein bands were carried out using the ImageJ software.

Data analysis

Results are expressed as the mean ± standard error of at least 5 independent experiments using samples from different animals for each experiment. Statistical differences of the data ($P<0.05$) were determined with two-way ANOVA, followed by multiple comparisons performed with post hoc Tukey's test, using Sigma Plot software v11.0.

Results

Effects of avocado oil on serum biochemical parameters and liver histology of rats with NAFLD

The relative liver weight (liver weight/body weight) at the end of the treatments increased significantly in all the experimental groups in comparison to the control group (Fig. 1a), except by the H1+AvoD group. However, the relative weight ratio of the H1+D group decreased with respect to the H1 group. Likewise, this parameter decreased also in the H4+Avo group in comparison to the H4 group. The effects of avocado oil on serum triglycerides and cholesterol are shown in the Figs. 1c and 1d, respectively. In comparison to the H1 group, triglycerides and cholesterol levels decreased in the H1+AvoD group by 9.41% and 60%, respectively, and 40% for both lipids in the H1+D group. Furthermore, triglycerides

cholesterol levels increased 12%. Serum glucose increased in the groups supplemented with avocado oil (i.e. H1+AvoD and H4+Avo) and in the H4 group. In contrast, glucose levels decreased in the H1 and H1+D groups even below the levels of the control group (Fig. 1b).

H&E staining was used to estimate the histopathological changes of rat liver. As shown in Fig. 1e, the livers of the H1 and H4 groups exhibited macro and microsteatosis, ballooning, presence of Mallory's hyaline and inflammatory cell infiltration. These alterations decreased considerably in the groups supplemented with avocado oil, the H1+AvoD and H4+Avo groups. Liver alterations were slightly improved only when the HFHC+Fr diet was changed by the standard diet (H1+D).

Fig. 1

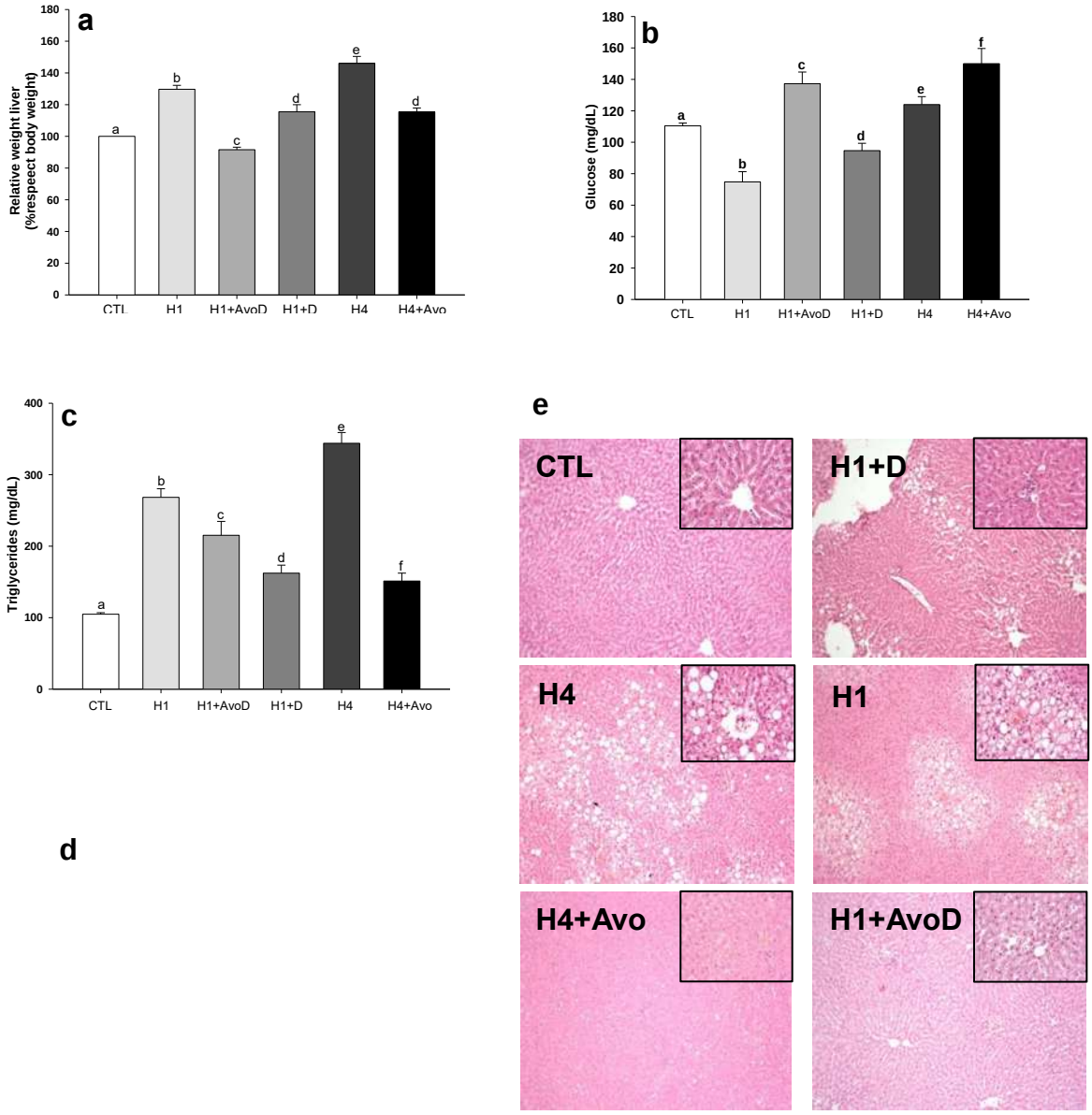

Fig. 1 Panel a: relative weight liver. Panel b-d: levels of blood glucose, triglycerides and cholesterol. The results are presented as the mean ± S.E. of n ≥ 4. Different letters indicate statistically significant differences at $P < 0.05$. Panel d: representative hematoxylin and eosin (H&E)-stained liver slices.

Avocado oil decreased the expression of pro inflammatory cytokines in rats with NAFLD

Accumulation of triglycerides as fat droplets within the cytoplasm of hepatocytes and inflammation precedes the progression of steatosis to NASH. To evaluate the progression of disease, the levels of pro-inflammatory cytokines were evaluated. Both H1 and H4 groups display increased expression of TNF-α, TNF-β and IL6 with respect to the control group (Fig 2A-C). In comparison to these groups, cytokines expression was decreased in the groups supplemented with avocado oil (i.e the H1+AvoD and H4+Avo groups) and when the HFHC+Fr diet was dropped (i.e. the H1+D group).

Fig. 2

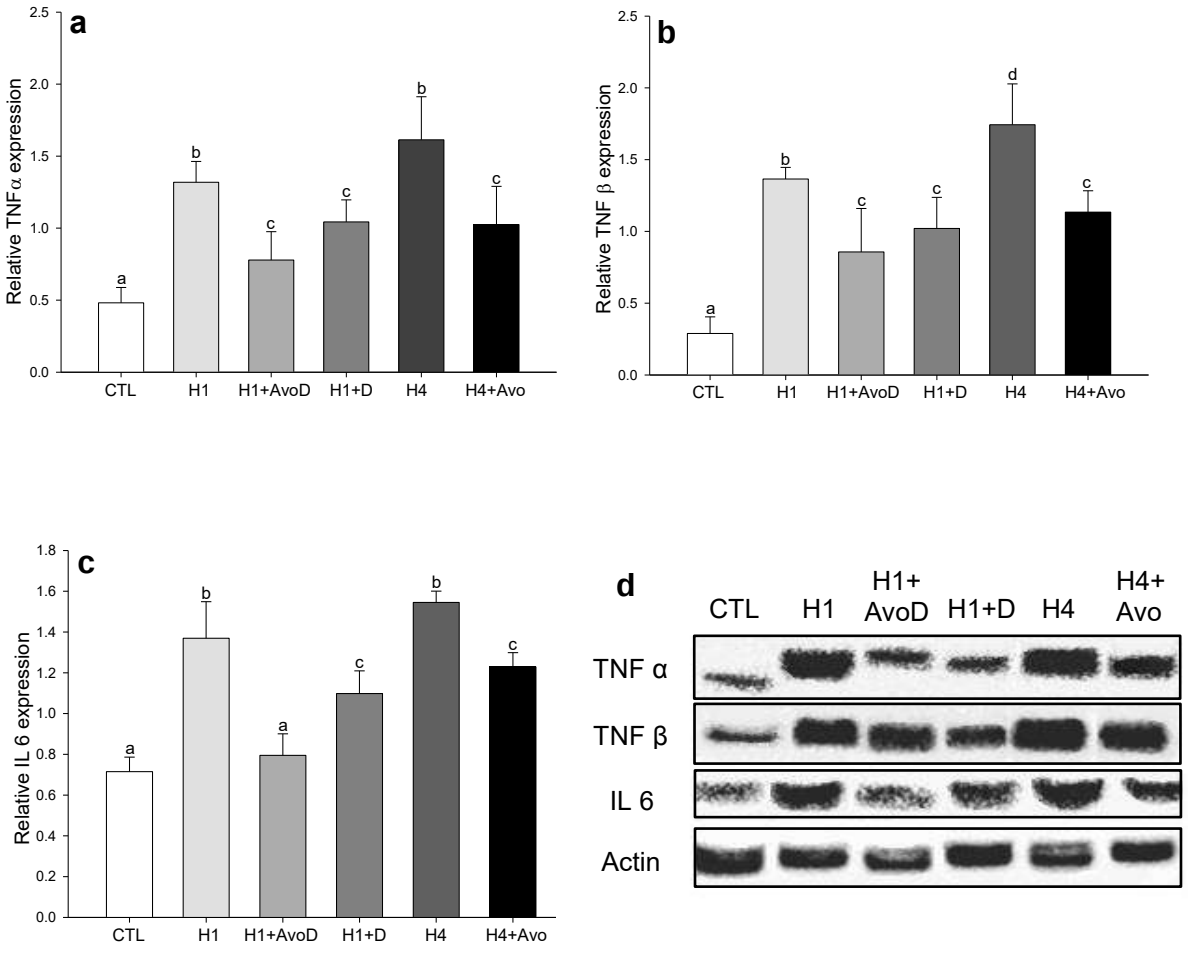

Fig. 2 Effects of avocado oil on the inflammation process during the development of hepatic steatosis. Quantified relative levels of pro inflammatory cytokines. The results are presented as the mean ± S.E. of n ≥ 4. Different letters indicate statistically significant differences at $P < 0.05$.Panel d: shown the representative immunoblots of liver mitochondria.

Avocado oil improves oxidative phosphorylation in rats with NAFLD

Basal (state 4) and phosphorylating (state 3) states of respiration were analyzed to evaluate the effect of avocado oil on mitochondrial function. Impaired respiration in both states was observed in mitochondria from H1, H4 and H1+D groups, indicating a severe impairment of oxidative phosphorylation as respiration rate did not respond neither to ADP nor to oligomycin addition in comparison to the control (Fig. 3a, blue, red and burgundy lines vs. black line, respectively). Respiration in both states was improved in both the H1+AvoD and H4+Avo groups (Fig. 3a, light gray and deep gray lines vs. blue and red lines), which were supplemented with avocado oil improved, as a response of respiration was observed when ADP and oligomycin were added. These data resulted in significant differences in respiratory control ratio (Fig. 3b), which is a parameter indicating the degree of coupling of oxidative phosphorylation. Impaired respiratory rates in the H1 and the H4 groups led to RCR ≤ 3, while control mitochondria had a RCR > 8. The H1+AvoD and H4+Avo groups, those with avocado oil supplementation, exhibited higher RCR values in comparison to both H1 and H4 groups.

Fig. 3

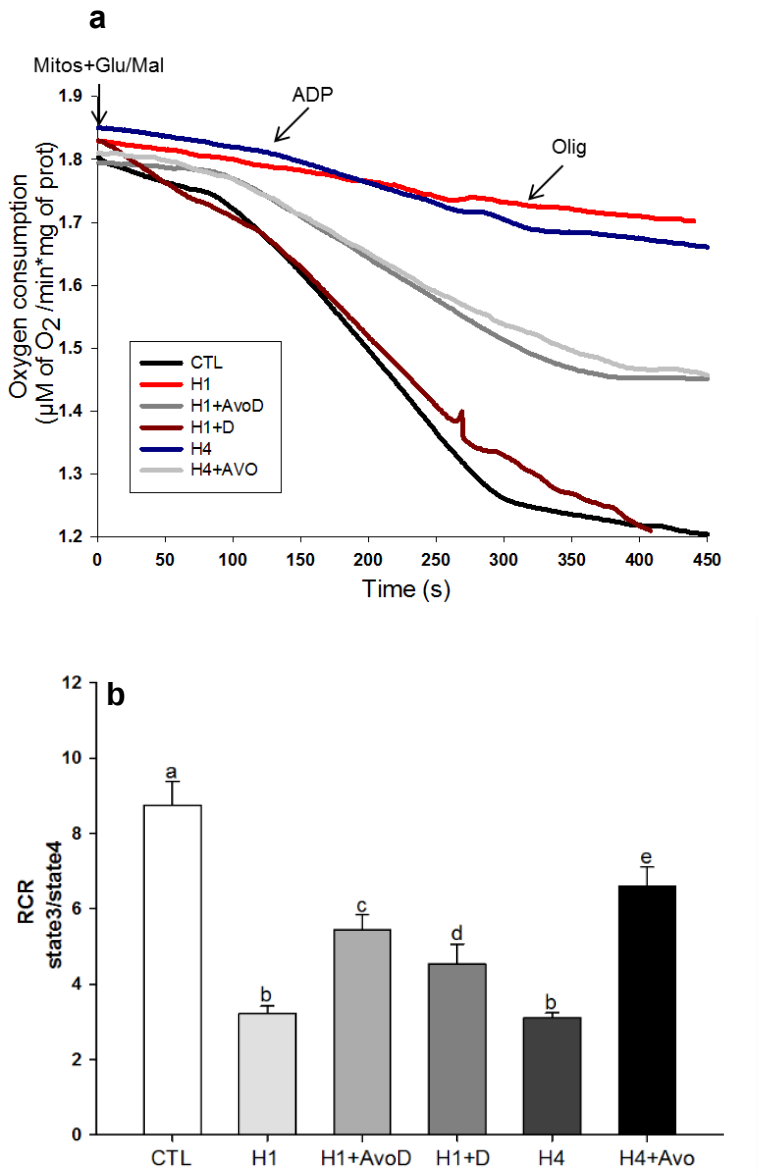

Fig. 3 Effects of avocado oil on mitochondrial respiration (panel a) and RCR (panel b). Panel a: representative traces of fresh isolated liver mitocondria. basal (state 2) respiration was stimulated in 0.5 mg/mL mitochondrial protein using glutamate-malate as substrate. State 3 was stimulated with ADP to stimulate respiration in phosphorylating state. State 4 was stimulated with oligomycin. The results are presented as the mean ± S.E. of n ≥ 4. Different letters indicate statistically significant differences at $P < 0.05$.

Effects of avocado oil on the activity of ETC complex of mitochondria from rats with NAFLD

The activity of the ETC complexes was evaluated to identify the sites in the ETC affected by NAFLD and if avocado oil correct such abnormalities. Complex I activity (Fig. 4a) was almost 50 % lower in mitochondria from the H1 group in comparison to the control group and this defect was not corrected by avocado oil or when the HFHC+Fr diet was dropped (H1+AvoD, H4+Avo and H1+D grops). Complex I activity was drastically diminished in the H4 group up to 80%; avocado oil supplementation (H4+Avo) partially corrected this abnormality when compared to control group. Complex II activity was 20% lower in both the H1 and H4 groups in comparison to the control group. Surprisingly, the H1+AvoD, H4+Avo and H1+D groups, those with avocado oil or when the HFHC+Fr diet was dropped, displays a lower activity, being 50% lower than the activities of the H1 and H4 groups (Fig. 4b). Regarding complex III, this activity was negligible in both the H1 and H4 groups (Fig. 4c), being 98% lower than the activity of the control. Avocado oil supplementation restored complex III activity up to a 60% and 40% in the H1 and the H4 groups, respectively, when compared to control, while a most discrete effect was observed in the H1+D group. Finally, complex IV activity (Fig. 4d) increased in the H1 and H1+AvoD groups in comparison to the control group and remained it unaffected in the H4 group. Complex IV was slightly inhibited in the H1+D and the H4+Avo with respect to control group.

Fig. 4

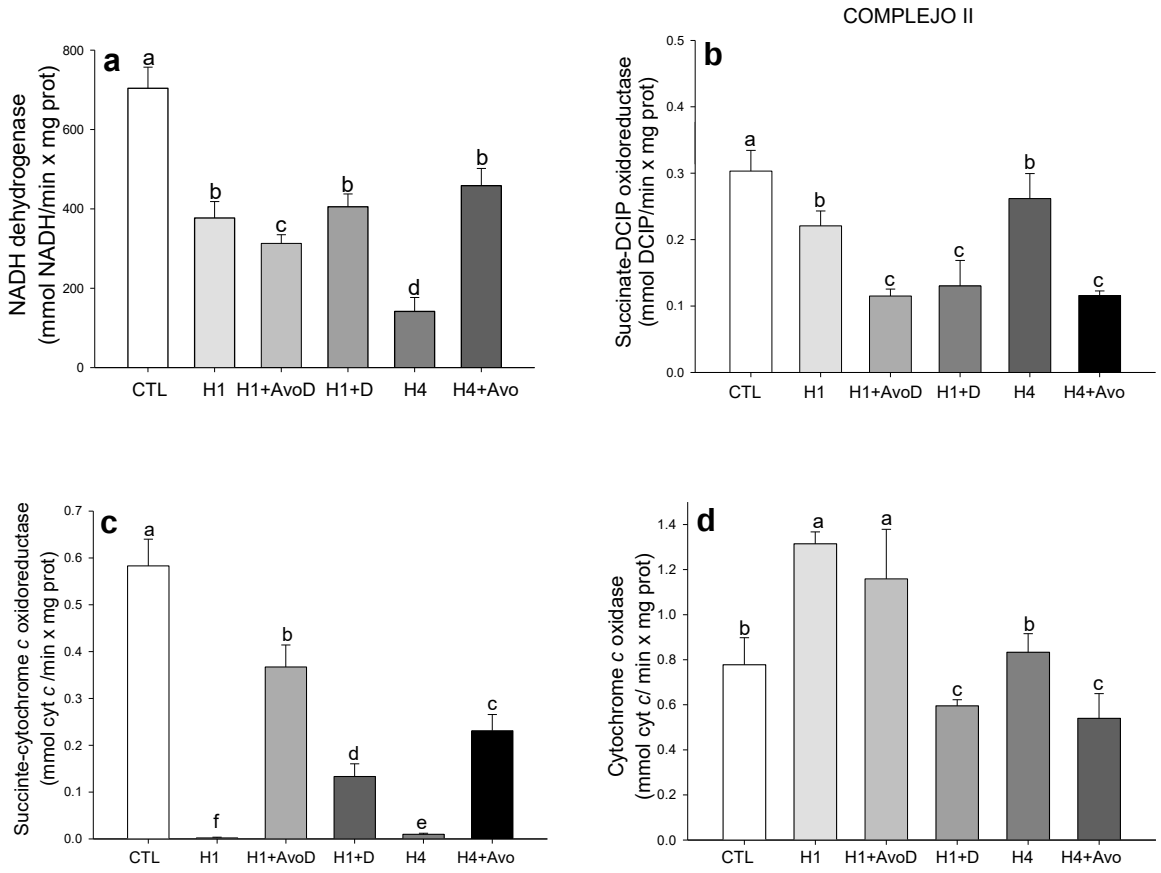

Fig. 4 Effect of avocado oil in complexes I (a); II (b); III; (c) and IV (d) activities in liver mitochondria. The results are presented as the mean ± S.E. of n ≥ 4. Different letters indicate statistically significant differences at $P < 0.05$.

Effects of avocado oil on the reduction of bc_1 cytochromes of complex III

Cytochrome reduction spectra of liver mitochondria were evaluated in order to investigate the defects responsible for complex III impairment in the H1 and H4 groups and whether avocado oil had positive effects in cytochromes reduction. Using succinate as electron donor, reduction of cytochromes c+c_1 at 550 nm was impaired in mitochondria from the H1, H4 and H1+D groups in comparison to the control group. Avocado oil supplementation restored to normality the reduction of cytochromes in the H1+AvoD and H4+Avo groups (Fig. 5). The deleterious effects of NAFLD were not only restricted to c+c_1 cytochromes, as cytochrome b reduction

counteracted this negative effect in the H1+AvoD group and improves cytochrome b reduction also in the H4+Avo in comparison to the H4 group. These results suggest that the severe impairment of complex III activity elicited by NAFLD is due to inhibited electron transfer to cytochromes c+c_1 and b, being this counteracted by avocado oil.

Fig. 5

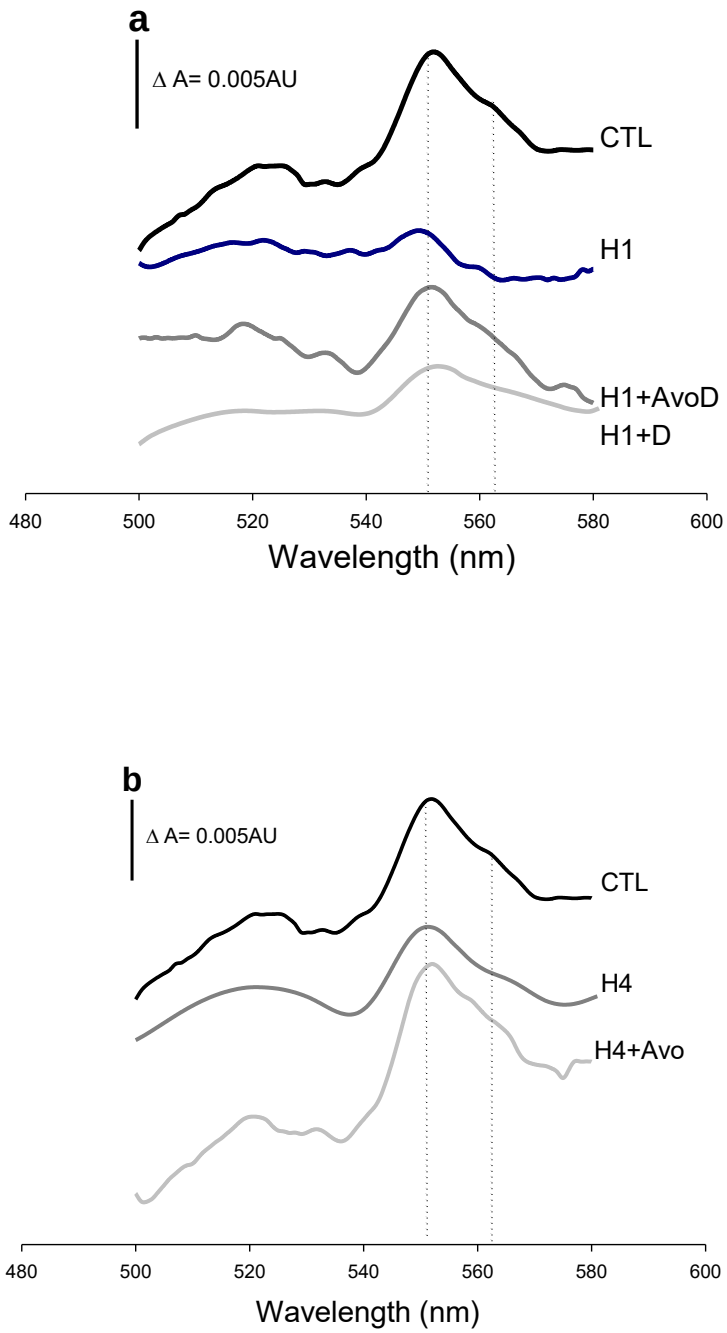

Fig. 5 Effects of avocado oil on difference absorption spectra of cytochromes in mitochondria from NAFLD rats. In dotted lines the absorption of cytochrome $c + c_1$ is indicated at 550 nm and at 562 nm cytochrome b. Cytochromes spectra were scanned as indicated in "Materials and methods" using succinate as electron donor. Data are representative of n≥4.

Avocado oil Increases the expression of proteins of mitochondrial fusion in rats with NAFLD

Western blot analyses revealed an increase in the levels of the fission proteins DRP1 and Fis 1 in all the experimental groups in comparison to the control group. Conversely, the H4+Avo group exhibited decreased levels of these proteins in comparison to all the groups, reaching an expression near to that observed in the control group (Fig. 6E and D). There was also a significant decrease in the fusion proteins Mfn1, Mfn2 and Opa1 (Fig. 6A to C) in the H1 and H4 groups in comparison to the control group. Avocado oil restored the levels of all the fusion proteins in the H1+AvoD group almost at the same levels of the control group. The levels of Mfn1 and Opa1 proteins increased in the H4+Avo group with respect to the H4 group, without reaching the levels of the control group. In the H1+D group, the levels of Mfn1 and Opa1 increased slightly in comparison to the H1 group, while the levels of Mfn2 and remained unchanged in this group.

Fig. 6

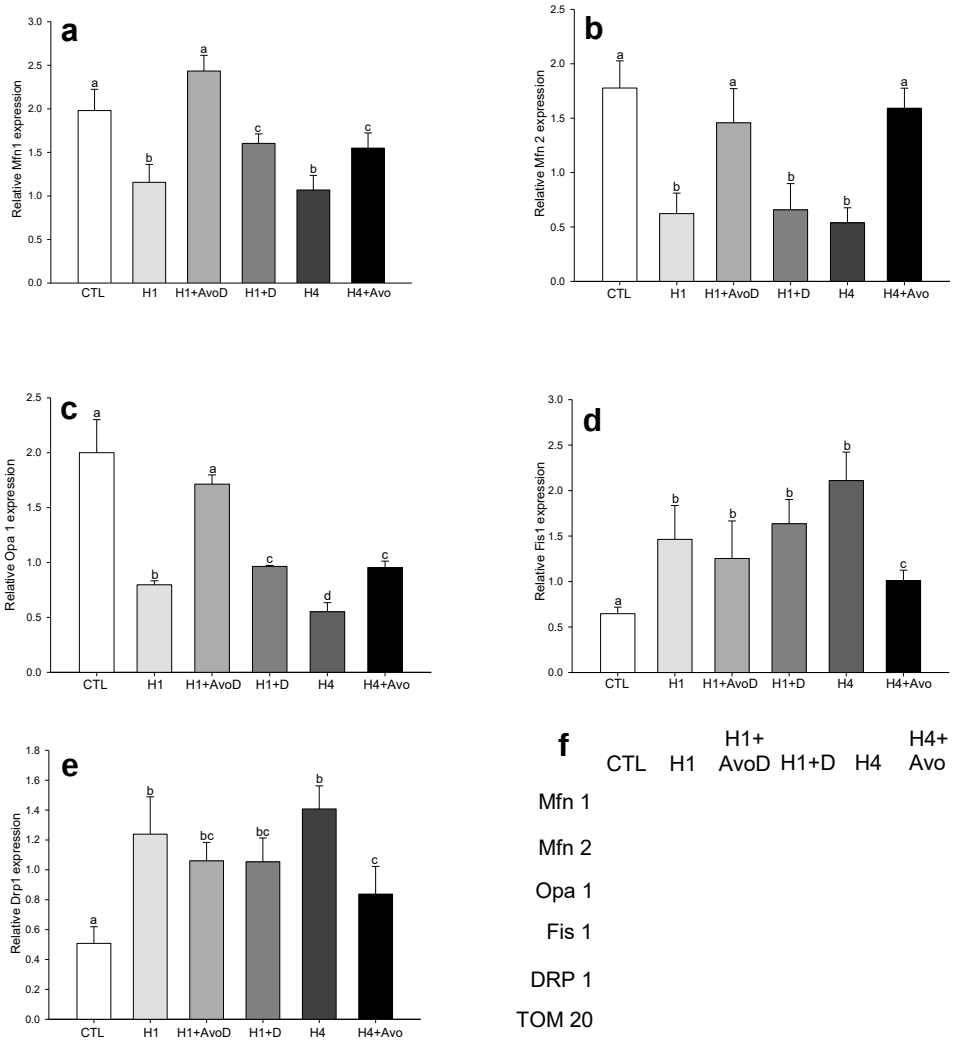

Fig. 6 Effects of avocado oil on the mitochondrial dynamics with the development of hepatic steatosis. Quantified relative levels of fusion proteins (a-c) and fission proteins (d-e). The results are presented as the mean ± S.E. of n ≥ 4. Different letters indicate statistically significant differences at $P < 0.05$.Panel f: shown the representative immunoblots of liver mitochondria.

Influenced of NAFLD and avocado oil in mitochondrial lipid peroxidation and ROS production

The mitochondria from H1, H4 and H1+D showed several fold increases in the levels of lipid peroxidation in comparison to the control group (Fig. 7a). Avocado oil decreased drastically the levels of lipid peroxidation in the H1 and H4 groups near to the levels of the control group. 25 μM Fe^{2+} was added in another experiment to induce the generation of hydroxyl radical and test the sensitivity of the different groups to lipid peroxidation. The H1 and the H4 groups were the most sensitive to lipid peroxidation (Fig. 7b), while the groups supplemented with avocado oil, the H1+AvoD and the H4 groups, and the H1+D group, that one where the HFHC+Fr diet was dropped, exhibited much lower sensitivity to lipid peroxidation, reflecting a better redox state of antioxidant systems.

Regarding ROS production, the H1 and H4 groups showed increase levels of mitochondrial ROS production in comparison to the control group when mitochondria was fueled with a complex I substrate. Avocado oil decreased ROS generation in the H1+AvoD and H4+Avo groups, while not an effect was observed in the H1+D group. Overall, these results suggest that avocado oil decreases mitochondrial oxidative stress induced by NAFLD.

Fig. 7

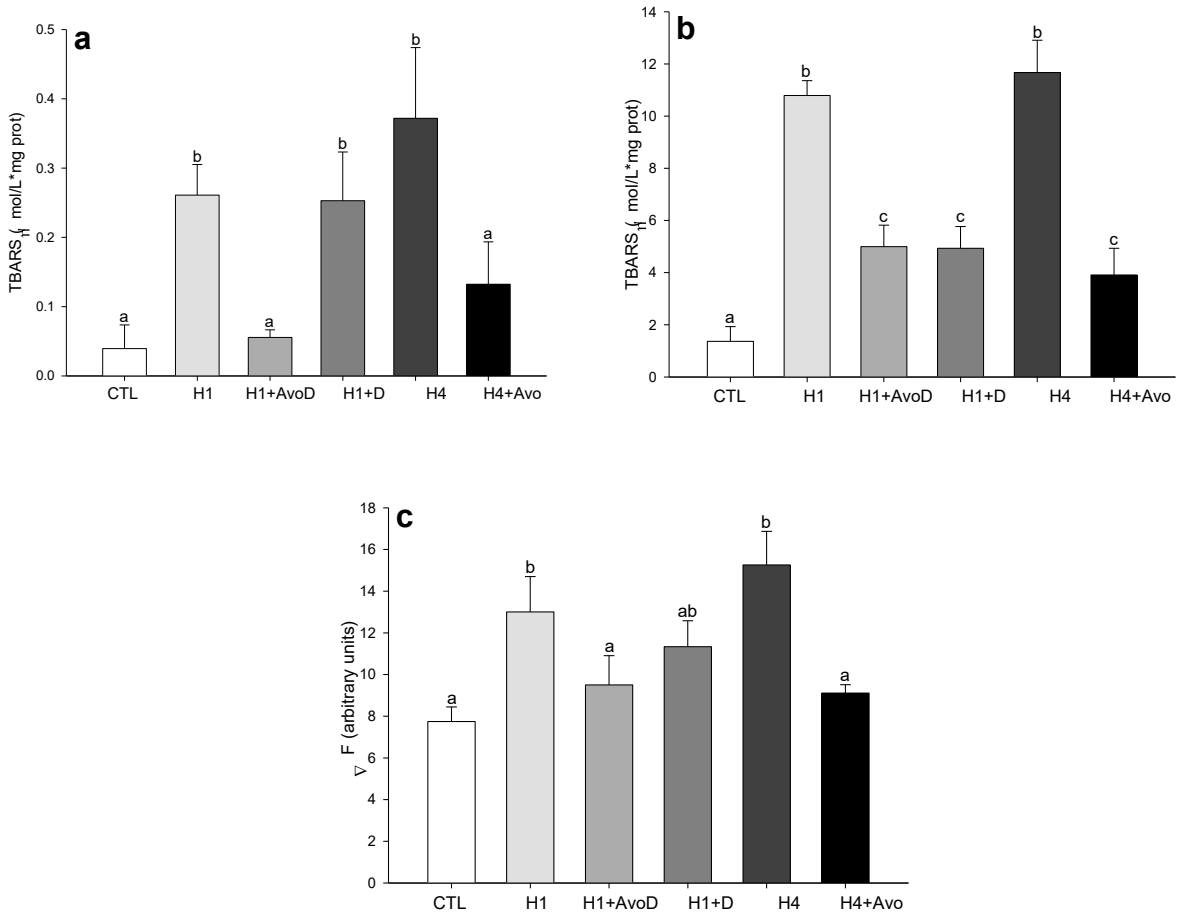

Fig. 7 Influence of avocado oil on lipid peroxidation (a, b) and ROS production (c) in liver rats. Panel a: mitochondria were incubated in phosphate buffer during 30 min at 4 °C. Panel b: mitochondria were incubated in phosphate buffer during 30 min at 4 °C with 50 μM Fe2+. Panel c: fluorescence changes stimulated by glutamate were monitored. Lipid peroxidation and ROS production were assayed as described in “Materials and methods” The results are presented as the mean ± S.E. of n ≥ 4. Different letters indicate statistically significant differences at $P < 0.05$.

Discussion

Non-alcoholic fatty liver disease (NAFLD) represents the most common chronic liver disease in western countries, being considered the hepatic manifestation of metabolic syndrome. The rapid increase in obesity worldwide is associated with an increase in the prevalence of non-alcoholic fatty liver disease (NAFLD) (21). Hepatic steatosis is defined as an intrahepatic accumulation of FFA in form of triglycerides. In parallel, abundant FAs cause lipotoxicity via the induction of ROS release, which causes inflammation, apoptosis, and thus the progression to NASH and fibrogenesis (22). Thus, more alternatives are needed to control this disease. Previously, it has been found that avocado oil supplementation decreases oxidative stress and protects mitochondrial function against the deleterious effects of diabetes and hypertension in different target organs (5, 30, 47, 48, 51). Since these two diseases are part of metabolic syndrome, we decided to evaluate if avocado oil has positive effects in NAFLD development via its beneficial effects in mitochondria.

Hepatic mitochondria are structurally and molecularly altered in NAFLD (25). Mitochondria are central to cellular metabolism, and a decrease in the function mitochondrial is enough to provoke metabolic disturbances and may potentially contribute to NAFLD progression. High-fat diets and the dysregulation of lipid metabolism cause the accumulation of hepatic FFA and triglycerides (23). This was evident in liver histology results like the presence of steatosis (Fig 1d). Modifications in mitochondrial structure and bioenergetics that are related with mitochondrial ROS production and lipid peroxidation induced by NAFLD (26,27) were also evident in the results of this work, with impaired oxidative phosphorylation (Fig. 3), increased ROS production and augmented lipid peroxidation (Fig 7) with the HFHC+Fr diet, which together, contributes to the oxidative stress developed in the pathology. In contrast, in the groups supplemented with avocado oil, it was observed increased resistance to lipid peroxidation, which agree with previous reports where avocado oil prevents oxidative stress by enhancing the GSH/GSSH ratio, decreases ROS production

and prevents lipid peroxidation (31). A probable explanation for these effects is that the antioxidants contained in avocado oil accumulate in mitochondrial membranes, protecting in this way from lipid peroxidation, which in turn may have benefic consequences for oxidative phosphorylation, since the activity of the ETC depends strongly in the intactness of inner mitochondrial membrane. Carotenoids such as violaxanthin have been described to possess a potent in vitro anti lipoperoxidative activity (33) and lutein, decreased lipid peroxidation and restores the levels of glutathione in the cerebral cortex of STZ induced diabetic rats (32).

Lipid peroxidation has been recognized as the major cause of mitochondrial dysfunction (28,29). Mitochondrial membranes are enriched in phospholipids and proteins that are required for mitochondrial biogenesis and for maintenance of mitochondrial morphology and the tubular network (30). Cardiolipin and phosphatidylethanolamine play an essential role in mitochondrial function, and proteins like Mfn1, Mfn2 and OPA1 depends on these phospholipids to carry out their functions. Increases in metabolic input such as elevated intake of glucose and/or free fatty acids, are associated with mitochondrial fragmentation in multiples tissues (33,34). In concordance with these antecedents, the groups with greater lipid peroxidation levels exhibited decreased expression of proteins for mitochondrial fusion such as Mfn1, Mfn2 and OPA1 and, consequently, increased expression of proteins for mitochondrial fission Fis1 and DRP1 was observed (fig. 6), suggesting an imbalance in mitochondrial dynamics towards mitochondrial fragmentation. Notably, the experimental groups supplemented with avocado oil displayed decreased lipid peroxidation and increased levels of fusion proteins, which suggest a change in mitochondrial dynamics towards the formation of mitochondrial networks, which correlates well with the improved mitochondrial function, lower ROS generation and decreased lipid peroxidation seen with avocado oil. This agree with the study of Galloway et al 2014, where palmitate was used as a therapeutic strategy against NFALD in a murine model, and where it was found inhibition of mitochondrial fission, increased proton leak acroos the inner mitochondrial membrane and decreased oxidative stress.

Lipid peroxidation has been associated to disruption of mitochondrial homeostasis in multiple tissues in diverse pathological conditions, including ischemia, hypothyroidism, aging (37), and has been identified as the fundamental cause of ETC dysfunction. Our results showed a severe decrease in the complex III activity during NAFLD (Fig. 4c), and, consequently, an augment of ROS production. Complex III has 11 subunits, with three of them, the Fe-S protein, the cytochrome *b* and cytochrome c_1 being its catalytic subunits. During the Q-cycle, ubiquinol, the complex III substrate, is oxidized by the Fe-S protein and one electron is transferred to cytochrome c_1 for cytochrome *c* reduction and electron transfer to the complex IV. The other electron is transferred simultaneously to cytochrome *b* for a reduction of a ubiquinone molecule. Inhibitor of these reactions induces ROS production. For example antimycin blocks re-oxidation of cytochrome *b*, leading to accumulation of electrons in *b* hemes and reverse transfer of electrons to quinone at the Q_o center to produce radical ubisemiquinone, which can reduce oxygen to superoxide (39, 40). Decrease reduction of cytochromes *c* and *b* was observed in the groups with NAFLD (I,e. the groups H1 and H4) (Fig 7). Complex I is another ETC site involved in ROS production and it is important in the regulation of mitochondrial respiration (41). Our results showed a decrease in the complex I activity in the H1 and the H4 groups as well as lower rate of state 3 respiration compared to the groups supplemented with avocado oil. Cardiolipin is emerging as an important factor that interacts with several proteins of inner mitochondrial membrane. It has been reported that cardiolipin is specifically required for electron transfer in the complexes I and III (38). The content of cardiolipin in the inner mitochondrial membrane may decrease as a consequence of alterations caused by oxidative damage by ROS. Thus it may be hypothesized that avocado oil might have a protective effect against cardiolipin damage, which is in agreement with another study from our group where exogenous cardiolipin reverted the inhibition in complex I activity elicited by diabetes at a same degree that avocado oil (unpublished results).

ROS cause NF-kB activation, which in turn induces the synthesis of TNF-α (43, 44). The levels of TNF-α in the liver of obese ob/ob mice were much higher

than in normal mice. The treatment of ob/ob mice with anti-TNF- α had a beneficial effect on the activity of the ETC complexes, and liver histology (45). In addition, TNF-α - induced mitochondrial swelling causes a bursting of the membrane leading to interference between respiratory chain complexes I and III (46). This data are in agreement with our results, as in the rats with NAFLD, mitochondrial dysfunction at the levels of the complexes I and III and oxidative stress were paralleled with increases in proinflammatory cytokines such as TNF-α and IL6. Since avocado oil decreases the expression of these cytokines and improved the activity of these complexes and decreases oxidative stress, it can be hypothesized that avocado oil improves liver health by augmenting electron fluxes at the complexes I and III, in the latter case by preventing defective electron transfer at cytochromes. This, in turn, decreases electron leak and hence, ROS generation and lipid peroxidation, leading to decreased expression of TNF-α and IL6 since the expression of these cytokines is stimulated by ROS and end-products of lipid peroxidation. This might create a virtuous cycle as the attenuation of TNF-α would lead to lower mitochondrial damage, which in conjunction with diminished lipid peroxidation, would lead ultimately to the preservation of mitochondrial networks and improved function of hepatocytes, which would lead to lower lipid cytosolic accumulation and attenuated inflammation.

In conclusion, avocado oil decreases ROS generation and lipid peroxidation in liver mitochondria of NAFLD rats, in association with enhanced complex I and complex III activities, attenuation of ROS production. This may be driving to improvement of mitochondrial dynamics towards enhanced mitochondrial fusion. All these events may be related with decreased expression of inflammatory cytokines, whose expression is triggered by oxidative stress, causing an improvement of NAFLD of rats consuming a high fat, high carbohydrates and fructose diet, which of note, are the main components of Western diet that has been associated to the current metabolic syndrome pandemic. These observations suggest the avocado oil intakes might be a nutritional approach to attenuate the progression of NAFLD.

References

1. Saavedra-Molina, A., & Devlin, T. M. (1997). Effect of extra-and intra-mitochondrial calcium on citrulline synthesis. *Amino Acids*, *12*(3-4), 293-298.
2. Kleiner, D. E., Brunt, E. M., Van Natta, M. F., Behling, C., Contos, M. J., Cummings, O. W., ... & Yeh, M. (2005). Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*, *41*(6), 1313-1321.
3. Buege, J. A., & Aust, S. D. (1978). [30] Microsomal lipid peroxidation. In *Methods in enzymology* (Vol. 52, pp. 302-310). Academic Press.
4. Cortés-Rojo, C., Calderón-Cortés, E., Clemente-Guerrero, M., Estrada-Villagómez, M., Manzo-Avalos, S., Mejía-Zepeda, R., ... & Saavedra-Molina, A. (2009). Elucidation of the effects of lipoperoxidation on the mitochondrial electron transport chain using yeast mitochondria with manipulated fatty acid content. *Journal of bioenergetics and biomembranes*, *41*(1), 15.
5. Ortiz-Avila, O., Sámano-García, C. A., Calderón-Cortés, E., Pérez-Hernández, I. H., Mejía-Zepeda, R., Rodríguez-Orozco, A. R., ... & Cortés-Rojo, C. (2013). Dietary avocado oil supplementation attenuates the alterations induced by type I diabetes and oxidative stress in electron transfer at the complex II-complex III segment of the electron transport chain in rat kidney mitochondria. *Journal of bioenergetics and biomembranes*, *45*(3), 271-287.
6. Hatree, E. F. (1972). Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal Biochem.*, *48*, 422-427.
7. Ludwig, J., McGill, DB., Lindor, KD. (1997). Review: nonalcoholic steatohepatitis. *J Gastroenterol Hepatol*, *12*, 398–403.
8. Matteoni, C. A., Younossi, Z. M., Gramlich, T., Boparai, N., Liu, Y. C., & McCullough, A. J. (1999). Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology*, *116*(6), 1413-1419.
9. -Labate, A. M., Bianchi, G., Bugianesi, E., McCullough, A. J., ... & Melchionda, N. (1999). Association of nonalcoholic

fatty liver disease with insulin resistance. *The American journal of medicine*, *107*(5), 450-455.

10. Marchesini, G., Brizi, M., Bianchi, G., Tomassetti, S., Bugianesi, E., Lenzi, M., ... & Melchionda, N. (2001). Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes*, *50*(8), 1844-1850.
11. Pessayre, D., & Fromenty, B. (2005). NASH: a mitochondrial disease. *Journal of hepatology*, *42*(6), 928-940.
12. Dixon, J. B., Bhathal, P. S., & O'brien, P. E. (2001). Nonalcoholic fatty liver disease: predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese. *Gastroenterology*, *121*(1), 91-100.
13. Day, C. P., & James, O. F. (1998). Steatohepatitis: a tale of two “hits”?. *Gastroenterology*, *114*, 842–845.
14. Ashton, O. B., Wong, M., McGhie, T. K., Vather, R., Wang, Y., Requejo-Jackman, C., ... & Woolf, A. B. (2006). Pigments in avocado tissue and oil. *Journal of agricultural and food chemistry*, *54*(26), 10151-10158.
15. Musso, G., Gambino, R., Cassader, M., & Pagano, G. (2010). A meta-analysis of randomized trials for the treatment of nonalcoholic fatty liver disease. *Hepatology*, *52*(1), 79-104.
16. Arab, J. P., Candia, R., Zapata, R., Muñoz, C., Arancibia, J. P., Poniachik, J., ... & Contreras, J. (2014). Management of nonalcoholic fatty liver disease: an evidence-based clinical practice review. *World Journal of Gastroenterology: WJG*, *20*(34), 12182.
17. Sanyal, A. J., Chalasani, N., Kowdley, K. V., McCullough, A., Diehl, A. M., Bass, N. M., ... & Van Natta, M. (2010). Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *New England Journal of Medicine*, *362*(18), 1675-1685.
18. Vuppalanchi, R., & Chalasani, N. (2009). Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: Selected practical issues in their evaluation and management. *Hepatology*, *49*(1), 306-317.
19. Roth, C. L., Elfers, C. T., Figlewicz, D. P., Melhorn, S. J., Morton, G. J., Hoofnagle, A., ... & Kowdley, K. V. (2012). Vitamin D deficiency in obese

rats exacerbates nonalcoholic fatty liver disease and increases hepatic resistin and Toll-like receptor activation. *Hepatology*, *55*(4), 1103-1111.

20. Browning, J. D., Szczepaniak, L. S., Dobbins, R., Nuremberg, P., Horton, J. D., Cohen, J. C., ... & Hobbs, H. H. (2004). Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology*, *40*(6), 1387-1395.
21. Cheung, O., & Sanyal, A. J. (2008, November). Abnormalities of lipid metabolism in nonalcoholic fatty liver disease. In *Seminars in liver disease* (Vol. 28, No. 04, pp. 351-359).
22. Eccleston, H. B., Andringa, K. K., Betancourt, A. M., King, A. L., Mantena, S. K., Swain, T. M., ... & Bailey, S. M. (2011). Chronic exposure to a high-fat diet induces hepatic steatosis, impairs nitric oxide bioavailability, and modifies the mitochondrial proteome in mice. *Antioxidants & redox signaling*, *15*(2), 447-459.
23. Sato, N. (2007). Central role of mitochondria in metabolic regulation of liver pathophysiology. *Journal of gastroenterology and hepatology*, *22*, S1-S6.
24. Einer, C., Hohenester, S., Wimmer, R., Wottke, L., Artmann, R., Schulz, S., ... & Borchard, S. (2018). Mitochondrial adaptation in steatotic mice. *Mitochondrion*, *40*, 1-12.
25. Pérez-Carreras, M., Del Hoyo, P., Martín, M. A., Rubio, J. C., Martín, A., Castellano, G., ... & Solis-Herruzo, J. A. (2003). Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology*, *38*(4), 999-1007.
26. VIDELA, L. A., Rodrigo, R., Orellana, M., Fernandez, V., Tapia, G., Quinones, L., ... & Rojas, J. (2004). Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients. *Clinical science*, *106*(3), 261-268.
27. Paradies, G., Petrosillo, G., Pistolese, M., Di Venosa, N., Serena, D., & Ruggiero, F. M. (1999). Lipid peroxidation and alterations to oxidative metabolism in mitochondria isolated from rat heart subjected to ischemia and reperfusion. *Free Radical Biology and Medicine*, *27*(1 2), 42-50.

28. Petrosillo, G., Ruggiero, F. M., Di Venosa, N., & Paradies, G. (2003). Decreased complex III activity in mitochondria isolated from rat heart subjected to ischemia and reperfusion: role of reactive oxygen species and cardiolipin. *The FASEB Journal*, *17*(6), 714-716.
29. Gohil, V. M., & Greenberg, M. L. (2009). Mitochondrial membrane biogenesis: phospholipids and proteins go hand in hand. *The Journal of cell biology*, *184*(4), 469-472.
30. Ortiz-Avila, O., Gallegos-Corona, M. A., Sánchez-Briones, L. A., Calderón-Cortés, E., Montoya-Pérez, R., Rodriguez-Orozco, A. R., ... & Cortés-Rojo, C. (2015). Protective effects of dietary avocado oil on impaired electron transport chain function and exacerbated oxidative stress in liver mitochondria from diabetic rats. *Journal of bioenergetics and biomembranes*, *47*(4), 337-353.
31. Arnal, E., Miranda, M., Barcia, J., Bosch-Morell, F., & Romero, F. J. (2010). Lutein and docosahexaenoic acid prevent cortex lipid peroxidation in streptozotocin-induced diabetic rat cerebral cortex. *Neuroscience*, *166*(1), 271-278.
32. Fu, H., Xie, B., Ma, S., Zhu, X., Fan, G., & Pan, S. (2011). Evaluation of antioxidant activities of principal carotenoids available in water spinach (Ipomoea aquatica). *Journal of Food Composition and Analysis*, *24*(2), 288-297.
33. Gao, C. L., Zhu, C., Zhao, Y. P., Chen, X. H., Ji, C. B., Zhang, C. M., ... & Guo, X. R. (2010). Mitochondrial dysfunction is induced by high levels of glucose and free fatty acids in 3T3-L1 adipocytes. *Molecular and cellular endocrinology*, *320*(1-2), 25-33.
34. Jheng, H. F., Tsai, P. J., Guo, S. M., Kuo, L. H., Chang, C. S., Su, I. J., ... & Tsai, Y. S. (2012). Mitochondrial fission contributes to mitochondrial dysfunction and insulin resistance in skeletal muscle. *Molecular and cellular biology*, *32*(2), 309-319.
35. Galloway, C. A., Lee, H., Brookes, P. S., & Yoon, Y. (2014). Decreasing mitochondrial fission alleviates hepatic steatosis in a murine model of

nonalcoholic fatty liver disease. *American Journal of Physiology-Heart and Circulatory Physiology*.

36. Twig, G., Elorza, A., Molina, A. J., Mohamed, H., Wikstrom, J. D., Walzer, G., ... & Alroy, J. (2008). Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *The EMBO journal*, *27*(2), 433-446.
37. Gandhi, S., Wood-Kaczmar, A., Yao, Z., Plun-Favreau, H., Deas, E., Klupsch, K., ... & Duchen, M. R. (2009). PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. *Molecular cell*, *33*(5), 627-
38. Shi, Y., Buffenstein, R., Pulliam, D. A., & Van Remmen, H. (2010). Comparative studies of oxidative stress and mitochondrial function in aging. *Integrative and comparative biology*, *50*(5), 869-879.
39. Paradies, G., Paradies, V., Ruggiero, F. M., & Petrosillo, G. (2014). Oxidative stress, cardiolipin and mitochondrial dysfunction in nonalcoholic fatty liver disease. *World journal of gastroenterology: WJG*, *20*(39), 14205.
40. Muller, F. L., Liu, Y., & Van Remmen, H. (2004). Complex III releases superoxide to both sides of the inner mitochondrial membrane. *Journal of Biological Chemistry*, *279*(47), 49064-49073.
41. Muller, F. L., Roberts, A. G., Bowman, M. K., & Kramer, D. M. (2003). Architecture of the Qo site of the cytochrome bc 1 complex probed by superoxide production. *Biochemistry*, *42*(21), 6493-6499.
42. Petrosillo, G., Portincasa, P., Grattagliano, I., Casanova, G., Matera, M., Ruggiero, F. M., ... & Paradies, G. (2007). Mitochondrial dysfunction in rat with nonalcoholic fatty liver: involvement of complex I, reactive oxygen species and cardiolipin. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1767*(10), 1260-1267.
43. Demeilliers, C., Maisonneuve, C., Grodet, A., Mansouri, A., Nguyen, R., Tinel, M., ... & Fromenty, B. (2002). Impaired adaptive resynthesis and prolonged depletion of hepatic mitochondrial DNA after repeated alcohol binges in mice. *Gastroenterology*, *123*(4), 1278-1290.

44. Yin, M., Gäbele, E., Wheeler, M. D., Connor, H., Bradford, B. U., Dikalova, A., ... & Thurman, R. G. (2001). Alcohol-induced free radicals in mice: direct toxicants or signaling molecules?. *Hepatology*, *34*(5), 935-942.
45. García-Ruiz, I., Rodríguez-Juan, C., Díaz-Sanjuan, T., del Hoyo, P., Colina, F., Muñoz-Yagüe, T., & Solís-Herruzo, J. A. (2006). Uric acid and anti-TNF antibody improve mitochondrial dysfunction in ob/ob mice. *Hepatology*, *44*(3), 581-591.
46. Sánchez-Alcázar, J. A., Schneider, E., Martínez, M. A., Carmona, P., Hernández-Muñoz, I., Siles, E., ... & Solís-Herruzo, J. A. (2000). Tumor necrosis factor-α increases the steady-state reduction of cytochrome b of the mitochondrial respiratory chain in metabolically inhibited L929 cells. *Journal of Biological Chemistry*, *275*(18), 13353-13361.
47. Ortiz-Avila, O., del Consuelo Figueroa-García, M., García-Berumen, C. I., Calderón-Cortés, E., Mejía-Barajas, J. A., Rodriguez-Orozco, A. R., ... & Cortés-Rojo, C. (2017). Avocado oil induces long-term alleviation of oxidative damage in kidney mitochondria from type 2 diabetic rats by improving glutathione status. *Journal of bioenergetics and biomembranes*, *49*(2), 205-214.
48. Márquez-Ramírez, C. A., de la Paz, J. L. H., Ortiz-Avila, O., Raya-Farias, A., González-Hernández, J. C., Rodríguez-Orozco, A. R., ... & Cortés-Rojo, C. (2018). Comparative effects of avocado oil and losartan on blood pressure, renal vascular function, and mitochondrial oxidative stress in hypertensive rats. *Nutrition*, *54*, 60-67.
49. Kuzmicic, J., del Campo, A., López-Crisosto, C., Morales, P. E., Pennanen, C., Bravo-Sagua, R., ... & Parra, V. (2011). Dinámica mitocondrial: un potencial nuevo blanco terapéutico para la insuficiencia cardiaca. *Revista Española de Cardiología*, *64* -923.
50. Hallberg, E., Wozniak, R. W., & Blobel, G. (1993). An integral membrane protein of the pore membrane domain of the nuclear envelope contains a nucleoporin-like region. *The Journal of cell biology*, *122*(3), 513-521.

51. Ortiz-Avila, O., Esquivel-Martínez, M., Olmos-Orizaba, B. E., Saavedra-Molina, A., Rodriguez-Orozco, A. R., & Cortés-Rojo, C. (2015). Avocado oil improves mitochondrial function and decreases oxidative stress in brain of diabetic rats. *Journal of diabetes research*, *2015*.

6. Discusión

EHNA se considera como la manifestación hepática del síndrome metabólico, un conjunto de características clínicas estrechamente relacionadas con la obesidad visceral y caracterizada por resistencia a la insulina, dislipidemia e hipertensión. Con el rápido aumento de la prevalencia del síndrome metabólico en la población en general, la EHNA se ha convertido en la causa más común de enfermedad hepática en los países occidentales. Esto debido a los cambios negativos en el estilo de vida respecto a la alimentación y el ejercicio, como lo son el sedentarismo y el cambio de dieta al tipo "comida rápida", alta en grasa saturada, colesterol y fructosa. Los resultados mostraron que los efectos perjudiciales de la grasa se agravaron con la adición de fructosa, ya que el grupo HF exhibió menor grado de esteatosis que el grupo HF + Fr. En este sentido, se ha planteado la hipótesis de que la gravedad del daño hepático se correlaciona con el grado de estrés oxidativo en los hepatocitos. La fructosa es distinta de la glucosa en su capacidad para inducir características del síndrome metabólico tanto en humanos (Stanhope et al, 2009) como en animales de laboratorio (Nakagawa et al 2006).

El mecanismo por el cual la fructosa induce hígado graso parece ser independiente de la ingesta total de energía. Una diferencia clave entre la fructosa y la glucosa está en su metabolismo inicial. La fructosa se metaboliza en el hígado por la fructocinasa, que utiliza ATP para fosforilar la fructosa a fructosa-1-fosfato. A diferencia de las hexocinasas, que fosforilan la glucosa y tienen un sistema de retroalimentación negativa para prevenir la fosforilación excesiva, la fructocinasa fosforila la fructosa a un nivel tan alto que conduce al agotamiento de fosfato intracelular. Los niveles más bajos de fosfato intracelular dan como resultado la activación de la desaminasa de AMP, que convierte el AMP en IMP, inosina y, eventualmente a ácido úrico, el cual se ha demostrado puede inducir efectos inflamatorios y estrés oxidativo en células vasculares y adipocitos. Por lo tanto, la fructosa tiene la capacidad para causar agotamiento intracelular de fosfato, agotamiento de ATP y generación de ácido úrico en el hígado (Bode et al, 1973 y Cortez-Pinto et al, 1999). Esto explica la disminución considerable en cuanto al consumo de oxígeno en el estado 3, y por lo tanto la obtención de un bajo cociente respiratorio, además de un claro decremento en cuanto a la actividad de los complejos de la cadena transportadora de electrones, en los

grupos que consumieron una dieta alta en grasa y fructosa. La esteatosis inducida por la obesidad se asocia con un aumento de la expresión de la proteína de desacoplamiento-2 (UCP-2) en el hígado, que promueve el agotamiento de ATP (Chavin et al, 1999). Esto se debe a una mayor fuga de H^+ a través de la membrana mitocondrial interna, que disipa el potencial de membrana y disminuye la síntesis de ATP (Perez-Carreras et al, 2003). Así mismo se sugiere que dentro de los defectos moleculares responsables de la producción de ROS son los defectos en los complejos I y III (Bailey y Cunningham, 1998; y Fernandez-Checa et al, 1997)

Una de las características principales de la EHNA es la acumulación de AGL y TG en el hígado, generando con ello lipotoxicidad y por ende estrés hepático. Esta condición es muy evidente en los resultados de este estudio así como las modificaciones en la estructura y bioenergética mitocondrial las cuales están acompañadas por la producción mitocondrial de ERO (Videla et al, 2004 y Paradies et al, 1999), que contribuyen al estrés oxidativo

En los grupos que desarrollaron EHNA fueron evidentes un aumento en los niveles de ERO y en consecuencia de la peroxidación lipídica en ratas con EHNA. Por el contrario, en las mitocondrias de los grupos suplementados con el aceite de aguacate mostraron resistencia a la peroxidación de lípidos, lo que coincidió con resultados anteriores de nuestro grupo de trabajo donde el aceite de aguacate disminuye el estrés oxidativo al aumentar la relación glutatión reducido / glutatión oxidado, la producción de ERO y con ello, la peroxidación de lípidos (Ortiz-Avila et al. 2015). Una posible explicación de esto es que los antioxidantes que contiene el aceite de aguacate podrían acumularse en las membranas mitocondriales. Se ha descrito que los carotenoides como la violaxantina posee una potente actividad anti lipo peroxidativa *in vitro* (Gao et al, 2010) así como la luteína que disminuye la peroxidación lipídica y restablece los niveles de glutatión en la corteza cerebral de ratas diabéticas (Fu et al, 2011).

La presencia de esteatosis está estrechamente asociada con la inflamación hepática crónica, un efecto en parte mediado por la activación de la vía de señalización Ikk-b / NF-kB. En otros estudios en modelos murinos que siguieron una dieta alta en grasas (HFD) con la finalidad de inducir esteatosis, se observó aumento de la actividad de NF-kB la cual se

asocia con la expresión hepática elevada de citocinas inflamatorias tales como TNFα, interleucina-6 (IL-6) e interleucina 1-beta (IL-1b), y la activación de células de Kupffer (Caid et al, 2005). Los resultados del presente estudio mostraron un incremento en citosinas pro-inflamatorias, en los grupos con una dieta alta en grasa y fructosa. Esto a su vez podría ser debido al incremento en la producción de ERO, que pueden dañar el DNA mitocondrial, los polipéptidos de la cadena respiratoria, así como a la cardiolipina y a los fosfolípidos de membrana (Demeilliers et al, 2002).

Los fosfolípidos de membrana así como la cardiolipina son altamente susceptibles al daño por ERO. De la integridad de estos lípidos dependen diversos procesos y por tanto la homeostasis mitocondrial. Se ha sugerido que alteraciones en la cardiolipina favorece al incremento de mitocondrias fisionadas (Galloway et al, 2014 y Twig et al, 2008), por lo tanto un incremento de proteínas como Fis1 y Drp1, disminuyéndose proteínas como Mnf1 y 2 así como OPA1. Durante la EHNA el incremento en la peroxidación de lípidos fue evidente, por lo que con estos resultados se puede sugerir que existe un incremento de mitocondrias fisionadas que desencadenaría la disfunción mitocondrial, el estrés oxidativo, la inflamación y el daño hepático.

Las estrategias de tratamiento actuales para la EHNA se centran en mejorar los componentes del síndrome metabólico, como la obesidad y la resistencia a la insulina, sin sugerir tratamientos específicos para el hígado. Sin embargo, la modulación de cualquiera de los múltiples mecanismos involucrados en la patogénesis podría proporcionar alternativas útiles para prevenir el desarrollo de la EHNA. En el mismo sentido, se ha propuesto que debieran integrarse terapias antioxidantes para ayudar a combatir al estrés oxidativo como principal responsable desarrollo de la EHNA, como por ejemplo, el empleo de la vitamina E como un posible coadyuvante para la enfermedad. De modo similar el empleo de aceite de aguacate ejerció efectos benéficos contra el desarrollo de la EHNA al proteger contra el estrés oxidativo mitocondrial, mejorar la función y dinámica mitocondrial y disminuir la inflamación.

7. Conclusión

El aceite de aguacate disminuyó el estrés oxidativo y la peroxidación de lípidos en las mitocondrias de ratas con EHNA, en asociación con una mejora de las actividades de los complejo I y III de la cadena respiratoria, la disminución de la producción de ERO y la disminución de la peroxidación lipídica, lo cual favoreció el equilibrio de la dinámica mitocondrial. Además, la disminución de los peróxidos lipídicos y las ERO por el aceite de aguacate podrían ser responsables de la disminución de citocinas pro-inflamatorias involucradas y por lo tanto, del daño hepático. Estas observaciones sugieren que la ingesta de aceite de aguacate podría ser un coadyuvante en el tratamiento de la EHNA.

8. Perspectivas

1.- Esclarecer el mecanismo molecular por el cual el aceite de aguacate le confiere protección a la mitocondria en la esteatosis.

2.- Determinar la actividad de los componentes del aceite de aguacate de manera individual o en combinación en la protección mitocondrial.

3.- Evaluar los perfiles de expresión global de genes en tejidos sanos y en tejidos con la enfermedad hepática no alcohólica.

9. Referencias

Alberts B., Johnson A, Lewis J, Raff M, Roberts K, Walter P. (2002). *Molecular biology of the cell*. Omega Eds, España. pg. 767-769.

Ashton, O. B., Wong, M., McGhie, T. K., Vather, R., Wang, Y., Requejo-Jackman, C., ... & Woolf, A. B. (2006). Pigments in avocado tissue and oil. *Journal of agricultural and food chemistry*, *54*(26), 10151-10158.

Bach, D., Pich, S., Soriano, F. X., Vega, N., Baumgartner, B., Oriola, J., ... & Rabasa-Lhoret, R. (2003). Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism A novel regulatory mechanism altered in obesity. *Journal of Biological Chemistry*, *278*(19), 17190-17197.

Bailey, S. M., & Cunningham, C. C. (1998). Acute and chronic ethanol increases reactive oxygen species generation and decreases viability in fresh, isolated rat hepatocytes. *Hepatology*, *28*(5), 1318-1326.

Bode, J. C., Zelder, O., Rumpelt, H. J., & Wittkampy, U. (1973). Depletion of liver adenosine phosphates and metabolic effects of intravenous infusion of fructose or sorbitol in man and in the rat. *European journal of clinical investigation*, *3*(5), 436-441.

Bossy-Wetzel, E., Barsoum, M. J., Godzik, A., Schwarzenbacher, R., & Lipton, S. A. (2003). Mitochondrial fission in apoptosis, neurodegeneration and aging. *Current opinion in cell biology*, *15*(6), 706-716.

Cai, D., Yuan, M., Frantz, D. F., Melendez, P. A., Hansen, L., Lee, J., & Shoelson, S. E. (2005). Local and systemic insulin resistance resulting from hepatic activation of IKK-β and NF-κB. *Nature medicine*, *11*(2), 183.

Cortez-Pinto, H., Chatham, J., Chacko, V. P., Arnold, C., Rashid, A., & Diehl, A. M. (1999). Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. *Jama*, *282*(17), 1659-1664.

Chavin, K. D., Yang, S., Lin, H. Z., Chatham, J., Chacko, V. P., Hoek, J. B., ... & Wu, T. C. (1999). Obesity induces expression of uncoupling protein-2 in hepatocytes and promotes liver ATP depletion. *Journal of Biological Chemistry*, *274*(9), 5692-5700.

Chen, H., Chomyn, A., & Chan, D. C. (2005). Disruption of fusion results in mitochondrial heterogeneity and dysfunction. *Journal of Biological Chemistry*, *280*(28), 26185-26192.

Chen, H., Detmer, S. A., Ewald, A. J., Griffin, E. E., Fraser, S. E., & Chan, D. C. (2003). Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *The Journal of cell biology*, *160*(2), 189-200.

Day CP, James OF (1998). Steatohepatitis: a tale of two 'hits'? *Gastroenterology*, *114*, 842–845.

Demeilliers, C., Maisonneuve, C., Grodet, A., Mansouri, A., Nguyen, R., Tinel, M., ... & Fromenty, B. (2002). Impaired adaptive resynthesis and prolonged depletion of hepatic mitochondrial DNA after repeated alcohol binges in mice. *Gastroenterology*, *123*(4), 1278-1290.

Dufour, J. F., Oneta, C. M., Gonvers, J. J., Bihl, F., Cerny, A., Cereda, J. M., ... & Swiss Association for the Study of the Liver. (2006). Randomized placebo-controlled trial of ursodeoxycholic acid with vitamin e in nonalcoholic steatohepatitis. *Clinical Gastroenterology and Hepatology*, *4*(12), 1537-1543.

Feldstein, A. E., Werneburg, N. W., Canbay, A., Guicciardi, M. E., Bronk, S. F., Rydzewski, R., ... & Gores, G. J. (2004). Free fatty acids promote hepatic lipotoxicity by stimulating TNF-α expression via a lysosomal pathway. *Hepatology*, *40*(1), 185-194.

Fernandez-Checa, J. C., Kaplowitz, N., Garcia-Ruiz, C. A. R. M. E. N., Colell, A. N. N. A., Miranda, M. E. R. C. E., MARi, M. O. N. T. S. E. R. R. A. T., ... & Morales, A. L. B. E. R. T. (1997). GSH transport in mitochondria: defense against TNF-induced oxidative stress and alcohol-induced defect. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *273*(1), G7-G17.

Fu, H., Xie, B., Ma, S., Zhu, X., Fan, G., & Pan, S. (2011). Evaluation of antioxidant activities of principal carotenoids available in water spinach (Ipomoea aquatica). *Journal of Food Composition and Analysis*, *24*(2), 288-297.

Galloway, C. A., Lee, H., Brookes, P. S., & Yoon, Y. (2014). Decreasing mitochondrial fission alleviates hepatic steatosis in a murine model of nonalcoholic fatty liver disease. *American Journal of Physiology-Heart and Circulatory Physiology*.

Gao, C. L., Zhu, C., Zhao, Y. P., Chen, X. H., Ji, C. B., Zhang, C. M., ... & Guo, X. R. (2010). Mitochondrial dysfunction is induced by high levels of glucose and free fatty acids in 3T3-L1 adipocytes. *Molecular and cellular endocrinology*, *320*(1-2), 25-33.

Herrero, A., & Barja, G. (1997). ADP-regulation of mitochondrial free radical production is different with complex I-or complex II-linked substrates: implications for the exercise paradox and brain hypermetabolism. *Journal of bioenergetics and biomembranes*, *29*(3), 241-249.

James, J., Thomas, P., Cavan, D., & Kerr, D. (2004). Preventing childhood obesity by reducing consumption of carbonated drinks: cluster randomised controlled trial. *Bmj*, *328*(7450), 1237.

Keaney Jr, J. F., Larson, M. G., Vasan, R. S., Wilson, P. W., Lipinska, I., Corey, D., ... & Benjamin, E. J. (2003). Obesity and systemic oxidative stress: clinical correlates of

oxidative stress in the Framingham Study. *Arteriosclerosis, thrombosis, and vascular biology*, *23*(3), 434-439.

Leninger, A. L. (1964). *The mitochondrion; Molecular basis of structure and function*. Wa Benjamin.

Lehninger D., Michael N., Cox M. (2002). *Principles of Biochemistry*. Fourth Edition. pg. 659-670.

Liesa, M., Palacín, M., & Zorzano, A. (2009). Mitochondrial dynamics in mammalian health and disease. *Physiological reviews*, *89*(3), 799-845.

Marchesini, G., Bugianesi, E., Forlani, G., Cerrelli, F., Lenzi, M., Manini, R., ... & Rizzetto, M. (2003). Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology*, *37*(4), 917-923.

Mitchell, P. (1961). Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature*, *191*(4784), 144-148.

Muñoz, M. A., & Barrón, A. R. (2009). Efectos médicos del aguacate. *Medicina Interna de México*, *25*(5), 379-385.

Nakagawa, T., Hu, H., Zharikov, S., Tuttle, K. R., Short, R. A., Glushakova, O., ... & Patel, J. M. (2006). A causal role for uric acid in fructose-induced metabolic syndrome. *American Journal of Physiology-Renal Physiology*.

Olichon, A., Emorine, L. J., Descoins, E., Pelloquin, L., Brichese, L., Gas, N., ... & Ducommun, B. (2002). The human dynamin-related protein OPA1 is anchored to the mitochondrial inner membrane facing the inter-membrane space. *FEBS letters*, *523*(1-3), 171-176.

Ortiz-Avila, O., Sámano-García, C. A., Calderón-Cortés, E., Pérez-Hernández, I. H., Mejía-Zepeda, R., Rodríguez-Orozco, A. R., ... & Cortés-Rojo, C. (2013). Dietary avocado oil supplementation attenuates the alterations induced by type I diabetes and oxidative stress in electron transfer at the complex II-complex III segment of the electron transport chain in rat kidney mitochondria. *Journal of bioenergetics and biomembranes*, *45* -287.

Ortiz-Avila, O., Gallegos-Corona, M. A., Sánchez-Briones, L. A., Calderón-Cortés, E., Montoya-Pérez, R., Rodriguez-Orozco, A. R., ... & Cortés-Rojo, C. (2015). Protective effects of dietary avocado oil on impaired electron transport chain function and exacerbated oxidative stress in liver mitochondria from diabetic rats. *Journal of bioenergetics and biomembranes*, *47*(4), 337-353.

Paradies, G., Petrosillo, G., Pistolese, M., Di Venosa, N., Serena, D., & Ruggiero, F. M. (1999). Lipid peroxidation and alterations to oxidative metabolism in mitochondria isolated from rat heart subjected to ischemia and reperfusion. *Free Radical Biology and Medicine*, *27*(1-2), 42-50.

Pérez-Carreras, M., Del Hoyo, P., Martín, M. A., Rubio, J. C., Martín, A., Castellano, G., ... & Solis-Herruzo, J. A. (2003). Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology*, *38*(4), 999-1007.

Pessayre, D., & Fromenty, B. (2005). NASH: a mitochondrial disease. *Journal of hepatology*, *42*(6), 928-940.

Pich, S., Bach, D., Briones, P., Liesa, M., Camps, M., Testar, X., ... & Zorzano, A. (2005). The Charcot–Marie–Tooth type 2A gene product, Mfn2, up-regulates fuel oxidation through expression of OXPHOS system. *Human molecular genetics*, *14*(11), 1405-1415.

Promrat, K., Kleiner, D. E., Niemeier, H. M., Jackvony, E., Kearns, M., Wands, J. R., ... & Wing, R. R. (2010). Randomized controlled trial testing the effects of weight loss on nonalcoholic steatohepatitis. *Hepatology*, *51*(1), 121-129.

Ratziu, V., Giral, P., Jacqueminet, S., Charlotte, F., Hartemann–Heurtier, A., Serfaty, L., ... & Grimaldi, A. (2008). Rosiglitazone for nonalcoholic steatohepatitis: one-year results of the randomized placebo-controlled Fatty Liver Improvement with Rosiglitazone Therapy (FLIRT) Trial. *Gastroenterology*, *135*(1), 100-110.

Sugioka, K., Nakano, M., Totsune-Nakano, H., Minakami, H., Tero-Kubota, S., & Ikegami, Y. (1988). Mechanism of O− 2 generation in reduction and oxidation cycle of ubiquinones in a model of mitochondrial electron transport systems. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *936*(3), 377-385.

Scheffler, I. E. (2001). A century of mitochondrial research: achievements and perspectives. *Mitochondrion*, *1*(1), 3-31.

Stanhope, K. L., Schwarz, J. M., Keim, N. L., Griffen, S. C., Bremer, A. A., Graham, J. L., ... & McGahan, J. P. (2009). Consuming fructose-sweetened, not glucose-sweetened, beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. *The Journal of clinical investigation*, *119*(5), 1322-1334.

Teff, K. L., Elliott, S. S., Tschöp, M., Kieffer, T. J., Rader, D., Heiman, M., ... & Havel, P. J. (2004). Dietary fructose reduces circulating insulin and leptin, attenuates postprandial suppression of ghrelin, and increases triglycerides in women. *The Journal of Clinical Endocrinology & Metabolism*, *89*(6), 2963-2972.

Tetri, L. H., Basaranoglu, M., Brunt, E. M., Yerian, L. M., & Neuschwander-Tetri, B. A. (2008). Severe NAFLD with hepatic necroinflammatory changes in mice fed trans-fats and a high fructose corn syrup equivalent. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *295*, 987-995.

Thomas, K. J., & Cookson, M. R. (2009). The role of PTEN-induced kinase 1 in mitochondrial dysfunction and dynamics. *The international journal of biochemistry & cell biology*, *41*(10), 2025-2035.

Troy, C. M., Stefanis, L., Prochiantz, A., Greene, L. A., & Shelanski, M. L. (1996). The contrasting roles of ICE family proteases and interleukin-1beta in apoptosis induced by trophic factor withdrawal and by copper/zinc superoxide dismutase down-regulation. *Proceedings of the National Academy of Sciences*, *93*(11), 5635-5640.

Twig, G., Elorza, A., Molina, A. J., Mohamed, H., Wikstrom, J. D., Walzer, G., ... & Alroy, J. (2008). Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *The EMBO journal*, *27*(2), 433-446.
Uygun, A., Kadayifci, A., Isik, A. T., Ozgurtas, T., Deveci, S., Tuzun, A. H. M. E. T., ... & Dagalp, K. (2004). Metformin in the treatment of patients with non-alcoholic steatohepatitis. *Alimentary pharmacology & therapeutics*, *19*(5), 537-544.

Van Den Berghe, G., Bronfman, M., Vanneste, R., & Hers, H. G. (1977). The mechanism of adenosine triphosphate depletion in the liver after a load of fructose. A kinetic study of liver adenylate deaminase. *Biochemical Journal*, *162*(3), 601-609.

VIDELA, L. A., Rodrigo, R., Orellana, M., Fernandez, V., Tapia, G., Quinones, L., ... & Rojas, J. (2004). Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients. *Clinical science*, *106*(3), 261-268.

Westermann, B. (2002). Merging mitochondria matters: cellular role and molecular machinery of mitochondrial fusion. *EMBO reports*, *3*(6), 527-531.

Yakaryilmaz, F., Guliter, S., Savas, B., Erdem, O., Ersoy, R., Erden, E., ... & Ozenirler, S. (2007). Effects of vitamin E treatment on peroxisome proliferator-activated receptor-α expression and insulin resistance in patients with non-alcoholic steatohepatitis: results of a pilot study. *Internal medicine journal*, *37*(4), 229-235.

Zhang, L., Yu, L., & Yu, C. A. (1998). Generation of Superoxide Anion by Succinate-Cytochromec Reductase from Bovine Heart Mitochondria. *Journal of Biological Chemistry*, *273*(51), 33972-33976.