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"EFECTO DEL ACEITE DE AGUACATE EN LA FUNCIÓN MITOCONDRIAL Y EL ESTRÉS OXIDATIVO EN ÓRGANOS BLANCO DE LA DIABETES"

TESIS

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RESUMEN

La diabetes es uno de los problemas más graves que enfrentan los sistemas de salud en todo el mundo, debido a su elevada prevalencia e incidencia, las cuales irán en crecimiento durante las próximas décadas. La diabetes está asociada a un elevado índice de mortalidad debido a sus complicaciones en el riñón, la retina, el hígado, el sistema nervioso periférico, el corazón e incluso el cerebro, además de que la diabetes acarrea también alteraciones en el metabolismo de los lípidos. Todas estas complicaciones hacen que el tratamiento y el manejo de esta enfermedad sean muy complicados. En la mayoría de las complicaciones, el aumento en el estrés oxidativo tiene un papel importante tanto en su establecimiento como en su desarrollo. Se ha reconocido ampliamente que el incremento en la producción de especies reactivas de oxígeno (ERO) a nivel mitocondrial es fundamental en el desarrollo del estrés oxidativo en la diabetes. Por lo tanto, la disminución del estrés oxidativo mitocondrial ha sido propuesto como un blanco novedoso para prevenir y/o retardar la aparición de las complicaciones de esta enfermedad. A este respecto, el consumo de alimentos funcionales con propiedades antioxidantes podría ser una estrategia nutricional para tal efecto. Uno de ellos es el aceite de aguacate, el cual contiene una amplia variedad de compuestos bioactivos con diversos efectos benéficos a la salud. Por lo tanto el objetivo de este trabajo fue analizar el efecto del consumo de aceite de aguacate en la función mitocondrial y el estrés oxidativo en el hígado, el cerebro y el riñón durante la diabetes. Para ello, se utilizaron modelos de diabetes en ratas y se evaluaron cambios en parámetros bioquímicos como la glucosa y el perfil de lípidos, en los niveles de insulina y adiponectina, así como alteraciones en la función mitocondrial y diversos parámetros de estrés oxidativo y su impacto en la histopatología en dos de los órganos analizados. El tratamiento con el aceite de aguacate mejoró los niveles de glucosa sanguínea en la diabetes tipo 2 pero no en la tipo 1, mientras que el perfil lipídico mejoró substancialmente en la diabetes tipo 1. En contraste el aceite no tuvo efecto en los niveles de insulina. La diabetes produjo alteraciones en la función mitocondrial de hígado, cerebro y riñón; el tratamiento con el aceite normalizó dichas alteraciones en la mayoría de los casos. La diabetes incrementó el estrés oxidativo en las mitocondrias de todos los órganos analizados y esto fue contrarrestado por el consumo del aceite de aguacate debido a una mejora en el estado redox del glutatión. En conclusión, el aceite de aguacate mejora el perfil metabólico y la función mitocondrial del hígado, el cerebro y el riñón en la diabetes, además de disminuir el estrés oxidativo mitocondrial en dichos órganos. Lo anterior sugiere que el consumo regular del aceite de aguacate podría funcionar como una estrategia nutricional para disminuir el impacto de la diabetes en sus principales órganos blanco.

Palabras clave: diabetes,complicaciones, hígado, cerebro, riñón.

ABSTRACT

Diabetes is one of the most serious problems that face health systems worldwide because of its high prevalence and incidence, and it will growth over the coming decades. Diabetes is associated with a high mortality rate due to its complications in the kidney, retina, liver, peripheral nervous system, heart and even brain, in addition, diabetes also causes alterations in lipid metabolism. All these complications make the treatment and management of this disease very difficult. In most complications, the increase in oxidative stress plays an important role both in its establishment and in its development. It has been widely recognized that the increase in the production of reactive oxygen species (ROS) at the mitochondrial level is fundamental in the development of oxidative stress in diabetes. Therefore, the reduction of mitochondrial oxidative stress has been proposed as a novel target to prevent and / or delay the onset of complications of this disease. In this respect, consumption of functional foods with antioxidant properties could be a nutritional strategy for this purpose. One of them is avocado oil, which contains a wide variety of bioactive compounds with various beneficial effects to health. Therefore the objective of this work was to analyze the effect of avocado oil consumption on mitochondrial function and oxidative stress on the liver, brain and kidney during diabetes. To do this, we used diabetic models in rats and evaluated changes in biochemical parameters such as glucose and lipid profile, insulin and adiponectin concentrations, as well as alterations in mitochondrial function and various parameters of oxidative stress and its impact in histopathology in two of the tissues analyzed. Treatment with avocado oil improved blood glucose levels in type 2 diabetes but not type 1, while the lipid profile improved substantially in type 1 diabetes. In contrast the oil had no effect on insulin levels. Diabetes produced alterations in the mitochondrial function of liver, brain and kidney; the treatment with the oil normalized these alterations in the majority of the cases. Diabetes increased oxidative stress in the mitochondria of all organs analyzed and this was counteracted by the consumption of avocado oil due to an improvement in the redox status of glutathione. In conclusion, avocado oil improves the metabolic profile and mitochondrial function of the liver, brain and kidney in diabetes, in addition to decreasing mitochondrial oxidative stress in these organs. This suggests that regular consumption of avocado oil could works as a nutritional strategy to decrease the impact of diabetes on its main target tissues.

Keywords: diabetes, complications, liver, brain, kidney.

1. MARCO TEÓRICO

1.1. Diabetes mellitus

1.1.1. Definición y clasificación

Según la Organización Mundial de la Salud (OMS, 2017), la diabetes es una enfermedad crónica que aparece cuando el páncreas no produce suficiente insulina o cuando el organismo no utiliza eficazmente la insulina que produce. La insulina es una hormona que se produce en el páncreas, la cual estimula que la glucosa proveniente de los alimentos ingeridos pase del torrente sanguíneo al interior de las células dependientes del GLUT-4, como son las células musculares y los adipocitos. (IDF, 2017).

Existen tres tipos principales de diabetes:

Diabetes tipo 1: Anteriormente denominada diabetes insulinodependiente o juvenil, por lo general, es causada por una reacción autoinmune donde el sistema de defensa del cuerpo ataca a las células beta del páncreas que producen insulina. La razón del porqué ocurre esto aún no se entiende completamente. Las personas con diabetes tipo 1 producen muy poca o nula insulina. La enfermedad puede afectar a personas de cualquier edad, pero por lo general se desarrolla en niños o adultos jóvenes. Las personas con esta forma de diabetes necesitan insulina por vía parenteral con el fin de controlar los niveles de glucosa en la sangre (IDF, 2017).

Diabetes tipo 2: Llamada anteriormente diabetes no insulinodependiente o del adulto y representa al menos el 90% de todos los casos de diabetes. Se caracteriza por una resistencia a la insulina y la deficiencia relativa de insulina, una o ambas alteraciones pueden estar presentes en el momento en que se diagnostica la diabetes. El diagnóstico de la diabetes tipo 2 puede ocurrir a cualquier edad. La diabetes tipo 2 puede permanecer sin ser detectada durante muchos años y el diagnóstico se hace a menudo cuando aparece una complicación o se realiza una prueba de glucosa en sangre u orina de rutina. A menudo, pero no siempre, se asocia con sobrepeso u obesidad, los cuales por sí mismos pueden causar resistencia a la insulina y conducir a altos niveles de glucosa en la sangre. Las personas con diabetes tipo 2 a menudo pueden inicialmente controlar su condición a través del ejercicio y la dieta. Sin embargo, con el tiempo la mayoría de la gente requerirá medicamentos orales e insulina (IDF, 2017).

Diabetes gestacional: Es una forma de diabetes que consiste en niveles altos de glucosa en la sangre durante el embarazo. Se desarrolla en uno de cada 25 embarazos en todo el mundo y se asocia con complicaciones tanto para la madre como para el bebé. Por lo general desaparece después del embarazo, pero las mujeres con diabetes

gestacional y sus hijos están en un mayor riesgo de desarrollar diabetes tipo 2 más adelante en la vida. Aproximadamente la mitad de estas mujeres desarrollan diabetes tipo 2 entre cinco y diez años después del parto (IDF, 2017).

1.1.2. Epidemiología de la diabetes

La diabetes es un problema de salud alarmante en todo el mundo, en el "Global Status Report on Noncommunicable Diseases - 2014" la OMS declaró que la diabetes es la cuarta causa de muerte dentro de las enfermedades no transmisibles (OMS, 2014). Por su parte, la Federación Internacional de Diabetes publicó el Atlas de la Diabetes, el cual reporta que en el 2015 existían 415 millones de personas con diabetes en el mundo y se reportaron 5 millones de muertes asociadas a la diabetes (Fig. 1), (FID, 2015).

Figura 1.- Número de adultos que murieron a causa de la diabetes en comparación con VIH/SIDA, tuberculosis y malaria. Tomada y modificada del Atlas de la Diabetes de la Federación Internacional de Diabetes (FID, 2015).

Otros aspectos importantes de su epidemiología son el que una de cada once personas es diabética y que la mitad de las personas enfermas permanecen sin ser diagnosticadas. También es importante resaltar que esta enfermedad requiere el 12% del presupuesto para la salud en el mundo ($673 mil millones de dólares). Otro dato importante es que, debido a la diabetes, muere una persona cada 6 segundos, esto refleja la importancia que tiene esta enfermedad en la actualidad (FID, 2015). México ocupa el sexto lugar a nivel mundial en cuanto al número de personas adultas con diabetes, con un total de 11.5 millones de diabéticos (Fig. 2). Según la ENSANUT MC 2016 existe una prevalencia de diabetes del 9.4% en la población mexicana (ENSANUT, 2016).

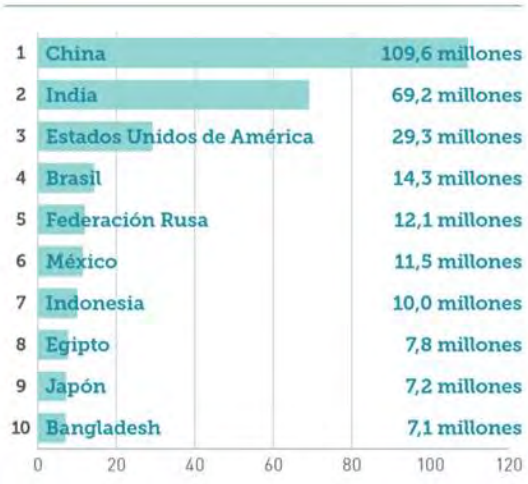

Figura 2.- Los diez primeros países según el número de adultos con diabetes en el 2015. Modificada del Atlas de la Diabetes de la Federación Internacional de Diabetes (FID, 2015).

1.1.3. Complicaciones de la diabetes

A pesar que en la actualidad se dispone de una gran variedad de herramientas de tratamiento efectivas para la diabetes, la amplia gama de complicaciones que se pueden presentar son responsables directas de su la alta morbimortalidad.

Las complicaciones macrovasculares que se pueden presentar durante la diabetes son las enfermedades cardiovasculares, como los ataques cardiacos, los accidentes cerebrovasculares y la insuficiencia circulatoria en los miembros inferiores (OMS, 2017). Esto es debido principalmente al aumento en la prevalencia de ateroesclerosis en los pacientes diabéticos (Shi *et al.*, 2017).

También es muy frecuente el desarrollo de retinopatía, neuropatía, pie diabético y nefropatía diabética las cuales son conocidas como complicaciones microvasculares. La más grave de estas complicaciones es la nefropatía diabética, la cual, es la causa individual más frecuente de insuficiencia renal terminal. La diabetes también se ha asociado con alteraciones hepáticas como es el caso de la esteatohepatitis no alcohólica y el hepatocarcinoma (El-Serag *et al.*, 2004). Además, la diabetes se ha descrito como un factor de riesgo para el deterioro cognitivo y el desarrollo de demencia en estos pacientes (Chung *et al.*, 2015). En la mayoría de estas complicaciones el estrés oxidativo juega un papel crucial tanto en su establecimiento como en el desarrollo de las mismas (Baynes *et al.*, 1991).

1.1.4. El estrés oxidativo durante la diabetes

La hiperglucemia prolongada que se presenta durante la diabetes produce un estrés oxidativo crónico, el cual empeora el estado diabético (Wolff *et al.*, 1991). El metabolismo anormal inducido por la hiperglucemia puede resultar en la sobreproducción de especies reactivas del oxígeno (ERO) tales como los radicales hidroxilo (OH•) y superóxido (O2•-) (Li *et al.*, 2017). Las ERO que se generan durante el estrés oxidativo pueden dañar diversas biomoléculas dentro de la célula como lípidos (Bandeira *et al.*, 2012), proteínas (Johar *et al.*, 2017) y el ADN (Vanitha *et al.*, 2017). Además, existen evidencias de una respuesta antioxidante alterada debido al estrés oxidativo que se genera en la diabetes, estas evidencias incluyen la disminución de las concentraciones de antioxidantes en plasma tanto en pacientes diabéticos (Bahmani *et al.*, 2016) como en modelos en animales (Rahimi-Madiseh *et al.*, 2017) lo que favorece aún más el estado de estrés oxidativo.

El estrés oxidativo juega un papel clave en el desarrollo de las complicaciones de la diabetes, tanto microvasculares como cardiovasculares. Las alteraciones metabólicas de la diabetes causan una sobreproducción de ERO en la mitocondria, lo cual es un evento muy importante para el desarrollo del estrés oxidativo ya que este aumento de la producción de ERO a nivel mitocondrial provoca la activación de 5 vías principales implicadas en la patogénesis de las complicaciones: 1) Un mayor flujo hacia la vía de polioles, 2) Un aumento de la formación de AGE (productos finales de glicosilación avanzada), 3) Un incremento de la expresión del receptor para AGE y sus ligandos de activación, 4) La activación de las isoformas de la protein cinasa c (PKC) y 5) El aumento en la actividad de la vía de la hexosamina (Giacco *et al.*, 2010), entre otras que son específicas de cada tejido afectado.

1.1.5. La disfunción mitocondrial durante la diabetes

En condiciones normales una de las principales funciones de la mitocondria es la producción de ATP mediante la fosforilación oxidativa, esto es posible mediante la generación de equivalentes reductores como el NADH y FADH2 en los procesos catabólicos, como la glucólisis, el ciclo de Krebs y la β-oxidación de ácidos grasos, los cuales alimentan a la cadena transportadora de electrones mitocondrial. El NADH dona sus electrones al complejo I, mientras que el FADH2 al complejo II, los electrones de ambos complejos son transferidos a la coenzima Q y después de la coenzima Q al complejo III, el cual reduce al citocromo c y transfiere su electrón al complejo IV, donde finalmente el oxígeno es reducido a H2O. Una parte de la energía de estos electrones es utilizada para traslocar protones de la matriz mitocondrial hacia el espacio intermembrana para generar un gradiente electroquímico (Δψm), el cual es utilizado por la ATP sintasa para producir ATP (Fig. 3).

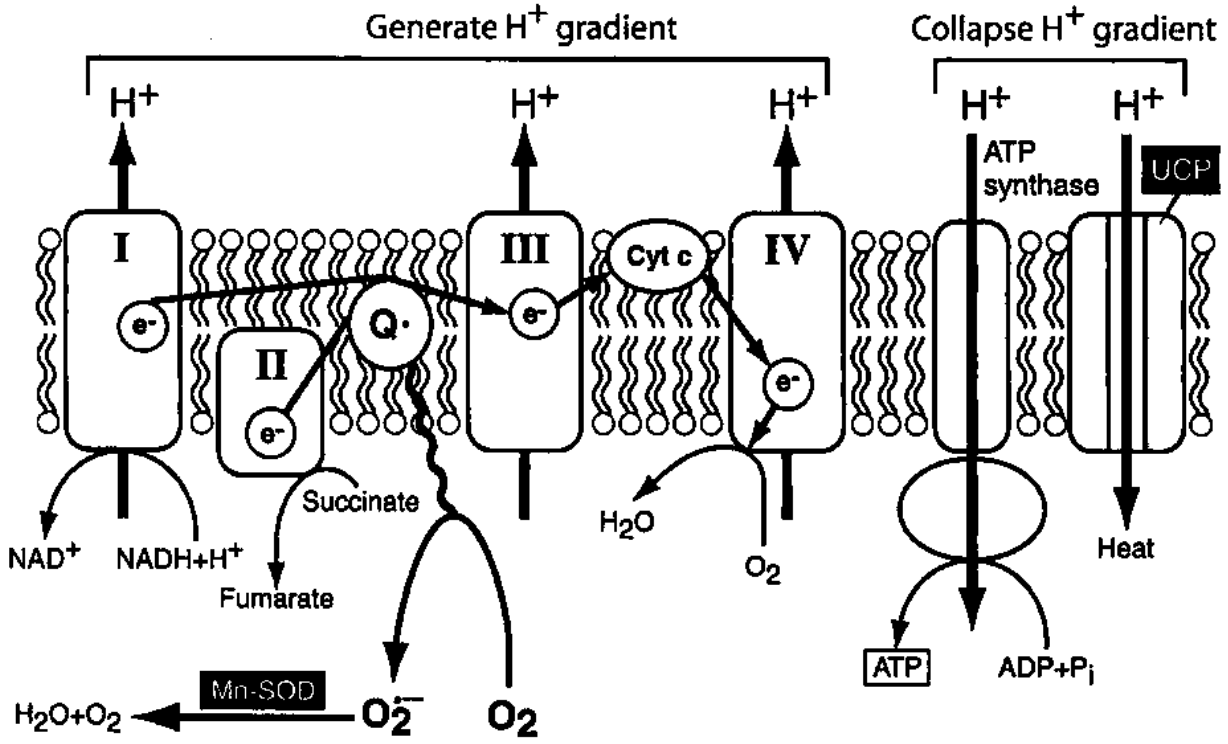

Figura 3.- Funcionamiento de la cadena transportadora de electrones, la fosforilación oxidativa y la generación de especies reactivas de oxígeno. Tomada y modificada de Brownle *et al.* 2005.

Sin embargo, durante la diabetes,se puede presentar una disfunción mitocondrial debido al estado de hiperglucemia, dentro de la célula aumenta la tasa de oxidación de la glucosa, lo cual aumenta las concentraciones de NADH y FADH2 en la cadena transportadora de electrones. Como resultado se produce un incremento en el Δψm, que al rebasar cierto límite produce una inhibición en el transporte de electrones en el complejo III, generando un intermediario parcialmente reducido de la coenzima Q (semiquinona), la cual puede donar un electrón al oxígeno para generar radical O2•- y, como ya se mencionó anteriormente, este aumento en la producción de ERO a nivel mitocondrial es un factor desencadenante para el desarrollo de complicaciones durante la diabetes. Hay algunos hallazgos, además del aumento en la generación de ERO, que enfatizan la participación de la disfunción mitocondrisl en la progresión de las complicaciones diabéticas. Por ejemplo, se ha reportado que la diabetes produce una descenso en la respiración mitocondrial y una pérdida del Δψm (Pieczenik *et al.*, 2007), una caída de la producción de ATP (Zhu *et al.*, 2011), una disminución en la biogénesis mitocondrial (Rovira-Llopis *et al.*, 2017) y la reducción en el número de copias de ADN mitocondrial (Irving *et al.*, 2007).

1.1.6. La terapia con antioxidantes durante la diabetes

Se ha estudiado el efecto de antioxidantes dirigidos hacia la mitocondria como una estrategia para disminuir el estrés oxidativo y mejorar la función mitocondrial y con esto controlar el desarrollo de complicaciones relacionadas con la diabetes (Smith *et al.*, 2010), uno de ellos es el MitoQ, que está formado por una molécula de ubiquinona unida a un catión trifenilfosfonio (TPP+), el cual permite su transporte y acumulación en la mitocondria (Pokrzywinski *et al.*, 2016). Existen reportes en modelos animales donde el MitoQ pudo prevenir el desarrollo de nefropatía diabética reduciendo la fibrosis glomerular (Kalyanaraman *et al.*, 2010). Sin embargo, estos antioxidantes aún están ensayos clínicos de Fase II por lo que su uso en humanos no ha sido aprobado.

En la actualidad la suplementación con antioxidantes se ha propuesto como una intervención preventiva y terapéutica promisoria para las patologías crónicas degenerativas como la diabetes. Sin embargo, las expectativas de este enfoque terapéutico a menudo han sido parcialmente decepcionantes en los ensayos clínicos (Conti *et al.*, 2016). Una gran parte de los estudios donde investigan la eficacia de la terapia de suplementación antioxidante en seres humanos mostraron resultados contrastantes (Bouayed *et al.*, 2010). Esto se debe a muchos aspectos entre ellos el poder estadístico frecuentemente limitado de los estudios, el fondo genético del paciente, la biodisponibilidad de las moléculas utilizadas y los efectos no específicos que los antioxidantes pueden tener en el cuerpo humano (Conti *et al.*, 2016). Sin embargo, como se puede observar en la Tabla 1 la terapia de suplementación con antioxidantes ha sido eficaz en ciertos parámetros en pacientes con diversas patologías cardiacas y crónico degenerativas.

Otro enfoque de la terapia con antioxidantes es el uso de alimentos funcionales y nutracéuticos. Un alimento funcional es todo aquel que contiene sustancias que proporcionan beneficios a la salud cuando se consumen regularmente, mientras que los nutracéuticos son aquellos extractos de alimentos que proporcionan beneficios a la salud (Volpe *et al.*, 2015).

En la literatura existen muchos datos que confirman la actividad antidiabética de varias plantas medicinales, que forman parte de nuestra dieta como verduras, frutas y especias (Bnouham *et al.*, 2006) (Gbolade *et al.*, 2009) (Modak *et al.*, 2007) (Singab *et al.*, 2014), por lo que diversos productos de origen vegetal han sido considerados valiosos suplementos dietéticos para mejorar el control de la glucosa en sangre y prevenir las complicaciones a largo plazo de la diabetes (Gallagher *et al.*, 2003). Entre los más utilizados durante la diabetes se encuentran el ácido alfa-lipoico, el cual se encuentra en vegetales como la espinaca, el brócoli y el tomate, así como en la carne. El ácido lipóico neutraliza varios radicales libres y puede reducir la forma oxidada de la vitamina C y el glutatión oxidado (GSSG) (Liu *et al.*, 2008). Los ácidos grasos

poliinsaturados omega-3 se encuentran principalmente en el pescado y algunos aceites vegetales (Calder *et al.*, 2013) y poseen múltiples propiedades, que incluyen funciones antiinflamatorias y antitrombóticas (Yaqoob *et al.*, 1995), también participan modulando la fluidez membranal y en la biogénesis mitocondrial (Gvozdjáková *et al.*, 2008), mejoran el perfil de lípidos y ayudan a controlar la presión arterial (Svensson *et al.*, 2008). El té verde y los frutos rojos contienen una diversidad de compuestos fenólicos (Redan *et al.*, 2016) cuyos efectos en la diabetes son atribuibles a sus propiedades antioxidantes (Higdon *et al.*, 2003), antiinflamatorias (Sarkar *et al.*, 2009) y de aumento de la sensibilidad a la insulina (Chang *et al.*, 2013).

Cuadro 1.- Estudios clínicos de la terapia de suplementación con antioxidantes en pacientes con enfermedades asociadas al envejecimiento. DM, diabetes mellitus; EAC, enfermedad arterial coronaria; ECV, enfermedad cardiovascular. Tomada y modificada de (Conti *et al.*, 2016).

Antioxidante	Enfermedad	Principales resultados	Referencia
Vitamina C	DM tipo 2 y EAC	↑ Respuesta vasodilatadora del antebrazo	Antoniades, 2004
	DM	↓ Presión arterial y mejora la rigidez arterial	Mullan, 2004
Vitamina E	ECV y DM	Ningún efecto sobre los resultados cardiovasculares	Yusuf, 2000
	EAC	↓ Biomarcadores de estrés oxidativo e inflamación en sangre	Devaraj, 2007
	Enfermedad Vascular y DM	↑ Riesgo de falla cardiaca	Lonn, 2005
Resveratrol	Hipertensión y Dislipidemia	↓ Disfunción endotelial	Carrizzo, 2013
	DM	Mejoría del control glucémico y sensibilidad a la insulina	Liu, 2014
Coenzima Q10	EAC	↑Actividad de enzimas antioxidantes y ↓ Inflamación	Lee, 2013
Vitamina E y C	Hipercolesterolemia	↓ Progresión de ateroesclerosis	Salonen, 2003
Vitaminas C, E y β-caroteno (solos o en combinación)	ECV	Ningún efecto sobre los eventos cardiovasculares	Cook, 2007

1.2. El aguacate: características y propiedades

El aguacate es el fruto del árbol *Persea americana*, el cual pertenece a la familia Lauraceae. El aguacate es un árbol originario de Mesoamérica, su origen tuvo lugar en la parte central de México y en algunas partes altas de Guatemala. El fruto del aguacate es una drupa, en forma de pera, de color verde claro a verde oscuro y de violeta a negro, cáscara rugosa con una pulpa verde amarillenta (SAGARPA, 2011). El aguacate es un fruto rico en ácidos grasos monoinsaturados (MUFAs) constituyendo un 71% del total de ácidos grasos, mientras que el contenido de ácidos grasos saturados (SFAs) es de 16% y el de ácidos grasos poliinsaturados (PUFAs) es de 13% (Dreher *et al.*, 2013).

Además del alto contenido de MUFAs, se ha reportado que el aguacate también posee una amplia variedad de antioxidantes tales como carotenos, tocoferoles, xantinas y clorofilas, además de beta-sitosterol (Tabla 2). El más abundante de estas moléculas antioxidantes es la luteína (Ashton *et al.*, 2006). Todos estos componentes del aguacate pueden tener efectos benéficos en la salud de quien lo consume.

En estudios clínicos, al aguacate se le han atribuido efectos tales como descenso del colesterol total, de las LDL y los triglicéridos, con aumento de las HDL y por lo tanto una mejoría del índice aterogénico en pacientes con colesterol normal, con hiperlipidemia, con hipertrigliceridemia y con diabetes mellitus tipo 2 (Alvizouri *et al.*, 2009). También en pacientes con diabetes tipo 2 el aguacate ayudó a lograr un control glucémico adecuado (Lerman *et al.*, 1994), permitió disminuir las dosis de agentes hipoglicemiantes orales y disminuyó la agregación plaquetaria, por tanto, se disminuyó el riesgo de trombosis (Carranza *et al.*, 1997).

1.2.1. El aceite de aguacate

Del fruto del aguacate se pude obtener el aceite y este mantiene como componente mayoritario al ácido linolénico (C16:0 5.92%, C16:1 0.2%, C18:0 1.86%, C18:1 55.9%, C18:2 24.9%, C18:3 10.1%) (Ortiz-Avila *et al.*, 2015). En nuestro grupo de trabajo se han realizado estudios respecto al efecto del aceite de aguacate sobre la disfunción mitocondrial en el riñón durante la diabetes, utilizando ratas inducidas con estreptozotocina (STZ) (Ortiz-Avila *et al.*, 2013). Se observó que la diabetes inhibió la actividad del complejo III debido a la alteración de la transferencia de electrones a nivel de los citocromos c + c1, causando una mayor generación de ERO sin cambios en los niveles de peroxidación lipídica. La administración durante 90 días del aceite de aguacate evitó estos efectos y aumentó la resistencia del complejo III a la inhibición por el estrés oxidativo inducido in vitro. Esto se atribuyó a la amplia variedad de antioxidantes lipofílicos presentes en el aceite.

Cuadro 2.- Compuestos presentes en la pulpa del aguacate. Tomada y modificada de Giacco *et al.*, 2010.

Compuestos que se pueden encontrar en la pulpa del aguacate por cada 100g de fruta:			
Vitamina C (mg)	8.80	Filoquinona (µg)	21.0
Tiamina (mg)	0.08	Ácidos grasos saturados (g)	2.13
Riboflavina (mg)	0.14	Ácidos grasos monoinsaturados (g)	9.80
Niacina (mg)	1.91	Ácidos grasos poliinsaturados (g)	1.82
Ácido pantotenoico(mg)	1.46	Colesterol (mg)	0.00
Vitamina B-6 (mg)	0.29	Estigmasterol (mg)	2.00
Folato (µg)	89.0	Campesterol (mg)	5.00
Colina (mg)	14.2	beta-sitosterol (mg)	76.0
Betaina (mg)	0.70	Calcio (mg)	13.0
Vitamina A (µg)	7.00	Hierro (mg)	0.61
alfa-caroteno (µg)	63.0	Magnesio (mg)	29.0
beta-caroteno (µg)	24.0	Fósforo (mg)	54.0
beta-criptoxantina (µg)	27.0	Potasio (mg)	507.0
Luteína + Zeaxantina (µg)	271.0	Sodio (mg)	8.00
alfa-tocoferol (mg)	1.97	Zinc (mg)	0.68
beta-tocoferol (mg)	0.04	Cobre (mg)	0.17
gamma-tocoferol (mg)	0.32	Manganeso (mg)	0.15
delta-tocoferol (mg)	0.02	Selenio (mg)	0.40

2. JUSTIFICACIÓN

Debido a que la diabetes es un grave problema de salud en todo el mundo y que su alta tasa de morbi-mortalidad está asociada a las diversas complicaciones que pueden afectar diversos órganos en estos pacientes, es de gran interés encontrar estrategias que puedan reducir la incidencia de estas complicaciones. Dado que el estrés oxidativo y la disfunción mitocondrial son elementos claves en la etiología de la mayoría de estas complicaciones, el aceite de aguacate podría ser una alternativa en la prevención y tratamiento de estas enfermedades asociadas a la diabetes, debido a la variedad de componentes como el ácido oleico y la diversidad de antioxidantes que lo componen, además de los efectos benéficos observados al proteger la actividad de los complejos de la cadena transportadora de electrones y disminuir la formación de ERO, lo cual mejora la función mitocondrial y disminuye el estrés oxidativo durante la diabetes.

3. HIPÓTESIS

El aceite de aguacate previene el daño en órganos blanco de la diabetes al disminuir el estrés oxidativo y mejorar la función mitocondrial.

4. OBJETIVO GENERAL

Determinar el efecto del aceite de aguacate sobre el daño en órganos blanco de la diabetes y su relación con el estrés oxidativo y la disfunción mitocondrial.

4.1. OBJETIVOS ESPECÍFICOS

- Analizar el efecto del aceite de aguacate sobre distintos parámetros fisiológicos que se alteran durante la diabetes.
- Evaluar el efecto del aceite de aguacate en la función mitocondrial en el hígado, cerebro y riñón de ratas diabéticas.
- Determinar el efecto del aceite de aguacate sobre el estrés oxidativo mitocondrial en el hígado, cerebro y riñón de ratas diabéticas.
- Analizar el efecto del aceite de aguacate en las alteraciones histopatológicas en el hígado y riñón de ratas diabéticas.

5. CAPÍTULO I. EFECTOS PROTECTORES DE LA INGESTA DE ACEITE DE AGUACATE EN EL DETERIORO DE LA FUNCIÓN DE LA CADENA DE TRANSPORTE DE ELECTRONES Y EL ESTRÉS OXIDATIVO EXACERBADO EN MITOCONDRIAS DEL HÍGADO DE RATAS DIABÉTICAS.

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Protective effects of dietary avocado oil on impaired electron transport chain function and exacerbated oxidative stress in liver mitochondria from diabetic rats

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Abstract Electron transport chain (ETC) dysfunction, excessive ROS generation and lipid peroxidation are hallmarks of mitochondrial injury in the diabetic liver, with these alterations also playing a role in the development of non-alcoholic fatty liver disease (NAFLD). Enhanced mitochondrial sensitivity to lipid peroxidation during diabetes has been also associated to augmented content of C22:6 in membrane phospholipids. Thus, we aimed to test whether avocado oil, a rich source of C18:1 and antioxidants, attenuates the deleterious effects of diabetes on oxidative status of liver mitochondria by decreasing unsaturation of acyl chains of membrane lipids and/or by improving ETC functionality and decreasing ROS generation. Streptozocin-induced diabetes elicited a noticeable increase in the content of C22:6, leading to augmented mitochondrial peroxidizability index and higher levels of lipid peroxidation. Mitochondrial respiration and complex I activity were impaired in diabetic rats with a concomitant increase in ROS generation using a complex I substrate. This was associated to a more oxidized state of glutathione. All these alterations were prevented by avocado oil except by the changes in mitochondrial fatty acid composition. Avocado oil did not prevented hyperglycemia and polyphagia although did normalized hyperlipidemia. Neither diabetes nor avocado oil induced steatosis. These results suggest that avocado oil improves mitochondrial ETC function by attenuating the deleterious effects of oxidative stress in the liver of diabetic rats independently of a hypoglycemic effect or by modifying the fatty acid composition of mitochondrial membranes. These findings might have also significant implications in the progression of NAFLD in experimental models of steatosis.

Keywords Diabetes · *Persea americana* · Respiratory chain · Liver disease · Fatty acids · Oxidative damage

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Introduction

Diabetes is characterized by a generalized state of ROS overproduction, leading to oxidative damage in biomolecules and triggering signaling pathways that ultimately lead to the development of diabetic complications in target organs (Giacco and Brownlee 2010). In the liver of diabetic models, ROS overproduction is associated, along with other factors, to impaired function of the mitochondrial electron transport chain (ETC) (Lukivskaya et al. 2007; Satav and Katyare 2004;

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Bouderba et al. 2012) and increased lipid peroxidation (Kristal et al. 1997). Moreover, diabetes is a risk factor for the development of nonalcoholic fatty liver disease (NAFLD), which includes the appearance of steatosis, nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis (Musso et al. 2011). Diabetes and NAFLD shares features of mitochondrial dysfunction and oxidative stress, being lipid peroxidation highlighted as a key factor that activates signaling pathways leading to inflammation and fibrosis (Dey and Swaminathan 2010). On this basis, it has been hypothesized that protection of liver mitochondrial function from the deleterious effects of oxidative damage may be beneficial to slow the progression of NAFLD. Hence, the improvement of mitochondrial function and oxidative stress may also be valuable against hepatic injury in diabetes (Grattagliano et al. 2012).

Some efforts have been done to improve liver mitochondrial function and oxidative stress during diabetes through the use of compounds with antioxidant properties. For example, ursodeoxycholic acid, a secondary bile acid used for the treatment of cholestatic liver diseases, prevents the decrease in the mitochondrial content of reduced glutathione and the generation of intermediate products of lipid peroxidation in alloxan-induced diabetic rats with an improvement of mitochondrial respiration (Lukivskaya et al. 2007). Moreover, the administration of boldine, an alkaloid extracted from *Peumus boldus*, restores the activity of glutathione peroxidase and decreases lipid peroxidation and ROS generation in liver mitochondria from STZ-induced diabetic rats, although these effects might be related with the partial hypoglycemic effect of that alkaloid (Jang et al. 2000). These two studies exemplify the potential of natural products against the deleterious effects of diabetes on liver mitochondrial bioenergetics and oxidative stress, supporting its usefulness as a complimentary strategy to conventional pharmacological approaches to prevent and/or delay liver damage during diabetes.

Our group has recently reported the properties of avocado oil against the harmful effects of diabetes on mitochondrial oxidative stress and ETC function in kidney from STZ-induced diabetic rats (Ortiz-Avila et al. 2013). It was observed that diabetes inhibited the activity of the complex III due to impaired electron transfer at cytochrome $c+c_1$, causing increased ROS generation without changes in the levels of lipid peroxidation. 90-days of dietary avocado oil intake prevented these effects and augmented the resistance of the complex III to the inhibition by in vitro-induced oxidative stress. This was attributed to the wide variety of lipophilic antioxidants present in the oil (Ashton et al. 2006). Oleic acid (C18:1) is the main fatty acid in avocado oil, constituting 50–70 % of its total fatty acids (Ozdemir and Topuz 2004). Besides the favorable effects of monounsaturated fatty acids (MUFA) from avocado on serum lipid profile and cardiovascular risk (Alvizouri-Muñoz et al. 1992; Carranza et al. 1995), MUFA sources like olive oil decreases oxidative damage in some components of the ETC from liver mitochondria by increasing MUFA content of mitochondrial membranes and enhancing its resistance to peroxidative damage (Quiles et al. 2006).

In view of the role of mitochondrial ROS generation and lipid peroxidation in liver damage during diabetes, the high content of MUFA and antioxidants in avocado oil and its protective effect against kidney mitochondrial dysfunction in diabetic rats, the main goal of this study was to explore whether avocado oil consumption decreases mitochondrial oxidative stress in liver mitochondria of diabetic rats in association with improved function of the ETC, lower ROS generation and remodeling of the fatty acid composition of liver mitochondrial membranes. In addition, we explored whether avocado oil promotes hepatic steatosis in control and diabetic rats.

Materials and methods

Animals and experimental design

Male Wistar rats weighing 300–350 g were used and kept in a room under controlled temperature and cycles of 12 h light/dark. Animals were feed with a standard rodent diet and water ad libitum. For rat care, we followed the recommendations of the Mexican Federal Regulations for the Use and Care of Animals (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico). This research was also approved by the Institutional Committee for Use of Animals of the Universidad Michoacana de San Nicolás de Hidalgo. Diabetes was induced by intraperitoneal administration of STZ (45 mg/kg) to rats subjected to 12-h fasting. Five days after STZ injection, blood glucose levels were determined and the rats exhibiting glucose levels higher than 300 mg/dL were considered diabetic. Control rats were treated in the same way except that it were administered only with vehicle.

After the determination of glucose levels, the animals were randomly assigned to 4 groups of 8 rats each: Group 1, consisting of normoglycemic rats feed only with rodent diet; group 2, consisting of diabetic rats feed only with rodent diet; group 3, consisting of normoglycemic rats feed with rodent diet plus avocado oil; group 4, consisting of diabetic rats feed with rodent diet plus avocado oil. Avocado oil was orally administered daily at a dose of 1 mL/250 g weight during 90 days, using a bottled, commercial presentation of avocado oil (Ahuacatlan, DIRI COM, S.A. de C.V., México), purchased from a local grocery. The fatty acid composition of this oil was assessed by gas chromatography and consisted of 5.92 ± 0.1 % C16:0, 0.2±0.02 % C16:1, 1.86±0.02 % C18:0, 55.9 %±0.2 C18:1, 24.9±0.1 % C18:2, 10.1±0.1 % C18:3 and 0.92±0.1 % of a non-identified fatty acid. Food and water intake, as well as the weight of the

animals, were recorded before the beginning and at the end of the treatment with avocado oil.

Determination of serum glucose and lipids

The animals were fasted 12 h before the sacrifice, after which blood was recollected and serum was obtained for the enzymatic determination of glucose and total cholesterol and triglycerides with kits from BioSystems (Barcelona, Spain), according to the manufacturer's instructions.

Histological evaluation of liver

Immediately after the sacrifice of the animals, liver sections were fixed in 10 % formalin, embedded in paraffin blocks and sectioned for hematoxylin-eosin staining in glass slides. Preparations were blindly examined by an independent researcher by light microscopy 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).

Isolation of mitochondria

Mitochondria were isolated by differential centrifugation of liver homogenates as described elsewhere (Saavedra-Molina and Devlin 1997). The mitochondrial pellets were stored at −80 °C until used, except for assays of mitochondrial respiration, where mitochondria were used immediately after isolation. Protein concentration was assessed before each assay by the Biuret method.

Determination of mitochondrial fatty acid composition

Lipids from mitochondrial samples were obtained by the method of Bligh and Dyer (1959) and the resultant fatty acids were derivatized for its analysis according to the method of Morrison and Smith (1964). Subsequently, the methyl-esters of fatty acids were analyzed by gas chromatography under the conditions previously described by Ortiz-Avila et al. (2013).

Evaluation of lipid peroxidation levels

This determination was carried out in 0.1 mg/mL of mitochondrial protein by measuring the levels of thiobarbituric acid reactive substances (TBARS), according to the protocol of Buege and Aust (1978). To avoid false positive results due to the interaction of thiobarbituric acid with the carbohydrates present in mitochondria isolation buffers, mitochondrial pellets were washed twice and resuspended with 50 mM KH_2PO_4 buffer (pH 7.6) 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 Fe^{2+} μM in 50 mM KH_2PO_4 buffer at the concentrations indicated in the Fig. 5b.

Evaluation of oxidative stress

This parameter was estimated by measuring the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). First, to measure the concentration of total glutathione (GSH+GSSG), mitochondrial samples (0.1 mg/mL) were deproteinized through the addition of 5 % sulfosalicilic acid and centrifuged at 7840 g during 10 min. Then, the pellet was discarded and 90 μL of the supernatant were added to a reaction mixture containing 0.1 M phosphate buffered solution, 3 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.1 M EDTA and 0.115 U/mL glutathione reductase. After 5 min of incubation at room temperature, 2 mM NADPH was added and DTNB reduction was followed spectrophotometrically at 412 nm in a Shimadzu UV2550 spectrophotometer, being the rate of DTNB reduction directly proportional to the concentration of GSH (Akerboom and Sies 1981). GSSG was determined by the procedure described above, except that GSH was derivatized with 3 % 4-vinylpyridine during 60 min. Finally, the amount of reduced glutathione was obtained by subtracting the concentration of GSH+GSSG minus the concentration of GSSG.

Measurement of ROS production

ROS generation was determined by evaluating the oxidation of the ROS probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) into the fluorescent compound 2′,7′-dichlorofluorescein (DCF) after its intramitochondrial hydrolysis into dichlorodihydrofluorescein (H_2DCF). Briefly, 0.5 mg/mL freeze-thawed mitochondrial protein and 500 mM H_2DCFDA were added to a buffer containing 100 mM KCl, 10 mM HEPES, 3 mM KH_2PO_4 and 3 mM $MgCl_2$ (pH 7.4) and incubated during 20 min with shaking at 4 °C. This mixture was placed in a quartz cuvette and basal fluorescence was recorded in a Shimadzu RF5301PC spectrofluorometer (λ_{ex} 491 nm; λ_{em} 518 nm). After 1 min, 10 mM glutamate-malate (Complex I-linked ROS) or 10 mM succinate plus rotenone (Complex II-linked ROS) were added and the changes in H_2DCF fluorescence were further followed by 20 min. Alternatively, to catalyze the reaction of H_2DCF with the oxidants produced by the activity of the ETC, mitochondria were incubated during 30 min with 25 μM of Fe^{2+}. All the determinations were done at room temperature. The results were expressed as the difference of the fluorescence (ΔF) in arbitrary units, calculated by subtracting the maximum fluorescence reached 20 min after substrate addition minus the basal fluorescence (i.e., the fluorescence before substrate addition).

Determination of the activities of the ETC complexes and mitochondrial respiration

To evaluate the activities of ETC complexes, mitochondria were solubilized with Triton X-100 (Hallberg et al. 1993) before each assay in order to enhance the accessibility of substrates and inhibitors to the redox sites of the ETC complexes. Complex I activity was assayed by a modification of the technique reported by Chomova et al. (2012). Briefly, 0.1 mg/mL solubilized mitochondria were resuspended in 1 mL 50 mM KH_2PO_4 buffer and incubated with 1 μg antimycin A plus 1 mM KCN. After 5 min, 5 mM $K_3Fe(CN)_6$ 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 16.3 mM^{-1} cm^{-1} for NADH. No changes in absorbance were detected in the absence of 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. (2009).

Mitochondrial respiration was assayed polarographically in intact, freshly isolated mitochondria with a Clark-type electrode connected to a YSI5300 biological oxygen monitor and a computer for data acquisition. 0.5 mg/mL mitochondrial protein was placed in a sealed glass chamber with constant stirring containing 2 mL respiration buffer (100 mM KCl, 10 mM HEPES, 3 mM KH_2PO_4, 3 mM $MgCl_2$, pH 7.4). 10 mM glutamate-malate was added as substrate to start oxygen consumption registration in basal, state 2. After 5 min, 300 μM ADP was added to stimulate state 3 respiration and oxygen consumption was further registered during 5 min.

Evaluation of F_1F_0-ATPase activity

This activity was estimated by quantifying the release of phosphorus from ATP hydrolysis following the methodology described by Fiske and SubbaRow (1925). To distinguish the phosphorus released by F_1F_0-ATPase activity from the phosphorus from other sources, this experiment was also carried out in mitochondria pre-treated before ATP addition with the F_1F_0-ATPase inhibitor oligomycin (50 μg) as a negative control. The phosphorous released by the activity of the enzyme was calculated by subtracting the concentration of phosphorous in the presence of oligomycin to that obtained in the absence of the inhibitor.

Statistical analyses

Results are expressed as the mean ± standard error. Statistical differences of the data were determined with Student's *t* test using Sigma Plot software v11.0. Statistically significant differences were defined as $P<0.05$.

Results

Effects of diabetes and avocado oil on physiological parameters and liver histology

At the end of the treatments, glucose levels of STZ-induced diabetic rats were 7.8-fold higher than in control rats. Avocado oil supplementation did not have any effect on this parameter, although normoglycemic rats treated in the same way exhibited a decrease of 23.8 % in glucose levels with respect to control animals (Fig. 1a). Diabetic rats also developed hyperlipidemia as serum levels of cholesterol and triglycerides were 1.3- and 4.4-fold higher, respectively, than in control rats, with avocado oil normalizing these alterations (Fig. 1b and c). Moreover, avocado oil did not modify the levels of these lipids in control rats.

Signs of polyphagia and polydipsia were detected in diabetic rats at the beginning of the treatment since food and water intake were almost two and three-fold higher, respectively, in comparison to control animals (Table 1) and these differences remained constant at the end of the study. No changes in polyphagia and polydipsia were observed when avocado oil was supplemented in diabetic rats. Regarding to the body weight of the groups, diabetic rats exhibited a loss weight of 68 g at the end of the treatment, while the opposite was observed in control rats with a weight gaining of 163.3 g. The treatment with avocado oil induced an apparent attenuation in loss weight in diabetic animals, as loss weight was 34 g lower in diabetic animals treated with avocado oil, although the differences between the final values of weight of the diabetic group and the diabetic group plus avocado oil were not statistically significant. In the same way, avocado oil caused an apparent higher weight gaining in control rats, but the differences in the final weight between the rats from the control group and the control group plus avocado oil were not statistically significant.

Histological analyses were carried out in order to assess whether avocado oil intake induced liver injury during the experimental period tested. No signs of steatosis, hepatocyte ballooning or lobular inflammation were observed due to avocado oil treatment in control or diabetic rats. Moreover, diabetes did not induce histological alterations although the elevated levels of serum lipids and glucose in that group (Fig. 2). No alterations were detected even when microscopic analysis were done at higher magnification (Fig S1).

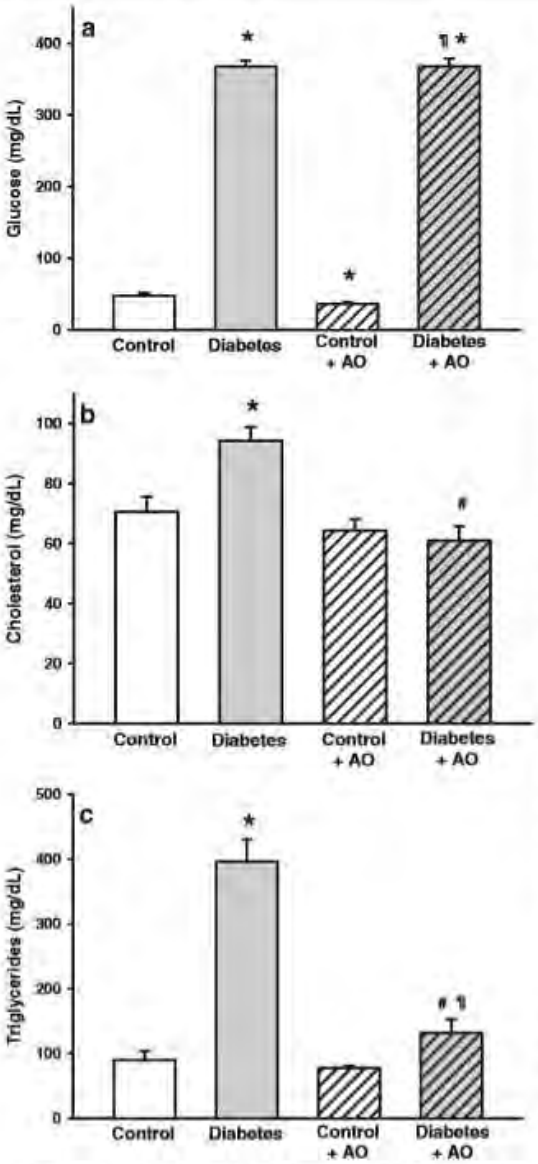


Fig. 1 Serum levels of glucose (**a**), cholesterol (**b**) and triglycerides (**c**) at the end of 90-days of treatment with avocado oil. The results are presented as the mean±S.E. of $n>5$. $^{*}P<0.05$ vs. control; $^{\P}P<0.05$ vs. control + AO; $^{\#}P<0.05$ vs. diabetes (student's *t*-test)

Effects of diabetes and avocado oil on mitochondrial fatty acid composition and fatty acid indexes

Profound alterations in fatty acid composition were detected in diabetic rats (Table 2). Changes were detected in the content of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), with decrements of 10, 87.5 and 40.4 % in palmitic (C16:0), palmitoleic (C16:1) and oleic (C18:1) acids, respectively, while the content of stearic acid (C18:0) exhibited an increment of 33.9 %. Regarding to the changes in PUFA, the content of linolenic acid (C18:3) decreased 44.4 %, whereas among all the fatty acid species, docosahexaenoic acid (22:6) underwent the most dramatic change with an increment in 104.4 %. Avocado oil intake failed to prevent these alterations and no significant changes were neither observed in mitochondria from control animals consuming avocado oil.

Concerning to fatty acid indexes, increased C18:0 content and the diminution in both C16:1 and C18:1 led to a slight increase in the total content of SFA (Fig. 3a) and a ~50 % decrease in the total content of MUFA, respectively (Fig. 3b). In contrast, the percentage of total polyunsaturated fatty acid (PUFA) remained without changes despite the prominent increase in the percentage of C22:6 (Fig. 3c). Avocado oil did not have any effect on these parameters. On the other hand, peroxidizability index (PI), a parameter that estimates membrane sensitivity to lipid peroxidation on the basis of its fatty acid composition, was 21 % higher in mitochondria from diabetic rats than in mitochondria from control rats, irrespectively of avocado oil treatment (Fig. 3d).

Influence of avocado oil over the effects of diabetes on liver mitochondrial oxidative stress and lipid peroxidation

Mitochondrial GSH/GSSG ratios were assessed in order to determine the degree of oxidative stress in liver mitochondria in response to diabetes and avocado oil (Fig. 4a). Mitochondria from diabetic rats displayed exacerbated oxidative stress as the GSH/GSSG ratio was 2.2-fold lower than in control mitochondria. Avocado oil fully prevented this effect without having any influence in control mitochondria. Lower GSH/GSSG ratio induced by diabetes may not be attributed to impaired glutathione synthesis since there were no statistically significant differences in the concentration of total glutathione among all the experimental groups, except by the diabetic + avocado oil group, which exhibited a 19.2 % increment with respect to the diabetic group (Fig. 4b). Regarding to the levels of lipid peroxidation (Fig. 5), a similar behavior with respect to oxidative stress levels was observed, with mitochondria from diabetic rats displaying a two-fold increase in TBARS levels and avocado oil fully inhibiting this effect (panel a). Moreover, avocado oil also aided to prevent excessive TBARS production when mitochondria were challenged against an acute oxidative stimulus (Fig. 5b), since mitochondria from animals supplemented with avocado oil were several-fold less sensitive to TBARS generation at concentrations up to 100 μM Fe^{2+}, independently of diabetes.

Table 1 Effect of diabetes on food and water intake and weight before and after 90-days of avocado oil supplementation

		Control	Control + AO	Diabetes	Diabetes + AO
Weight	Before treatment	339.0±13.8	333.5±8.3	314.3±2.5	316.0±8.3
	After treatment	502.3±9.6	547.8±15.3	246.3±16.0*	282.0±19.3*
Food	Before treatment	16.5±0.8	18.3±2.3	32.9±2.4*	33.9±0.9*
	After treatment	17.7±1.7	19.6±1.0	36.0±0.8*	38.6±1.7*
Water	Before treatment	51.3±2.6	48.7±2.1	156.2±12.3*	166.1±9.4*
	After treatment	49.0±1.6	43.6±2.7	171.5±4.4*	166.6±2.0*

These parameters were determined 5 days after STZ injection, just before the start of the treatment with avocado oil and after 90-days of the treatment. Data are presented as the mean±S.E. of $n=4$. *$P<0.05$ vs. control (student's t-test)

Effects of avocado oil on ROS generation and ETC functionality

Aimed to test whether increased oxidative stress and lipid peroxidation are related to enhanced ROS production at the ETC, this parameter was measured in the presence of glutamate-malate, a complex I-linked substrate (Fig. 6a) or succinate, a complex II-linked substrate (Fig. 6b). In the first case, liver mitochondria from diabetic animals produced 1.7-fold more ROS than mitochondria from normoglycemic rats. Avocado oil intake inhibited ROS generation to the levels of control mitochondria. In contrast, when succinate was used as substrate (Fig. 6b), ROS generation was marginal in comparison with that observed with glutamate-malate (Fig. 6a) and no differences were observed between all the experimental groups. These experiments were also carried out in the presence of 25 μM Fe^{2+} to catalyze the reaction of the oxidants produced at the ETC with H_2DCF. As observed in the Fig. 6c and d, a similar trend was observed under this condition, although the levels of DCF fluorescence were 10-fold higher than in the absence of exogenously added Fe^{2+}, which reflects the catalyzing effect of Fe^{2+}.

The activity of the complexes from the ETC was determined to further characterizing the site of ROS generation in liver mitochondria of diabetic rats and the effects of avocado oil. As expected from the results of ROS generation with glutamate-malate (Fig. 6a), the activity of the complex I from mitochondria of diabetic animals was almost 50 % lower in comparison to the activity of mitochondria from control animals (Fig. 7a). Moreover, this effect was fully prevented by avocado oil supplementation. In contrast, complex II activity increased 1.6 times in the mitochondria from the diabetic rats and a similar effect was observed in mitochondria from control animals supplemented with avocado oil (Fig. 7b). On the contrary, the activities of the complexes III and IV (Fig. 7c and d, respectively), remained without significant changes.

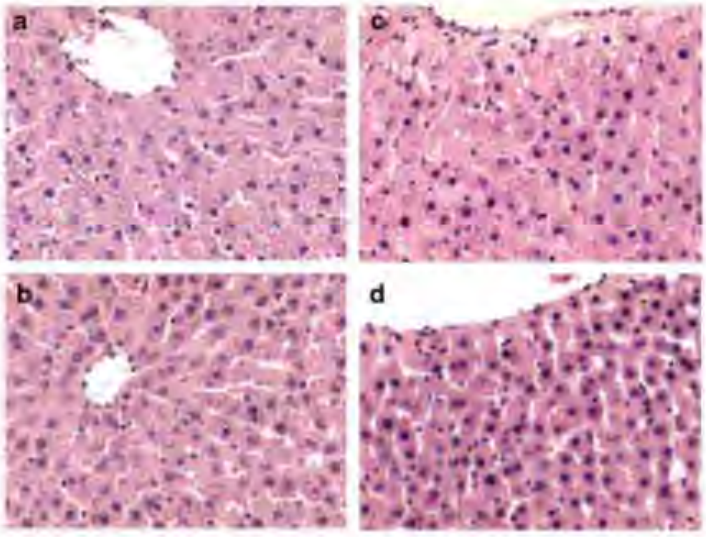


Fig. 2 Histological analyses by hematoxylin-eosin staining of liver sections of rats from control (**a**), Diabetes (**b**), control + AO (**c**) and diabetes + AO (**d**) groups. Pictures were taken at 400× magnification and are representative of $n=4$

Table 2 Effect of avocado oil on fatty acid composition of liver mitochondria from control and diabetic rats

	C16:0	C16:1	C18:0	C18:1	C18:2	γ-C18:3	C18:3	C20:4	C20:5	C22:1	C22:6	N.I.
Control	21.2±0.5	0.8±0.1	15.3±0.4	9.9±0.3	25.2±0.3	0.4±0.1	0.9±0.0	18.7±0.4	0.4±0.0	0.2±0.1	4.5±0.2	2.3±0.4
Diabetes	19.2±0.6*	0.1±0.1*	20.5±0.8*	5.9±0.2*	22.9±1.8	0.3±0.0	0.5±0.1*	18.0±0.7	0.2±0.1	0.4±0.3	9.2±1.2*	2.8±0.3
Control + AO	19.8±0.5	0.7±0.1	15.4±1.3	11.5±0.9	23.6±1.1	0.3±0.0	0.9±0.1	20.1±1.4	0.3±0.1	0.7±0.5	4.8±0.2	1.9±0.3
Diabetes + AO	19.2±0.2	0.2±0.1¶	20.6±0.8¶	6.3±0.2¶	22.3±1.0	0.3±0.0	0.4±0.0¶	17.5±0.7	0.2±0.1	0.1±0.1	10.3±0.4¶	2.6±0.4

Data are presented as the mean±S.E. of $n=5$. *$P<0.05$ vs control; ¶ $P<0.05$ vs control + AO (student's t-test)

Basal (state 2) and phosphorylating (state 3) states of respiration were evaluated in order to determine whether complex I inhibition have an impact in the overall function of the ETC and its ability to modulate oxygen consumption in response to exogenous ADP addition. Decreased respiration in both state 2 and state 3 was observed in mitochondria from diabetic rats when compared to control (Fig. 8a, gray line vs. black line, respectively, and Fig. 8b). As observed in the gray dashed trace, supplementation with avocado oil improved the decline in respiration observed in diabetic rats in both states, being statistically significant this effect only in state 3 (Fig. 8b). In contrast, avocado oil becomes mitochondria from control rats unresponsive to ADP addition (black dashed trace), although respiration rate in state 2 was similar to control mitochondria (Fig. 8b).

To test whether decreased stimulation of state 3 respiration was the result of impaired F_1F_0-ATP synthase in mitochondria from either diabetic rats or control rats supplemented with avocado oil, we measured the activity of this enzyme in the sense of ATP hydrolysis. As depicted in the Fig. 8c, ATPase activity remained unaltered in mitochondria from control rats treated with avocado oil; however, diabetes induced a ~40 %

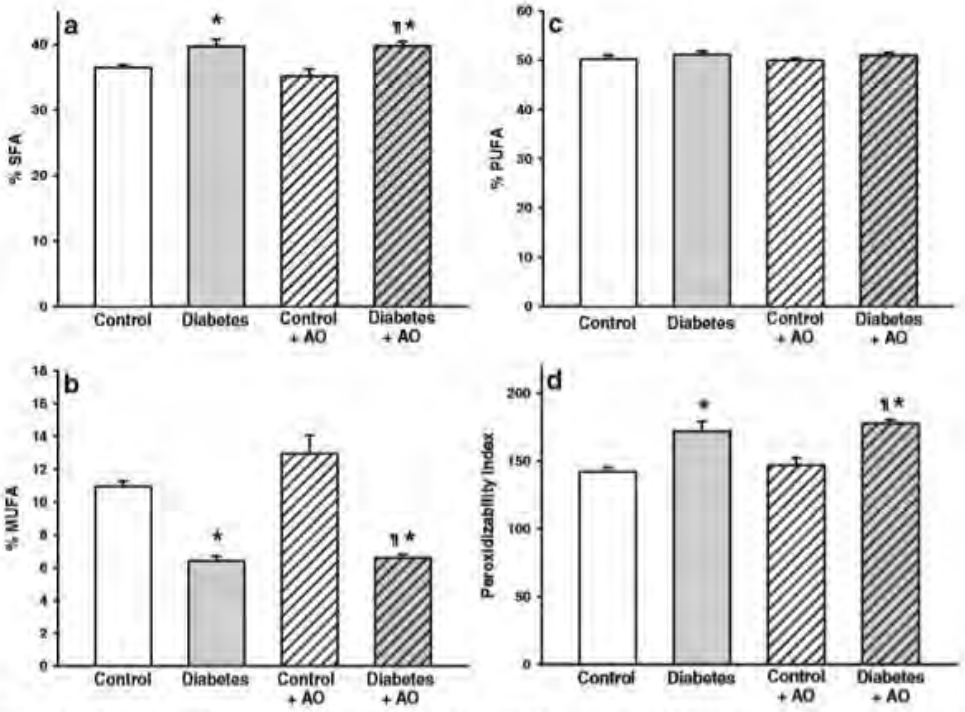


Fig. 3 Mitochondrial fatty indexes: Percentages of saturated fatty acids (SFA) (**a**), monounsaturated fatty acids (MUFA) (**b**), polyunsaturated fatty acids (PUFA) (**c**), and peroxidizability indexes (PI) (**d**). Indexes were calculated using the data from the Table 1. PI were calculated with the following equation reported by Pamplona et al. (1998): PI = [(%Monoenoic × 0.025) + (%Dienoic × 1) + (%Trienoic × 2) + (%Tetraenoic × 4) + (%Pentaenoic × 6) + (%Hexaenoic × 8)]. Data are presented as the mean±S.E. of $n=5$. *$P<0.05$ vs. control; ¶ $P<0.05$ vs. control + AO (student's t-test)

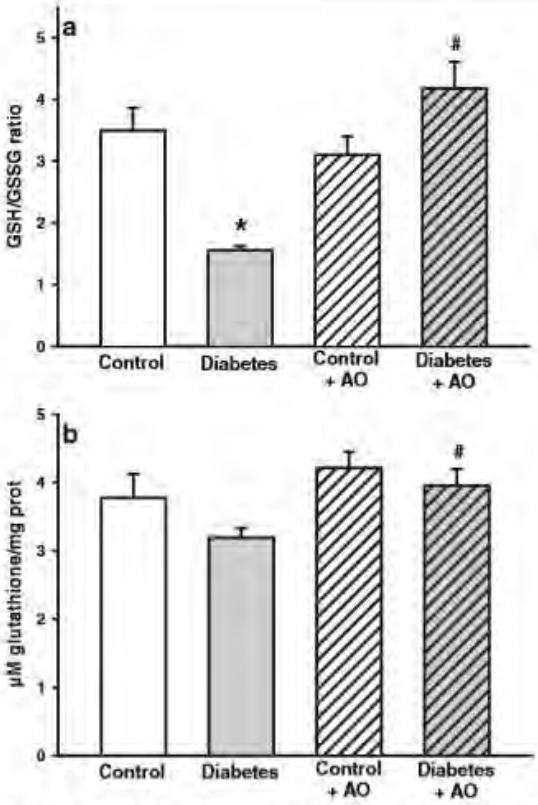


Fig. 4 Influence of avocado oil and diabetes on mitochondrial GSH/GSSG ratios (**a**) and total levels of glutathione (**b**). The results are presented as the mean±S.E. of $n=4$. $^{*}P<0.05$ vs. control; $^{\#}P<0.05$ vs. diabetes (student's *t*-test)

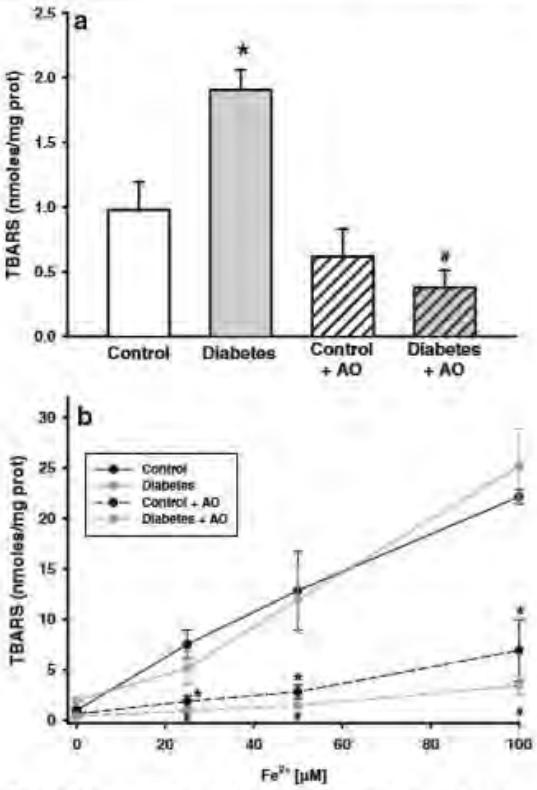


Fig. 5 Influence of avocado oil over the alterations induced by diabetes on mitochondrial lipid peroxidation. **a** Basal levels of lipid peroxidation. **b** Mitochondrial sensitivity to in vitro-induced lipid peroxidation with increasing concentrations of Fe^{2+} during 30 min. Data are represented as thiobarbituric acid reactive substances (TBARS) and presented as the mean±S.E. of $n=4$. $^{*}P<0.05$ vs. control; $^{\#}P<0.05$ vs. diabetes (student's *t*-test)

increase in this activity, which was statistically significant only in mitochondria from the diabetic animals treated with avocado oil.

Discussion

Global prevalence and incidence of diabetes are in sharp growth, which has urged for the implementation of novel strategies for the management of this disease and their devastating complications (Danaei et al. 2011). Glycemic control is fundamental for the management of diabetes; however, due to the complexity of this disease, diabetes care is more complex and requires strategies beyond to only addressing glycemic control (Standards of medical care in diabetes-2014 2014). The results from this study shows that avocado oil intake did not improved hyperglycemia (Fig. 1a) and, consequently, polyphagia and polydipsia remained unaltered (Table 1). However, avocado oil intake decreased liver mitochondrial oxidative stress and improved ETC function in diabetic rats, which might be the result of the antioxidant actions of the components of avocado oil independently of glycemic control. Alternatively, since mitochondrial function and ROS production also depends on other processes like mitophagy or mitochondrial fusion/fission (Lee et al. 2012; Westermann 2012), the possibility remains that the antioxidants constituting avocado oil may modulate signaling pathways favoring these processes, as it has been shown that some components from avocado modify intracellular signaling independently of their antioxidants properties (D'Ambrosio et al. 2011).

Despite the lack of effects on hyperglycemia, avocado oil normalized the rise in serum lipids caused by diabetes (Fig. 1b and c). This is in concordance with the hypocholesterolemic and hypotriglyceridemic effects of diets enriched with avocado in subjects with type-2 diabetes (Lerman-Garber et al. 1994) or hypercholesterolemia (Carranza et al. 1995; López

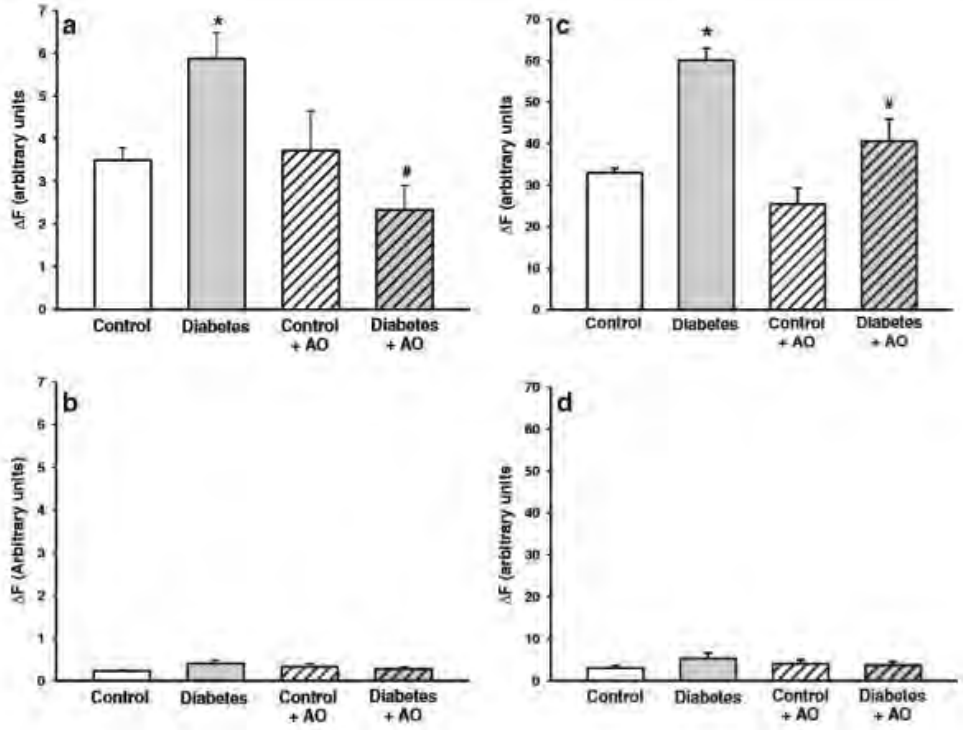


Fig. 6 Influence of avocado oil over the alterations induced by diabetes on mitochondrial ROS generation. H_2DCF oxidation was monitored in the absence (panels **a** and **b**) or the presence of 25 μM Fe^{2+} (panels **c** and **d**) to catalyze H_2DCF oxidation by ETC-generated oxidants. Glutamate-malate (panels **a** and **c**) or succinate (**b** and **d**) were used as substrates. Data are presented as the mean±S.E. of $n>3$. $^{*}P<0.05$ vs. control; $^{\#}P<0.05$ vs. diabetes (student's *t*-test)

Ledesma et al. 1996). It has been demonstrated that C18:1 promotes intracellular cholesterol esterification and increased expression of low-density lipoprotein (LDL) receptors (Rumsey et al. 1995). Moreover, enriched diets with C18:1 decrease serum triglycerides in mice by a mechanism involving lower hepatic triglyceride production and inhibition of hormone-sensitive lipase (Kotake et al. 2004). Thus, as C18:1 constitutes more than 50 % of the fatty acids from avocado oil, increased LDL uptake and decreased hepatic triglyceride synthesis may be underlying causes of normalization of serum cholesterol and triglycerides, respectively, by avocado oil. The molecular mechanism of avocado oil for controlling lipids levels might be related with peroxisomes proliferator activated receptors (PPARs). It has been reported that C18:1 is capable to induce PPAR-δ (Wu et al. 2012), which has been associated with degradation of lipids by enhancing fatty acid catabolism (Wang et al. 2003). Another interesting characteristic of avocado oil is the relative high concentration of linolenic acid (around 10.1 %), an omega-3 fatty acid precursor of long chain fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Omega-3 fatty acids are widely recognized as PPARs activators with several effects on lipids metabolism, leading to increased fatty acid oxidation not only in liver but also in skeletal muscle, regulating in this way lipid concentrations on serum (Grygiel-Górniak 2014).

Another probable mechanism accounting for the effects of avocado oil on elevated lipids levels of diabetic rats might reside in the protective effects of avocado oil over the complex I activity of these animals. In this regard, utilization of triglycerides through mitochondrial β-oxidation requires a constant influx of NAD^+ to sustain the activity of the 3-hydroxyacyl-CoA dehydrogenase. NADH equivalents produced by this enzyme are re-oxidized by complex I and its inhibition lead to diminished β-oxidation flux and accumulation of 3-hydroxyacyl-CoA and carnitine esters (Eaton et al. 1994). Therefore, the rise in triglycerides levels detected in diabetic rats may be related with the inhibition of complex I activity (Fig. 7a), leading to limited β-oxidation and enhanced fatty acid efflux from liver to circulation. Therefore, it is possible to suggest that normalized complex I activity by avocado oil may enhance mitochondrial β-oxidation by favoring NADH reoxidation, leading to lowered levels of triglycerides as observed in the Fig. 1c. This is in agreement with the notion that

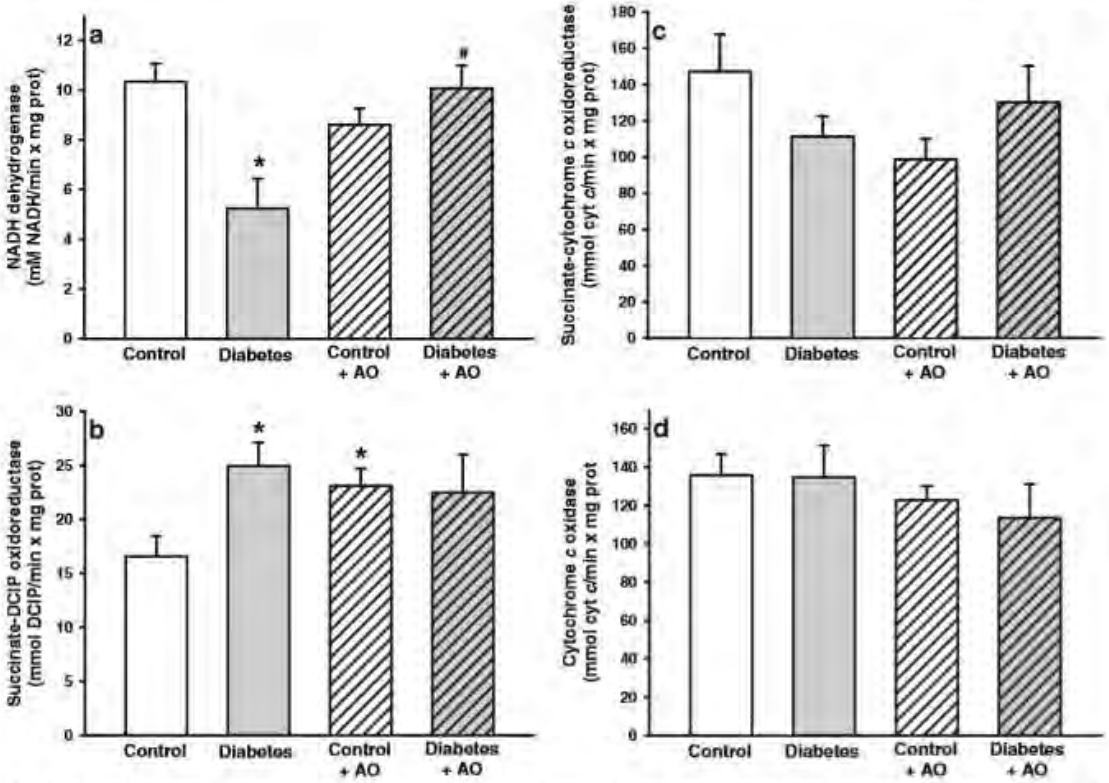
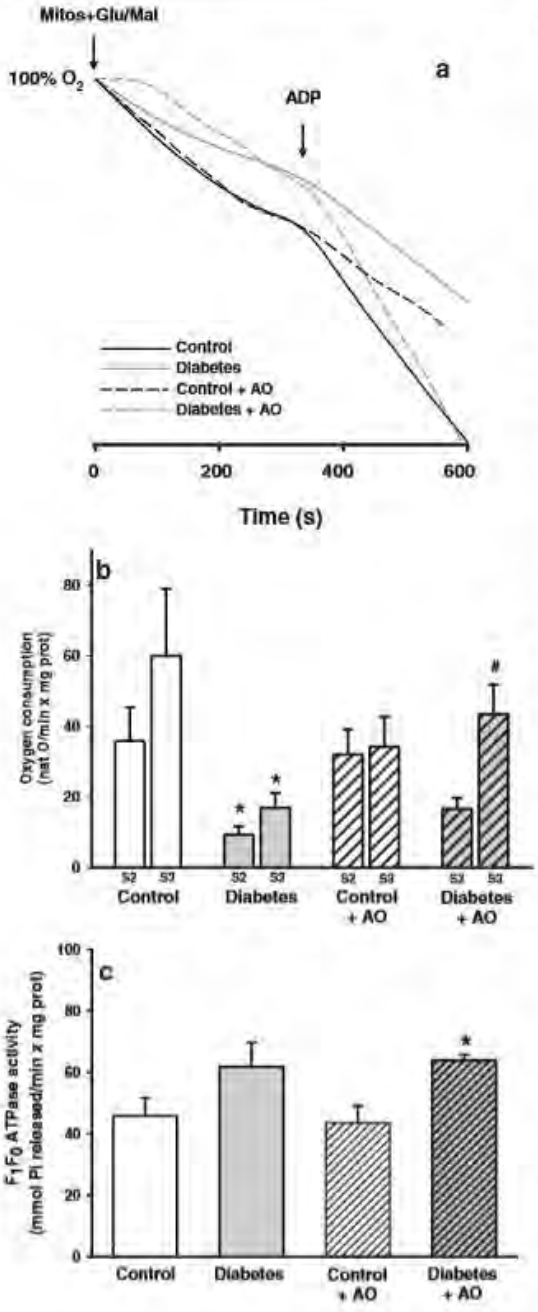


Fig. 7 Effects of diabetes and avocado oil on the activities of the ETC complexes. Complex I (**a**), complex II (**b**), complex III (**c**), complex IV (**d**). Data are presented as the mean ± S.E. of $n>4$. $^{*}P<0.05$ vs. control; $^{\#}P<0.05$ vs. diabetes (student's *t*-test)

the augment of the catabolism of lipids causes a diminution in the circulating levels of triglycerides and free fatty acids (Wang et al. 2003), and is also related with activation of PPARs as was discussed above. Thus, the role of avocado oil consumption in PPARs activation, mitochondrial β-oxidation flux and $NADH/NAD^{+}$ ratios deserves further investigation.

No evidence for steatosis or inflammation was observed in the diabetic animals, even considering the "extra load" of fat when avocado oil was supplemented (Fig. 2). Although it has been paid more attention to hepatic steatosis in type-2 diabetes than in type-1 diabetes, there is substantial evidence about the occurrence of this condition in the latter case (Regnell and Lernmark 2011). Even though this may be contradictory with our results about the absence of steatosis in diabetic rats (Fig. 2b), a previous study reported that STZ-induced type-I diabetes lead to a very low degree of steatosis in Sprague-Dawley rats, being this attributed to an absence of hyperinsulinemia despite elevated levels of serum free fatty acids and hyperglycemia (Wang et al. 2011). Thus, the probable hypoinsulinemia induced by STZ in diabetic rats due to the destruction of pancreatic β-cells, along with the different rat strain used in this study (i.e., Wistar rat), the lower dose of STZ (45 vs. 55 mg/Kg) and the shorter time of diabetes duration (12.8 vs. 14 weeks), may account together for the absence of steatosis seen in the diabetic animals.

Regarding to lipid peroxidation, diabetic rats exhibited a prominent augment in this parameter (Fig. 5), which is in agreement with a similar effect observed also in STZ-induced (Kristal et al. 1997) or alloxan-induced (Lukivskaya et al. 2007) diabetic rats. In order to elucidate if this effect was due to a remodeling of the fatty acid composition of mitochondrial membranes leading to enhanced sensitivity to peroxidation, we analyzed mitochondrial fatty acid composition and calculated some fatty acid indexes. Accordingly, peroxidizability index (PI) was higher in diabetic rats (Fig. 3d). This can be explained mainly as a consequence of an inferior percentage of MUFA (Fig. 3b) and higher amounts of n-3 PUFA, which in turn was the result of a diminution in C16:1 and C18:1 proportions and a noticeable increment in C22:6, the more unsaturated fatty acid present in the membranes (Table 2). Furthermore, the changes in fatty acid composition induced by STZ are in concordance with the suggestion by Kristal et al. (1997) about the influence of probable



◀ **Fig. 8** Effects of diabetes and avocado oil on mitochondrial respiration (panels **a** and **b**) and F_1F_0-ATPase activity (panel **c**). In the panel **a**, basal (state 2 (S2)) respiration was stimulated in 0.5 mg/mL mitochondrial protein using glutamate-malate as substrate. Where indicated, 300 μM ADP was added to stimulate respiration in phosphorylating state (state 3 (S3)). The quantification of oxygen consumption rates is shown in the panel **b**. Data of oxygen consumption and F_1F_0-ATPase activity are presented as the mean+S.E. of $n \geq 4$. $^{*}P<0.05$ vs. control; $^{\#}P<0.05$ vs. diabetes (student's *t*-test)

changes in fatty acid composition on augmented rates of lipid peroxidation in STZ-induced diabetic rats.

Avocado oil fully abrogated the effect of diabetes on lipid peroxidation (Fig. 5) but this was not attributable to an influence of avocado oil on fatty acid composition of diabetic rats (Table 2) and, hence, on the PI of membranes, as this parameter and the fatty acid indexes remained without changes, even in control, normoglycemic rats. This is contrary to a previous report from our group where avocado oil supplemented under the same conditions, elicited an increase in the percentage of MUFA in kidney mitochondrial membranes of both normoglycemic and STZ-induced diabetic rats (Ortiz-Avila et al. 2013). The latter is in agreement with the notion that fatty acid residues of mitochondrial membranes are modified at some extent according to the type of fat supplemented in the diet, in such way that rats supplemented with a source of MUFA exhibited higher proportions of MUFA in their mitochondrial membranes in comparison with animals supplemented with a source of PUFA (Ochoa et al. 2003). However, as stated by the same authors, mitotic tissues like the liver are more resilient to the influence of exogenous fatty acids on membrane lipid composition than post-mitotic tissues (Quiles et al. 2002). Therefore, this may explain the differences observed in MUFA accumulation between the membranes of liver and kidney mitochondria from rats supplemented with avocado oil. Avocado oil also lowered ostensibly the sensitivity to lipid peroxidation induced by a short-term, exogenous, oxidant challenge, which suggest that its administration enables a more reduced redox state of the mitochondrial milieu that aid to attenuate the deleterious effect of oxidative stress. The results of the later experiment were also important to conciliate the use of the TBA technique with some concerns about the suitability of this assay for measurements of lipid peroxidation levels in biological samples (Janero 1990; Trevisan et al. 2001). Although it is true that TBA exhibits a high unspecificity as react with many aldehyde different from malondialdehyde (i.e., one of the final products of lipid peroxidation), it has been recognized that methods measuring free MDA like the TBA assay are applicable to short-term in vitro preparations of biological materials, in which free MDA accumulates, (Draper et al. 1993), which would be applicable for the experiment of the Fig. 5b. In support for the use of TBA assay in this study, the usefulness of this method has been recognized when lipid peroxidation is estimated in isolated membrane systems (i.e., isolated mitochondria) (Halliwell and Gutteridge 1999). Furthermore, when the extent of lipid peroxidation in biological samples measured by the TBA assay has been compared to more refined assays like

gas chromatography-mass spectroscopy (GC-MS), it has been concluded that both methods yields parallel results, although the TBA assay exhibits a lower sensitivity (Liu et al. 1997). In summary, all these arguments, along with our findings under Fe^{2+} stress, suggests that avocado oil might decrease the in vivo damage to mitochondrial lipids as reflected by the lower levels of TBARS found in mitochondria from diabetic rats consuming avocado oil. Nevertheless, the need to confirm this result by a more sophisticated technique is not denied.

Mitochondria from diabetic rats underwent a condition of severe oxidative stress as reflected by a twofold decrease in GSH/GSSG ratio (Fig. 4a). Thus, increased mitochondrial lipid peroxidation in diabetic rats may also be the result of diminished availability of GSH. In mitochondrial membranes, phospholipid hydroperoxide glutathione peroxidase 4 (GPX4) protects from lipid peroxidation by reducing lipid hydroperoxides at the expense of GSH (Ursini and Bindoli 1987; Arai et al. 1996). Therefore, the mechanism by which avocado oil decreased lipid peroxidation in diabetic rats might also involve enhanced reduction of lipid hydroperoxides by GPX4, as increased availability of GSH (Fig. 4a) may improve the ability of this enzyme to counteract lipid peroxidation. Nonetheless, it cannot be discarded that some lipophilic antioxidants from avocado oil may also protect membrane phospholipids against peroxidative damage by directly scavenging ROS at the lipophilic core of the membranes. In this regard, lutein and zeaxanthine, two xanthophyll carotenoid reported to be present in avocado oil (Ashton et al. 2006), possess notable protective effects against membrane peroxidative injury without altering the mechanical properties of the membranes (Sujak et al. 1999), which in turn may be advantageous for the preservation of the ETC functionality. In summary, the relative contributions of enhanced GPX4 activity or the direct actions of xanthophyll carotenoids against increased lipid peroxidation in the liver of diabetic animals supplemented with avocado oil deserves further research.

Besides GSH/GPX4 system, mitochondrial oxidative stress is also under the control of the thioredoxine (Trx) system (Stanley et al. 2011; Aon et al. 2012). In mitochondria, the Trx system is constituted by peroxiredoxin 3 (Prx3), which scavenges H_2O_2 produced at the ETC and its activity is dependent on the reduced form of thioredoxin 2 (Trx2), which in turn, is converted back to its reduced form by the action of thioredoxin reductase 2 (TrxR2) using NADPH as reducing power (Ribas et al. 2014). When ETC operates in forward mode (i.e., as tested in this work when glutamate-malate was used as complex I substrate), it is believed that Trx2 is the main controller of ROS production by virtue of the low Trx2 concentrations in comparison with GSH concentrations and the prominent increase of H_2O_2 production when Trx2 is selectively inhibited (Stanley et al. 2011). However, these systems exhibits an interdependence between them since when GSH is depleted, Trx2 becomes oxidized and enhanced susceptibility to ROS is observed (Zhang et al. 2007; McCommis et al. 2011). Recently, it was confirmed that both systems act in a concerted and continuous way by relieving each other when the antioxidant capacity of one of these system becomes saturated during mitochondrial H_2O_2 overproduction (Aon et al. 2012). Based on these reports, it can be proposed that a probable mechanism by which avocado oil decreases ROS production in mitochondria from diabetic rats is by enhancing the activity of Trx2 system through augmented availability of reduced GSH, as reflected by the increased GSH/GSSG ratio observed with avocado oil (Fig 4a), although this possibility was not directly demonstrated. Nonetheless, in agreement with this idea, it has been shown that exogenous addition of GSH to cardiac mitochondria from diabetic rats reverts lowered activity of Trx2 system (Tocchetti et al. 2012).

Importantly, in the aforementioned study, it was shown that diabetic rats exhibited impaired bioenergetics function as reflected by altered state 2–state 3 respiratory transition, which is in concordance with our results (Fig. 8a and b). Moreover, it was also found that diabetic mitochondria exhibited a decrease in the reduction of Trx2 stimulated by addition of respiratory substrates, which was related with impaired activity of the ETC (Tocchetti et al. 2012). Reduced status of GSH in liver mitochondria is also dependent on respiration by increasing the levels of NADPH (Garcia et al. 2010). Therefore, the decreased GSH/GSSG ratio in mitochondria from diabetic rats and the prevention of this effect by avocado oil (Fig. 4a) may be also explained by decreased respiratory capacity in states 4 and 3 and the prevention of this effect by avocado oil (Fig. 8a and b), respectively. These speculations reflect the need to evaluate the target(s) of avocado oil in mitochondria by further determining its effect on the redox couples $Trx2(SH_2)$/Trx2SS, $NADH/NAD^+$ and $NADPH/NADP^+$, as well as the identity of the molecule(s) from avocado oil possibly targeting these systems. Exacerbated oxidative stress in mitochondria from diabetic rats seems to be related to a remarkable increment in ROS production when the ETC oxidizes complex I substrates (Fig. 6a). Of note, a marginal production of ROS was observed when succinate, a complex II substrate, was used in combination with rotenone (an inhibitor of complex I) (Fig. 6b), confirming that ROS generation proceeds exclusively at the complex I. This is in full agreement with the twofold inhibition of the complex I activity observed in mitochondria from diabetic rats (Fig. 7a). Moreover, the treatment with avocado oil abolished both the exacerbation in ROS production and the inhibition of the complex I activity observed in diabetic rats, which further supports the hypothesis that complex I was the solely site of ROS production in these animals. Diabetes also decreases NADH dehydrogenase and complex I activity in liver mitochondria from alloxan-treated rats (Lukivskaya et al. 2007) and diabetic, obese, *Psammomys obesus* gerbils (Bouderba et al. 2012), respectively, further

suggesting that complex I is an important target in the liver of diabetic models. However, our data of ETC complex activities in diabetic rats seems to be in conflict with some reports about this topic. For example, Raza et al. (2011) have found that 8 weeks after STZ administration, liver mitochondria displayed declined activities of both complex III and complex IV along with an upregulation of complex II, the latter being in agreement with our results. However, another discrepant result from this report is that complex I activity was also increased. We ignore the reasons explaining those differences with our results, and it can be only conjectured that the different strain of rat (Sprague Dawley vs, Wistar in that paper and our manuscript, respectively) and a shorter time of treatment with STZ may account for these differences. The issue about the duration of treatment with STZ and the effects on ETC function was addressed by other study (Satav and Katyare 2004), where it was found that respiration with complex I and complex II-linked substrates was inhibited at the end of 1-month STZ treatment, but longer times were not tested. Moreover, the diabetes-prone *Psammomys obesus* gerbil, a rodent model of nutritional diabetes, displayed an inhibition in the activity of the complexes I and III and upregulation of complex II activity after 18 weeks of feeding with a hypercaloric diet (Bouderba et al. 2012). From this report and our results, it seems that after several weeks of hyperglycemia (~8–12, according to the report of Raza et al. 2011 and our results), complex II is upregulated probably to increase the flux of electrons towards ETC to compensate an eventual decrease in electron transfer at complex I. Thus, the kinetics of ETC impairment at longer times than 3-months needs to be studied to explore this hypothesis and to verify whether avocado oil has beneficial effects on mitochondrial function throughout the entire lifespan of the rat.

It has been argued that H_2DCFDA may not be the more convenient probe to measure ROS generation due to the inability of the hydrolyzed probe (i.e., H_2DCF) to cross the inner mitochondrial membrane and its dependence on catalyst such as transition metals or cytochrome *c* to detect H_2O_2. Moreover, under certain circumstances, it has been considered that H_2DCF fluorescence may reflects catalyst level as much as ROS production (Karlsson et al. 2010; Dikalov and Harrison 2014; Wardman et al. 2002). To circumvent all these disavantages, we addressed the issue of inner mitochondrial membrane impermeability to H_2DCF by doing all the experimentation in freeze-thawed mitochondria. On the other hand, to discard that higher H_2DCF oxidation in mitochondria from diabetic animals and the prevention by avocado oil simply reflect changes in the pool of either cytochrome c or iron instead estimating oxidants production at the ETC, we carried out additional experiments by incubating mitochondria with 25 μM Fe^{2+}. The rationale for this approach was that an excess of exogenous catalyst might circumvent any constrain in H_2DCF oxidation (and therefore, in ROS detection) imposed by variable amounts of endogenous catalyst. The Fig. 6c and d show that the differences in H_2DCF oxidation seen in the Fig. 6a and b were not due to parallel variations in endogenous catalyst since the same trend in fluorescence changes was observed than in the absence of added iron. Moreover, the parallel, ~10-fold increase in the levels of H_2DCF fluorescence reflect a higher catalyzing effect by exogenous iron.

The substrate-dependent rise in the fluorescent signal of H_2DCF constitute another line of evidence supporting that, under our experimental conditions, this parameter reflects the production of oxidants due to the function of the ETC instead of being a result of the method unspecificity. This is displayed in the representative time-traces of H_2DCF fluorescence in mitochondria from all the samples using glutamate-malate or succinate as substrate (Fig S2). The panel b of the Figure S2 is a "zoom" of the Fig S2a that allows a better appreciation of the effects of substrate addition on fluorescence. In mitochondria from diabetic rats, it can be observed that upon addition of glutamate-malate, there is an instantaneous increase in H_2DCF fluorescence, while in the rest of the samples there is a biphasic behavior, first, with a lag phase of different extent among all the samples and then, a phase of rapid rise after several seconds. In contrast, it was observed that H_2DCF fluorescence begins to increase several minutes after succinate addition in a discrete fashion. Additional control experiments were done to discard that fluorescence changes elicited by substrate were the results of an artifact and not by mitochondrial electron transport (Fig S2, panels c and d). In the panel c it is observed that fluorescence remained unchanged in mitochondria from control rats when substrate addition was omitted. The same behavior was observed for heat-denatured mitochondria or when substrates were added in a reaction mix not containing mitochondria. These results are better observed in the Fig S2d. These data demonstrate that rising in H_2DCF fluorescence is dependent on both the availability of substrates for electron transport and a functional ETC.

On the other hand, in concordance with the notable inhibition of complex I, respiration in both state 2 and state 3 exhibited a notable repression, which was prevented in an important extent by avocado oil (Fig. 8a and b). Therefore, it seems that complex I is the main step limiting the flux of electrons through the ETC in the diabetic animals. Repression of respiration in state 3 might be attributable to decreased F_1F_0-ATP synthase activity; however, this scenario was discarded as this enzyme showed a tendency to increase its activity in mitochondria from diabetic animals independently of avocado oil treatment (Fig. 8c). This reinforces the idea that the overall activity of the ETC in diabetic rats was impaired at the level of complex I and that avocado oil prevented this effect by preserving the activity of this respiratory complex. The tendency of incremented F_1F_0-ATPase activity might be interpreted as a probable compensatory effect to counteract the

deleterious effects of diabetes in bioenergetic status of mitochondria due to ETC dysfunction in order to supply adequate amounts of ATP to sustain the intense anabolic activity of liver. However, it is important to point out that such increment was not statistically significant.

The insensitivity to ADP addition seen in mitochondria from control rats supplemented with avocado oil was neither due to inhibition of F_1F_o-ATP synthase (Fig. 8c). An uncoupling of respiration does not seems the cause of this effect as respiration in state 2 was similar to mitochondria from control animals. Thus, suppressed activity of adenine nucleotide translocase or limited availability of inorganic phosphate may be probable candidates responsible for this effect. However, it is important to note that neither ROS generation, lipid peroxidation nor oxidative stress was higher in mitochondria from control animals supplemented with avocado oil despite its inhibitory effect on state 3 respiration.

Cardiolipin plays a crucial role in both the structure and function of the complex I (Lenaz et al. 2006). Peroxidative damage to this phospholipid has been linked to impaired complex I activity and exacerbated ROS generation during in vitro-induced oxidative stress (Paradies et al. 2002), in hepatic steatosis (Petrosillo et al. 2007) and cardiac isquemia-reperfusion injury (Paradies et al. 2004). Despite these data, it is difficult to envisage that lipid peroxidation inhibition has a primary role on the inhibition of complex I during diabetes neither in the mechanism by which avocado oil prevented this effect and excessive ROS generation. In this regard, it is well known that cardiolipin is essential for the activity of a myriad of proteins embedded in mitochondrial membranes, including all the ETC complexes (Chicco and Sparagna 2007). By this reason, lipid peroxidation has an inhibitory role in all the ETC complexes (Forsmark-Andrée et al. 1997; Cortés-Rojo et al. 2009). Thus, the lack of inhibitory effects by diabetes on the activity of the complexes II and IV, along with the weak, non-statistically decline in complex III activity, argues against the idea about a central participation of lipid peroxidation in the effects of diabetes on the ETC. Instead, selective complex I inhibition and enhanced ROS generation at this site fits better with probable glutathionylation of this enzyme due to a more oxidized state of glutathione, as reflected by decreased GSH/GSSG ratio in diabetic mitochondria (Fig. 4a). Protein glutathioylation occurs in response to decreased GSH/GSSG ratio and consist in the reaction of oxidized glutathione (GSSG) formed during oxidative stress with protein thiols ($Pr\text{-}S^-$) to yield mixed disulfides (Pr-S-SG), inhibiting in this way the activity of a variety of proteins. This reaction occurs in a non-enzymatic fashion when GSH/GSSG approach to a value of 1.0 and ROS generation is high. Alternatively, glutaredoxin enzymes (Grx) can catalyze this process (Ribas et al. 2014). In mitochondria, complex I is the solely enzyme from the ETC that becomes persistently glutathionylated, being its activity inhibited by ~50 % when the GSH/GSSG ratio has a value of 2.0 (Beer et al. 2004). Besides, glutathionylation increases ROS production in complex I and this has been associated with anomalous ROS production during pathologic states (Taylor et al. 2003). These numbers are in good agreement with the obtained in this study, since in diabetic rats, GSH/GSSG ratio decreased to 1.5±0.06 and complex I activity was inhibited in 49.3 % with a concomitant augment in ROS production only with a complex I-linked substrate (i.e., glutamate-malate). Thus, the mechanism underlying the beneficial effects of avocado oil in mitochondria from diabetic rats might consist in the maintenance of a more reduced GSH/GSSG ratio similar to that observed in control mitochondria (4.1±0.4 vs 3.5±0.3, respectively), which would prevent and/or revert complex I inhibition as high GSH/GSSG ratio enables Grx2-mediated deglutathionylation (Ribas et al. 2014). Despite this mechanism is merely speculative because we have not measured complex I gluthationylation, there is increasingly evidence supporting the pathologic importance of complex I inhibition by glutahionylation, as demonstrated in a recent report where complex I inhibition due to homozygotic Grx2 deletion caused cardiac impairment and hypertension and this was prevented by restoration of mitochondrial redox environment (Mailloux et al. 2014).

Although under our experimental conditions diabetic rats did not developed liver steatosis, some consideration may be done in relation with the probable beneficial effects of avocado oil in the context of NAFLD. The two-hits hypothesis of NAFLD pathogenesis states that lipid accumulation in liver constitutes a "first" hit that sensitize this organ to a "second" hit where oxidative stress and inflammation lead to the progression of steatosis to NASH and fibrosis (Day and James 1998). Despite this theory has been subjected to several modifications (Dowman et al. 2010; Takaki et al. 2013), mitochondrial dysfunction and lipid peroxidation remain as central players of this hypothesis, with these processes contributing to the activation of inflammatory pathways (Day 2002; Jaeschke et al. 1996; Bacuerle and Henkel 1994) and the development of fibrosis (Letteron et al. 1996), respectively. As indicated above, diabetic rats did not exhibited the first hit (i.e., steatosis); nevertheless, some components of the second hit such as mitochondrial dysfunction, ROS generation and increased lipid peroxidation does. Thus, it can be hypothesized that avocado oil or some of its fractions might delay the progression of NAFLD in diabetic models where steatosis is observed (e.g., Zucker diabetic rats (Forcheron et al. 2009)), by inhibiting the factors involved in the second hit. Further research is underway in our laboratory to test this hypothesis.

Regarding to the utility of avocado oil to improve liver health during diabetes, the possible implications of our findings is that the antioxidant properties of avocado oil at mitochondrial level might be useful to delay the deterioration of liver function during this disease, mainly the aspects related

with ROS-activated signaling pathways inducing tissue degeneration. However, considering its high fat load, this suggestion must be taken with caution because we still do not know whether avocado oil consumption can be deleterious due to lipotoxicity when steatosis is already established. Thus, one of the next steps in this research is to test the effects of avocado oil on liver function in animals with steatosis or steatohepatitis. Another possibility is that the probable disadvantages of fat from avocado oil may be bypassed by administering a fatty acid-free extract. In these regards, intensive work is being carried out in our group to answer these questions. Finally, other possibility is that regular consumption of avocado oil in the diet may delay the development of liver dysfunction in people with predisposition to suffer metabolic syndrome before the clinical and biochemical manifestations of this disease begin.

In conclusion, avocado oil decreases oxidative stress and lipid peroxidation in mitochondria from STZ-induced diabetic rats in association with enhanced complex I activity and attenuation of ROS production, independently of a hypoglycemic effect or by prevention of the alterations in mitochondrial fatty acid composition elicited by diabetes, but with amelioration of hyperlipidemia.

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6. CAPÍTULO II. EL ACEITE DE AGUACATE MEJORA LA FUNCIÓN MITOCONDRIAL Y DISMINUYE EL ESTRÉS OXIDATIVO EN EL CEREBRO DE RATAS DIABÉTICAS.

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Research Article

Avocado Oil Improves Mitochondrial Function and Decreases Oxidative Stress in Brain of Diabetic Rats

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Diabetic encephalopathy is a diabetic complication related to the metabolic alterations featuring diabetes. Diabetes is characterized by increased lipid peroxidation, altered glutathione redox status, exacerbated levels of ROS, and mitochondrial dysfunction. Although the pathophysiology of diabetic encephalopathy remains to be clarified, oxidative stress and mitochondrial dysfunction play a crucial role in the pathogenesis of chronic diabetic complications. Taking this into consideration, the aim of this work was to evaluate the effects of 90-day avocado oil intake in brain mitochondrial function and oxidative status in streptozotocin-induced diabetic rats (STZ rats). Avocado oil improves brain mitochondrial function in diabetic rats preventing impairment of mitochondrial respiration and mitochondrial membrane potential ($\Delta\Psi_m$), besides increasing complex III activity. Avocado oil also decreased ROS levels and lipid peroxidation and improved the GSH/GSSG ratio as well. These results demonstrate that avocado oil supplementation prevents brain mitochondrial dysfunction induced by diabetes in association with decreased oxidative stress.

1. Introduction

Diabetes is characterized by a constant stage of hyperglycemia leading in the long term to severe damage to several systems [1], including the central nervous system (CNS). Diabetes has been involved in several brain conditions such as cerebral ischemia, macrovascular disease, microangiopathy, cognitive decline, and brain atrophy [2]. However, the mechanisms underlying neuronal damage in CNS, known as diabetic encephalopathy, are still unclear [3].

Mitochondrial dysfunction has been hypothesized to be a key factor in the progression of hyperglycemia-mediated neuronal damage [4, 5]. This is related to the large demand of the cells from the CNS for ATP to allow neurotransmission. For this reason, the maintenance of oxidative phosphorylation capacity is extremely important in the CNS since about 90% of the ATP required for the normal function of neurons is provided by mitochondria [6]. Thus, mitochondrial dysfunction may contribute to the loss of neuronal metabolic control and, consequently, to neurodegeneration [7]. This notion is supported by data demonstrating mitochondrial function decline with aging and in age-related diseases, such as diabetes [8].

Mitochondrial alterations related to diabetic encephalopathy include increased mitochondrial fission, excessive ROS levels [9], augmented levels of both lipid peroxidation and nitrite, and decreased levels of total antioxidant [10]. In addition, it has been suggested that diabetes-induced oxidative stress increases the levels of proinflammatory cytokines, which enhances neuronal degeneration [3]. Therefore, mitochondrial oxidative damage contributes, at least in part, to the development of diabetic encephalopathy [11].

Further studies are required about these issues for the development of therapeutic strategies to ameliorate the impact of diabetic encephalopathy and other complications of diabetes. In this regard, nutraceuticals with antioxidant properties have been used as alternative treatments to slow and/or prevent the inherent complications of diabetes [12–14]. A candidate belonging to this group of nutraceuticals is avocado, as this fruit contains a wide variety of antioxidants including carotenoids, tocopherols, chlorophylls, vitamins, and oleic acid (C18:1) as the main fatty acid [15]. Moreover, improvement in glycemic control, plasma lipid profile, and atherogenic index has been observed in diabetic patients consuming avocado in their diets [16]. Regarding the alterations in mitochondrial function and oxidative stress in diabetes, we have previously reported that avocado oil prevented renal mitochondrial dysfunction in streptozotocin-induced type I diabetic rats by preserving the activity of the complex III of the electron transport chain (ETC) and attenuating ROS levels due to protection of the integrity of cytochromes $c + c_1$ [17].

In this work, we aimed to evaluate the effects of avocado oil on brain mitochondrial function and oxidative status in STZ-induced type I diabetic rats. For this purpose, several parameters of mitochondrial function were analyzed such as respiratory control ratio (RCR), activity of the ETC complexes, transmembrane potential ($\Delta\Psi_m$), lipid peroxidation levels, ROS levels, and (GSH/GSSG) ratios.

2. Materials and Methods

2.1. Animals and Experimental Design. Male Wistar rats weighing between 300 and 350 g were used and kept under controlled temperature and 12 hours cycles of light/dark. Rats were feed with a rodent diet and water ad libitum. For the management of the animals, we followed the recommendations from Mexican Federal Regulations for the Use and Care of Animals (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico). This research was also approved by the Institutional Committee for Use of Animals of the Universidad Michoacana de San Nicolás de Hidalgo. Diabetes was induced by intraperitoneal administration of STZ (45 mg/kg), in rats subjected to 12-hour fasting conditions. Five days after diabetes induction, the glucose levels were determined and rats exhibiting blood glucose levels higher than 300 mg/dL were included in the experimental trial.

Rats were randomly divided in four groups: (1) normoglycemic rats (Control); (2) normoglycemic rats plus avocado oil (Control + AO); (3) diabetic rats (Diabetic); (4) diabetic rats plus avocado oil (Diabetic + AO). Avocado oil was administered orally at a dose of 1 mL/250 g weight in a daily basis for a period of 90 days. A commercial presentation of avocado oil (Ahuacatlan, DIRICOM, S.A. de C.V., México), purchased from a local grocery, was used in the experimental trial.

2.2. Isolation of Mitochondria. At the end of the treatment, animals were fasted overnight and sacrificed by decapitation. Brain was quickly removed and mitochondria were isolated by differential centrifugation in a Percoll gradient as previously described [18]. Briefly, the entire brain, without the cerebellum, was extracted and placed in a cold medium containing 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 0.5% bovine serum albumin, and 10 mM MOPS (pH 7.4). The brain was homogenized manually in a glass homogenizer and centrifuged at 400 g. The supernatant was centrifuged at 9000 g. Centrifugations were carried out during 10 min at 4°C. Mitochondrial protein concentration was measured by a modification of the Biuret method [19] calibrated with bovine serum albumin.

2.3. Oxygen Consumption. To measure mitochondrial respiration, freshly isolated brain mitochondria were resuspended in a final volume of 2 mL of buffer for measuring oxygen consumption (10 mM HEPES, 100 mM KCl, 3 mM $MgCl_2$, and 3 mM KH_2PO_4 at pH 7.4) in a sealed glass chamber with constant stirring. The rate of oxygen consumption was determined at room temperature using a Clark-type oxygen electrode coupled to an oxygen monitor YSI 5300 and a computer for data acquisition. The determinations started immediately after adding 10 mM glutamate/malate as respiratory substrate for complex I (state 4) and after 3 minutes, 1 mM ADP was added to determine oxygen consumption in the phosphorylating state (state 3). Finally, inhibitors of complex III (1 μg antimycin A) and complex IV (1 mM KCN) were added to inhibit mitochondrial respiration. The respiratory control ratio (RCR) was calculated from the ratio of the state 3/state 4 respiratory rates.

2.4. Determination of Mitochondrial Membrane Potential ($\Delta\Psi_m$). $\Delta\Psi_m$ was estimated by a spectrofluorometric assay using Safranin O [20]. 0.5 mg/mL mitochondria were resuspended in a medium containing of 100 mM KCl, 75 mM mannitol, 25 mM sucrose, and 0.05 mM EDTA (pH 7.4). $\Delta\Psi_m$ traces were started by measuring basal Safranin O fluorescence during 1 min. Later, mitochondria were energized with 10 mM glutamate/malate and fluorescence changes were followed by additional 4 min. Finally, 5 μM of the uncoupler CCCP (carbonylcyanide-chlorophenylhydrazone) was added to dissipate $\Delta\Psi_m$. The changes in Safranin O fluorescence were measured at λ_{ex} 495 nm and λ_{em} 586 nm in a Shimadzu RF5301PC spectrofluorometer.

2.5. Evaluation of Lipid Peroxidation Levels. This determination was carried out in brain mitochondria by measuring the levels of thiobarbituric acid reactive substances (TBARS) [21]. Absorbance was measured at 532 nm with a Perkin Elmer Lambda 18 UV/VIS spectrophotometer. Data were expressed as nanomoles of TBA reactive species (TBARS)/mg protein.

2.6. Measurement of the Activity of the ETC Complexes. To determine the activities of the ETC complexes I, II, III, and IV, intact mitochondria were permeabilized with Triton X-100 as previously described [22]. Enzymatic activities were assayed using 0.1 mg/mL permeabilized mitochondria resuspended in 50 mM KH_2PO_4 buffer. NADH-oxidoreductase (complex I) activity was assayed in brain mitochondria incubated

with 1 μg antimycin A plus 1 mM KCN. After 5 min, 5 mM $K_3Fe(CN)_6$ 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 16.3 $mM^{-1}cm^{-1}$ for NADH [23]. Succinate-DCIP oxidoreductase (complex II) activity was measured spectrophotometrically at 600 nm by following the reduction of 2,6-dichlorophenolindophenol (DCIP). Antimycin A-sensitive succinate-cytochrome *c* oxidoreductase (complex III) activity was followed by measuring at 550 nm the reduction of cytochrome *c*. Cytochrome *c* oxidase (complex IV) activity was evaluated by measuring the oxidation of reduced cytochrome *c* at 550 nm [24].

2.7. Measurement of ROS Levels. ROS levels were determined by measuring the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). 0.5 mg/mL intact mitochondria and 1.25 mM H_2DCFDA were incubated in a buffer containing 10 mM HEPES, 100 mM KCl, 3 mM $MgCl_2$, and 3 mM 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 1 min, 10 mM glutamate/malate was added and the changes in H_2DCFDA fluorescence were further followed by 20 min [17]. Fluorescence changes were detected in a Shimadzu RF-5301PC spectrofluorophotometer (λ_{ex} 485 nm; λ_{em} 520 nm).

2.8. Glutathione Assay. Mitochondrial samples were treated with 5% (v/v) sulfosalicylic acid and centrifuged at 7800 g for 10 min to remove denatured proteins, and reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined by an enzymatic method. The total glutathione (GSH + GSSG) content was assayed in a cuvette containing 90 μL of the supernatant in 0.1 M sodium phosphate buffer (pH 7.5), 3 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and 0.115 unit/mL glutathione reductase in a final volume of 1 mL. After 5 min of incubation at room temperature, 2 mM NADPH was added and the kinetics of the reaction was monitored for 5 min. The increment in absorbance at 412 nm was converted to GSH concentration using a standard curve with known amounts of GSH [25]. For determination of GSSG, the same DTNB recycling assay was performed after using 3% (v/v) 4-vinylpyridine to remove reduced GSH followed by incubation at room temperature for 1 h before the beginning of the assay.

2.9. Data Analysis. Results are expressed as the mean ± standard error of at least 3 independent experiments using samples from different animals for each experiment. Statistical differences of the data were determined with Student's *t*-test using Sigma Plot software v11.0.

3. Results

3.1. Body Weight and Levels of Serum Glucose and Lipids. The brains used for the experiments of this study were dissected from the same rats used for other study reporting the effects of avocado oil on liver mitochondrial function of diabetic rats [26]. As reported in that study, control and STZ rats exhibited at the end of the trial fasting serum glucose levels of 47.5 ± 4.1 and 367.6 ± 8.0 mg/dL, respectively. Cholesterol and triglycerides levels reached 70.5 ± 5.0 and 89.8 ± 13.99 mg/dL, respectively, in control animals and 94.2 ± 4.5 and 396.5 ± 33.6 mg/dL, respectively, in STZ rats. Avocado oil normalized cholesterol and decreased triglyceride levels in STZ-treated rats, as the levels of these lipids were 60.9 ± 4.6 and 131.8 ± 21.2 mg/dL, respectively. Avocado oil had not any effect on glucose levels of STZ-treated rats. Moreover, control and STZ-treated rats displayed at the end of the study body weight values of 502.3 ± 9.6 and 246.3 ± 16.0 g, respectively, without avocado oil treatment significantly altering this parameter neither in control nor in STZ-treated rats. Together, these results confirm that STZ treatment induced diabetes and demonstrate that avocado oil corrects only diabetic dyslipidemia.

3.2. Effects of Diabetes and Avocado Oil on Respiratory Function of Brain Mitochondria. Oxygen consumption rates (OCR) in resting state (state 4) and phosphorylating state (state 3) were measured with the objective to evaluate whether diabetes impaired mitochondrial function and determine the protective effects of avocado oil. Diabetes had a notable impact on brain mitochondria respiration (Figures 1(a) and 1(b)) as OCR in states 4 and 3 decreased 41.3% and 54.5%, respectively. Importantly, avocado oil prevented these effects, being this protective effect more prominent in state 3 respiration (Figure 1(b)). Impaired respiratory rates led to lower RCR in diabetic rats (Figure 1(c)), although, in this case, RCR values were not different in a statistically significant way.

3.3. Effects of Diabetes and Avocado Oil on Mitochondrial Transmembrane Potential ($\Delta\Psi_m$). Further characterization of impaired brain mitochondrial function in diabetic rats was carried out by analyzing $\Delta\Psi_m$. As observed in Figure 2, the energization of mitochondria from the control group (black line) with glutamate/malate elicited a large, instantaneous decrease in Safranin O fluorescence, which reflects the establishment of the $\Delta\Psi_m$. Moreover, $\Delta\Psi_m$ remained stable after 2 min and the addition of an uncoupler (CCCP) induced an increase in Safranin fluorescence at initial levels before substrate addition, which is indicative of full dissipation of the $\Delta\Psi_m$. In contrast, the changes elicited by glutamate/malate in Safranin fluorescence were of a considerably lower magnitude in mitochondria from diabetic rats (gray line) and occurs at a slower rate than in mitochondria from control rats (black line), which together indicates that diabetes impaired brain mitochondrial functionality. Notably, avocado oil intake fully prevented the alterations in the $\Delta\Psi_m$ observed in diabetic rats (gray pointed line) and did not alter this parameter in the control group (black discontinuous line). Therefore, these results confirm that diabetes induced brain mitochondrial dysfunction and that avocado oil fully prevented this alteration.

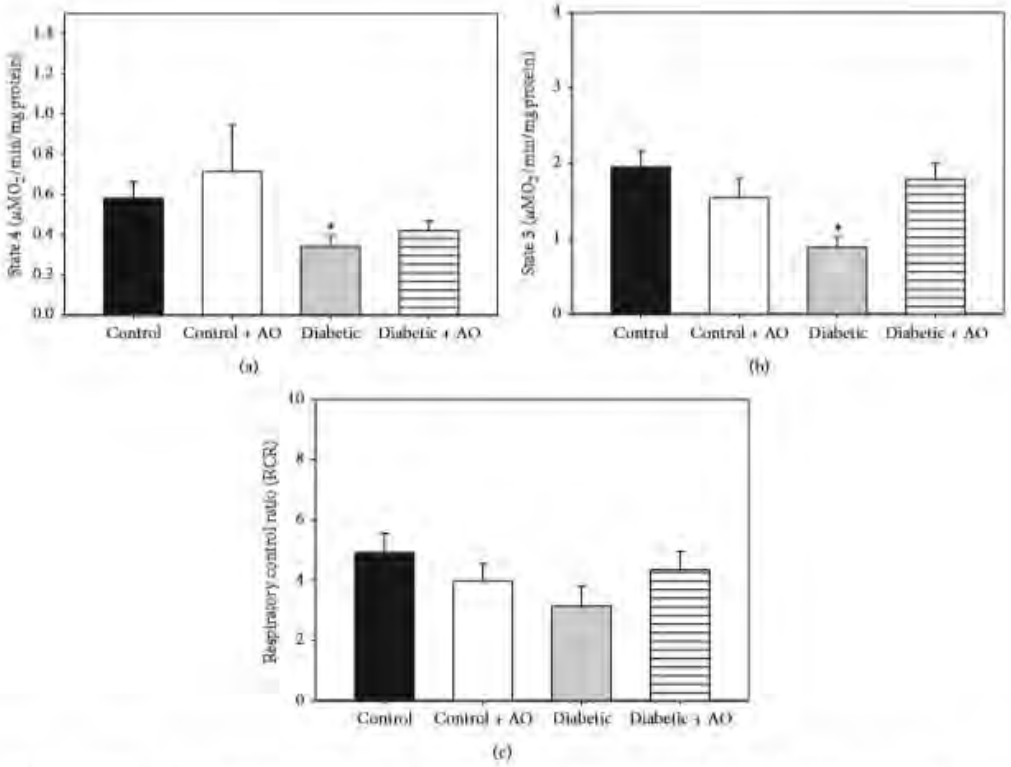

FIGURE 1: Effects of avocado oil treatment in brain mitochondrial respiratory chain parameters: (a) state 3 respiration; (b) state 4 respiration, and (c) respiratory control ratio (RCR). Data are the mean ± EE of $n = 5$. $^*P < 0.05$ compared with brain control mitochondria.

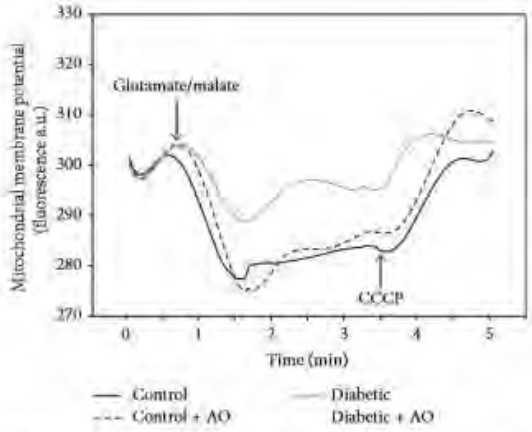

FIGURE 2: Effect of avocado oil on mitochondrial membrane potential ($\Delta\Psi_m$). Representative traces of fresh isolated brain mitochondria from diabetic rat. Membrane potential was expressed in fluorescence arbitrary units (a.u.). The traces are typical of four experiments.

3.4. Analysis of the Effects of Diabetes and Avocado Oil on ETC Functionality. The activities of the complexes from the ETC are shown in Figure 3. Regarding complex I, no differences in activity (Figure 3(a)) were observed between the control and diabetic groups; however, avocado oil intake decreased 40.5% of this activity in comparison with the control group. Diabetes did not provoked changes in complex II activity (Figure 3(b)), but avocado oil intake in control rats induced an increase of 3.2-fold in this activity. Regarding the activity of the complex III (Figure 3(c)), diabetes enhanced this activity by 44.2% when compared to control group. Avocado oil also augmented the activity of complex III in mitochondria from diabetic and normoglycemic rats in 125.7% and 87.1% respectively. Complex IV activities were similar in the mitochondrial from all groups, except by the control group treated with avocado oil, which exhibited a decrease of 54.9% in complex IV activity.

3.5. Influence of Diabetes and Avocado Oil on ROS Levels. In order to explore whether the impairment in both $\Delta\Psi_m$ and respiration was related to enhanced ROS levels, we evaluated the changes in the fluorescence of H_2DCFDA

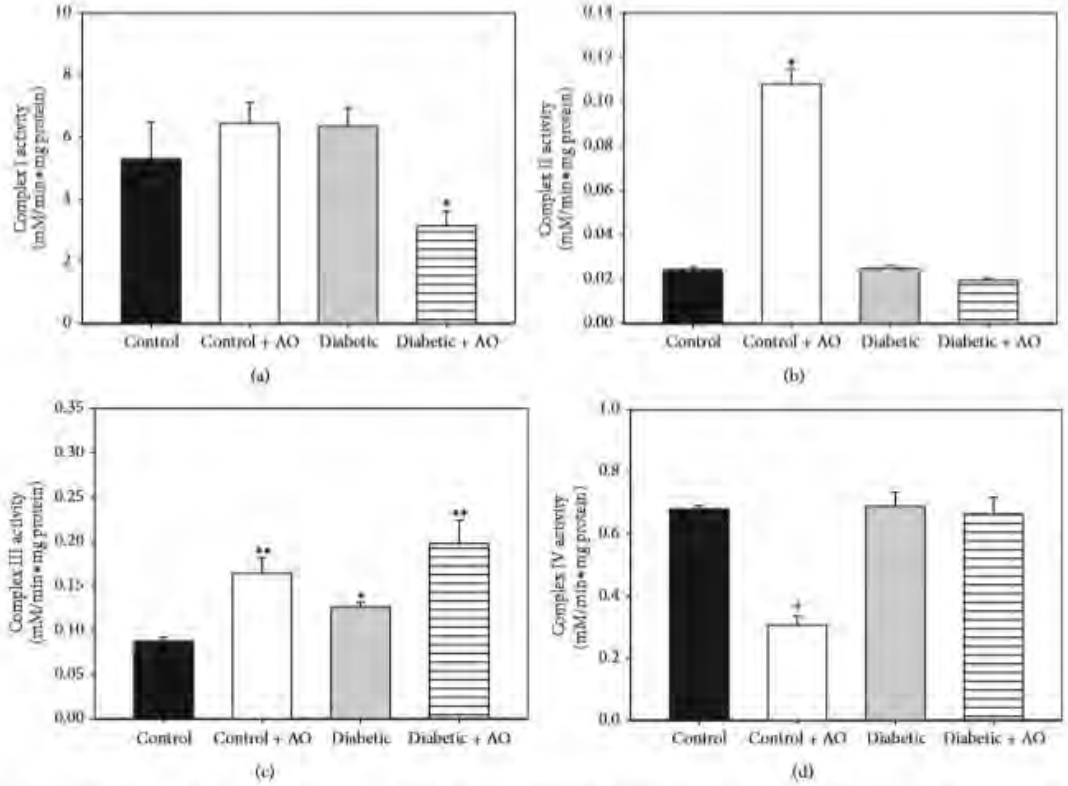

FIGURE 3: Effect of avocado oil in complexes I (a); II (b); III; (c) and IV (d) activities in brain mitochondria. Data are the mean ± EE of ($n = 4$–8). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with brain control mitochondria.

in response to the addition of glutamate/malate. The data presented in Figure 4 shows that ROS levels were increased by 64.5% in brain mitochondria from diabetic rats when compared to control rats. It was also observed that avocado oil administration fully prevented this effect in mitochondria from diabetic rats, while in mitochondria from control rats, avocado oil did not altered ROS levels.

3.6. Effects of Diabetes and Avocado Oil on Brain Mitochondria Oxidative Stress. Lipid peroxidation and GSH/GSSG ratios were analyzed as markers of oxidative stress to test whether enhanced ROS levels in diabetic rats and the protection conferred by avocado oil were parallel to changes in oxidative stress. In comparison to control rats, the levels of TBARS were similar in mitochondria from diabetic rats (Figure 5). However, avocado oil decreased the levels of lipid peroxidation in both control and diabetic groups, although this effect was statistically significant only in the diabetic group (65%).

Regarding to the redox status of glutathione, the GSH/GSSG ratio of mitochondria from diabetic rats was 38.3% lower with respect to mitochondria from control animals, indicating a state of higher oxidative stress in mitochondria from diabetic animals. Avocado oil prevents this effect and even augmented by 3.1-fold the GSH/GSSG ratio in comparison to mitochondria from control animals. Besides, avocado oil also produced a more discrete, 1.3-fold increase in this parameter in normoglycemic rats.

4. Discussion

Diabetes complications are associated with end-stage damage in the eyes, kidneys, peripheral nerves, and the brain [27–29]. In the CNS, type 1 diabetes encephalopathy is manifested like cognitive dysfunction characterized by a slowing of mental speed and a diminished mental flexibility [30]. Furthermore, the risk of dementia appears to be almost doubled in diabetic patients [31]. The exact mechanisms underlying the complications in CNS occurring in diabetes are not fully understood [32], as it seems to be a complex, multifactorial process including physiological, molecular, and metabolic alterations [28]. Due to the fact that avocado oil administration decreases oxidative stress and protects mitochondrial function in kidney mitochondria [16], it was decided to evaluate its effect on

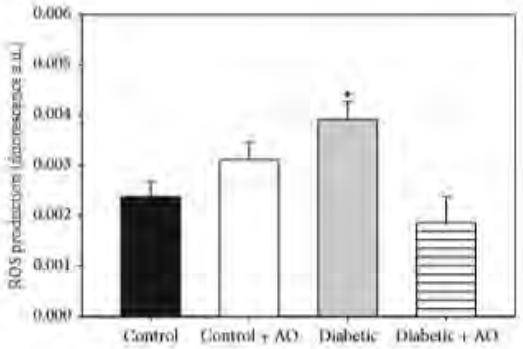

FIGURE 4: Effect of avocado oil in brain mitochondria ROS levels. Experiment was carried out using 10 mM glutamate/malate as a mitochondria ETC substrate. ROS levels were expressed in fluorescence arbitrary units (a.u.). Data are the mean ± EE of (n = 5-6). $^{*}p < 0.05$ compared with brain control mitochondria.

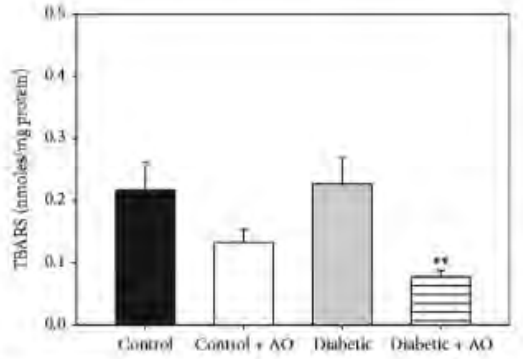

FIGURE 5: Effect of avocado oil on TBARS of brain mitochondria. Data are the mean ± EE of (n = 4). $^{**}p < 0.01$ compared with brain control mitochondria.

the CNS, with a focus on bioenergetics and oxidative stress of brain mitochondria from type I diabetic STZ-induced rats.

Mitochondrial dysfunction has been proposed to mediate development of diabetes complications in many tissues including neurons [33]. Our results show that diabetes impairs brain mitochondrial respiration in both state 4 and state 3 (Figure 1), which may be interpreted as diabetes induced alterations in the functioning of the ETC and/or in oxidative phosphorylation like in other tissues such as cardiac muscle, liver, and kidney [34–36]. This is consistent with reports demonstrating mitochondrial dysfunction in the brain of diabetes models [37, 38]. A probable factor leading to decreased respiration might be declined expression of nuclear respiratory factor 1 (NRF-1) and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), which regulates mitochondrial biogenesis and the expression of several components of the ETC [39]. Proteomic studies also have shown a reduction in the expression and activity of some

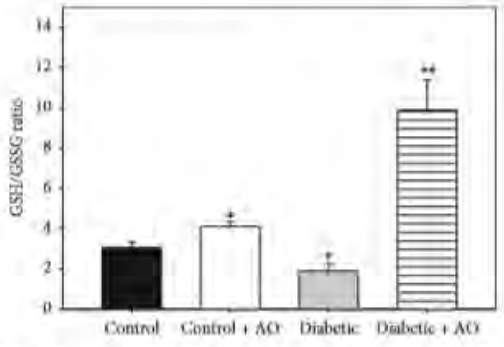

FIGURE 6: Effect of avocado oil on GSH/GSSG ratio in brain mitochondria. Data are the mean ± EE of (n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with brain control mitochondria.

components of the ETC, contributing further to neuronal mitochondrial dysfunction [40]. Another characteristic of diabetes is the increased oxidative stress, which also directly affects mitochondrial function by affecting lipids, membrane proteins, and mitochondrial DNA [41]. Our results of impaired respiration in state 3 imply a decrease in oxidative phosphorylation of ADP in mitochondria of diabetic rats. Oxidative phosphorylation depends importantly on $\Delta\Psi_m$ to drive the conversion of ADP into ATP. As $\Delta\Psi_m$ was severely impaired in mitochondria of diabetic rats (Figure 2), our results suggest that depolarization of the inner mitochondrial membrane leads to lower rates of respiration in state 3, although the possibility that other factors like impaired activity and/or expression of both the F_1F_0-ATP synthase and the adenine nucleotide translocator may be also involved in this phenomena remains.

The protective effects of avocado oil on mitochondrial respiration and $\Delta\Psi_m$ during diabetes may be attributed to some of the compounds present in the oil, such as lutein [15] as this carotenoid interacts with transcription factors counteracting mitochondrial dysfunction such as NRF-1 and PGC-1α [42, 43]. Another probable mechanism involved may be decreased mitochondrial oxidative stress, as avocado oil had an antioxidant effect in mitochondria by reducing the levels of lipid peroxidation (Figure 5) ROS levels (Figure 4) and by maintaining the mitochondrial redox state to similar levels that control brain mitochondria (Figure 6), which may further contribute to preserve adequate mitochondrial function.

Regarding the activities of the complexes from the ETC, the only significant change during diabetes was an increase in the activity of complex III (Figure 3), which has been reported for some brain regions such as the prefrontal cortex and striatum of diabetic rats [5]. This increase in activity may be related to a compensatory effect in an effort to counteract enhanced ROS levels and decreased expression of the proteins constituting the ETC [15], including some subunits of complex III [44] and diminished amounts of cytochrome b and $c + c_1$ in brain mitochondria-STZ rats [45].

Avocado oil further increased the activity of the complex III in the diabetic rats, which may contribute to improved electron flow through the redox centers of complex III, as evidenced by improved respiration in diabetic mitochondria treated with avocado oil. Enhanced complex III activity may reduce the half-life of semiquinone intermediates and decrease ROS levels as semiquinones are electron donors to oxygen to form superoxide anion ($O_2^{\bullet}$). This hypothesis is in fully agreement with ameliorated ROS levels (Figure 4) and decreased oxidative stress (Figure 6) observed in mitochondria from diabetic rats treated with avocado oil. On the other hand, decreased activity of complex I in diabetic rats supplemented with avocado oil might contribute to decreased ROS levels due to electron leak at the ETC by limiting the oxidation of NADH and decreasing the rate of semiquinone generation, which in turn agrees with the role of the complex I also in ROS generation besides complex III [46, 47]. On the other hand, it would be argued that the lack of inhibitory effects of diabetes on the activities of the ETC complexes does not fit well with decreased respiratory rates and partially dissipated $\Delta\Psi_m$ observed in mitochondria of the same animals. However, as pointed out by Brand and Nicholls, [48], mitochondrial function (i.e., substrate oxidation and ATP turnover) is not only under the control of the ETC enzymes but is widely shared between many processes, including adenine nucleotide exchange by adenine nucleotide translocase, phosphate availability due to phosphate carrier activity or F_1F_0-ATP synthase activity. For this reason, altered ETC functionality may indeed have a negligible effect on overall mitochondrial function. Based on these considerations, it may be suggested that mitochondrial dysfunction in brain mitochondria is independent of alterations on the activities of the ETC enzymes. Instead, decreased respiration in state 3 may be due to alterations in ADP phosphorylation as was discussed above.

Mitochondrial ROS levels were higher in the diabetic group (Figure 4). In other studies, increased ROS levels have been described to activate signaling pathways that lead to cell death of neurons and the development of diabetic encephalopathy [4, 49]. Moreover, avocado oil consumption prevented exacerbated ROS generation in diabetic rats, which raises the possibility that the onset of diabetic encephalopathy might be delayed in these animals. The observation that avocado oil decreases mitochondrial ROS levels confirms its antioxidant potential. Its antioxidant capacity may be also related to its constitution of fatty acids since monounsaturated fatty acids, as oleic acid (C18:1), which comprises ~60% of the fatty acids present in the oil, are less susceptible to damage by ROS that polyunsaturated fatty acids [50]. It is also possible that many of the antioxidant compounds in avocado such as vitamins, carotenoids, chlorophylls, and tocopherols are present in the oil and all these components could be responsible for the observed effect, where the lipophilic components allow accumulation in mitochondria. This feature has been used for the synthesis of new mitochondria-target antioxidants [51], for example, the SOD-mimics [52], which have been created to reduce ROS in oxidative stress related diseases like diabetes.

To estimate the extent of mitochondrial oxidative stress during diabetes and the ability of avocado oil to counteract this process, lipid peroxidation also was determined. According to different reports about the levels of lipid peroxidation in brain mitochondria, there is no consensus about whether this process remains unaltered or increases during diabetes [37, 41, 53]. A probable answer to this issue is that increased lipid peroxidation occurs differentially only in certain brain regions such as the prefrontal cortex and amygdala [10, 38]. In our experiments, an increase in TBARS levels in mitochondria of diabetic group was not observed (Figure 5), which might be related with the fact that we used a whole brain homogenate to isolate mitochondria. Avocado oil decreased lipid peroxidation of mitochondrial membranes of the brain from control and diabetic rats. This probably reflects an antioxidant effect in the entire brain. Diabetes also decreased the GSH/GSSG ratio, which, in contrast with the above controversy about lipid peroxidation, is consistent with other reports where, besides, the total glutathione content was found to be decreased in diabetic brain [3, 38]. Lower GSH/GSSG ratios impair the ability of some antioxidant enzymes that need GSH to regenerate its function, which further increases oxidative stress in brain diabetic mitochondria.

It is important to note that one important limitation of this work is that cognitive decline due to diabetes and the impact of avocado oil were not assessed in this study, which impedes to confer avocado oil a protective role on diabetic encephalopathy. As a first approach, it was intended in this study to explore whether avocado oil supplementation may protect from diabetes-induced brain mitochondrial dysfunction and exacerbated oxidative stress. However, we thought that the findings from this study pave the road to further research addressing if the administration of avocado oil may have beneficial effects in diabetic encephalopathy, since the attenuation of some features of oxidative stress observed in our study, such as increased lipid peroxidation and glutathione exhaustion, is believed to ameliorate cognitive deficits during diabetes [54].

5. Conclusion

Avocado oil improves brain mitochondrial function in diabetic rats by preventing the impairment in mitochondrial respiration and $\Delta\Psi_m$ induced by diabetes, besides increasing complex III activity. This may be related to decreased ROS levels and improved redox status in diabetic rats as reflected by a higher GSH/GSSG ratio. These effects might delay the onset of diabetic encephalopathy, but this possibility remains to be investigated.

Conflict of Interests

The authors declare that there is no conflict of interests.

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7. CAPÍTULO III. EL ACEITE DE AGUACATE INDUCE UN ALIVIO A LARGO PLAZO DEL DAÑO OXIDATIVO EN MITOCONDRIAS DE RIÑÓN EN RATAS DIABÉTICAS TIPO 2 AL MEJORAR EL ESTADO DEL GLUTATIÓN.

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Avocado oil induces long-term alleviation of oxidative damage in kidney mitochondria from type 2 diabetic rats by improving glutathione status

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Abstract Hyperglycemia and mitochondrial ROS overproduction have been identified as key factors involved in the development of diabetic nephropathy. This has encouraged the search for strategies decreasing glucose levels and long-term improvement of redox status of glutathione, the main antioxidant counteracting mitochondrial damage. Previously, we have shown that avocado oil improves redox status of glutathione in liver and brain mitochondria from streptozotocin-induced diabetic rats; however, the long-term effects of avocado oil and its hypoglycemic effect cannot be evaluated because this model displays low survival and insulin depletion. Therefore, we tested during 1 year the effects of avocado oil on glycemia, ROS levels, lipid peroxidation and glutathione status in kidney mitochondria from type 2 diabetic Goto-Kakizaki rats. Diabetic rats exhibited glycemia of 120–186 mg/dL the first 9 months with a further increase to 250–300 mg/dL. Avocado oil decreased hyperglycemia at intermediate levels between diabetic and control rats. Diabetic rats displayed augmented lipid peroxidation and depletion of reduced glutathione throughout the study, while increased ROS generation was observed at the 3rd and 12th months along with diminished content of total glutathione at the 6th and 12th months. Avocado oil ameliorated all these defects and augmented the mitochondrial content of oleic acid. The beneficial effects of avocado oil are discussed in terms of the hypoglycemic effect of oleic acid and the probable dependence of glutathione transport on lipid peroxidation and thiol oxidation of mitochondrial carriers.

Keywords Diabetic nephropathy · Reactive oxygen species · *Diabetes* · Oleic acid · Hypoglycemic · Goto-Kakizaki rats

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Introduction

Mitochondrial ROS overproduction has a predominant role in the development of diabetic renal damage, as revealed by the prevention of glomerular injury, tubulointerstitial fibrosis and mesangial expansion through the use of mitochondria targeted antioxidants, which blocks the signaling pathways involved in the development of these renal abnormalities (Chacko et al. 2010; Hou et al. 2016). Mitochondrial ROS generation is exacerbated during diabetes either by alterations in oxidative phosphorylation, by antioxidants depletion or both (Moreira et al. 2006; Coughlan et al. 2009; Raza et al. 2011; Sourris et al. 2012). In turn, antioxidant depletion, particularly of

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glutathione, may favor peroxidative damage in lipids from mitochondrial membranes (Maddaiah 1990; Madrigal et al. 2001), leading to impaired mitochondrial electron transfer and enhanced electron leak responsible for ROS generation, creating a vicious circle of antioxidant depletion and ROS production.

Previously, we have shown that avocado oil, which contains a wide variety of lipophilic antioxidants and important amounts of oleic acid, counteracts some defects responsible for high ROS production in kidney mitochondria from streptozotocin (STZ) - induced diabetes rats, such as loss of cytochrome $c + c_1$ and defective electron transport at complex III, besides it augments the resistance of that complex to the deleterious effects of in vitro oxidative stress induced by Fe^{2+} (Ortiz-Avila et al. 2013). Although these data suggest that avocado oil has a beneficial role against the deleterious effects of diabetes over mitochondria, the induction of diabetes with STZ raises some experimental issues that impede to evaluate properly other important aspects that may support the beneficial effects of avocado oil during diabetes. For example, STZ provokes a decrease in PUFA due to inhibited conversion of C18:2 into C20:4, lowering in this way the susceptibility of mitochondrial membranes of diabetic animals to lipid peroxidation, which is contradictory to other findings about an increase in this parameter in renal mitochondria during diabetes (Çelık et al. 2012). Thus, the protective effect of avocado oil against renal mitochondrial lipid peroxidation cannot be properly evaluated in this model. Another issue arises from the difficulty to evaluate the hypoglycemic effect of avocado oil on STZ-induced rats as this drug fully depletes pancreatic insulin production due to β-cells death (Szkudelski 2001). This is an important issue to be addressed since it has been reported that glucose control decreases the development of diabetic nephropathy by more than 50% in diabetic patients (Nathan et al. 2013), although the benefits of hypoglycemic therapy on the treatment of diabetic nephropathy have been put in doubt by other studies (Gross et al. 2005 and references therein). On the other hand, a glycemic control in type 2 diabetic patients has been achieved using a diet rich in oleic acid from avocado consumption (Lerman-Garber et al. 1994); therefore, the possibility remains that beneficial effects of avocado oil on kidney mitochondria might be also related to decreased concentrations of blood glucose. Besides, it is difficult to evaluate the long-lasting effects of avocado oil in the STZ model because the extremely high levels of blood glucose reached in these animals (~370 mg/dL in our previous studies) lead to decreased rat lifespan. This is also crucial for the evaluation of the long lasting effects of avocado oil on the mitochondrial content of glutathione, since it has been described that increased mitochondrial concentrations of this antioxidant can be achieved only transitorily through other experimental manipulations (Lash 2015).

The above issues may be addressed by studying the effects of avocado oil in a model of non-insulin dependent diabetes allowing analyzing the effects of avocado oil on prolonged hyperglycemia. In this regard, Goto-Kakizaki rats, a lean model of type 2 diabetes, is a more proper experimental model since these animals can live for more than 1 year (Moreira et al. 2003). Besides, it has been pointed out that this model resembles in a good degree the renal alterations seen in diabetic patients with prolonged hyperglycemia (Phillips et al. 2001), besides these animals do not exhibit full depletion of insulin synthesis but insulin resistance (Dadke et al. 2000). For all these reasons, we consider Goto-kakizaki rats as an adequate model to evaluate whether the mitochondrial benefits of avocado oil are long lasting and its relationship with an improved glycemic control.

Given the importance of glutathione on the maintenance of mitochondrial redox state and membrane integrity, the participation of mitochondrial ROS in the development of diabetic renal complications and the short-term mitochondrial protective effects of avocado oil on STZ-induced diabetic rats, we decided to test in type 2 diabetic Goto Kakizaki rats whether avocado oil supplementation decreases ROS levels in kidney mitochondria during prolonged diabetes and its relationship with the redox state and content of glutathione, the peroxidative damage to membrane lipids, the fatty acid composition of membranes and the levels of blood glucose.

Materials and methods

Animals and experimental design Male diabetic Goto-Kakizaki rats were obtained from Charles River Laboratories International, Inc. (Wilmington, MA, USA). Control animals were non-diabetic male Wistar rats of similar age, obtained from our local colony. Animals were kept under controlled light and humidity conditions and with free access to standard rodent chow (Laboratory Rodent Diet 5001, LabDiet) and water. For animal management, we followed the recommendations of the Federal Regulation for the Use and Care of Animals (NOM-062-ZOO-1999) from the Mexican Ministry of Agriculture. This research was also approved by the Institutional Committee for Use of Animals of the Universidad Michoacana de San Nicolás de Hidalgo.

Two months - old rats were randomly divided in four groups: 1) Wistar normoglycemic rats (control); 2) Wistar normoglycemic rats plus avocado oil (control + AO); 3) Goto-Kakizaki rats (diabetes); 4) Goto-Kakizaki rats plus avocado oil (diabetes + AO). The dose of avocado oil was 1 mL/250 g weight and the oral administration was performed daily for a period of 3, 6 and 12 months. A commercial presentation

of avocado oil was used in the study (Ahuacatlan, DIRICOM, S.A. de C.V., México), purchased from a local grocery.

Isolation of mitochondria Kidney mitochondria were isolated by differential centrifugation (Saavedra-Molina and Devlin 1997) from a homogenate of the whole kidney. The mitochondrial pellet was re-suspended in a buffer with 220 mM mannitol, 70 mM sucrose and 2 mM MOPS (pH 7.4). Mitochondrial protein concentration was measured by a modification of the Biuret method (Gornall et al. 1949) calibrated with bovine serum albumin.

Analysis of mitochondrial fatty acids composition Fatty acids from kidney mitochondria were derivatized for its analysis using boron trifluoride (BF_3) in a 14% methanol solution according to the method of Morrison and Smith (1964). Subsequently, the methyl-esters of fatty acids were analyzed by gas chromatography under the conditions previously described (Ortiz-Avila et al. 2013).

Evaluation of lipid peroxidation levels This determination was carried out in 0.1 mg/mL kidney mitochondria protein by measuring the levels of thiobarbituric acid reactive substances (TBARS) (Buege and Aust 1978). Absorbance was measured at 532 nm with a Perkin Elmer Lambda 18 UV/VIS spectrophotometer. Data were expressed as nanomoles of TBA reactive species (TBARS) per mg of mitochondrial protein.

Glutathione determinations Mitochondria samples (0.3 mg/mL) were treated with 5% (v/v) sulfosalicilic acid and centrifuged at 7800 g for 10 min to remove denatured proteins. Reduced (GSH) and oxidized (GSSG) glutathione were determined in the supernatant by an enzymatic method (Akerboom and Sies 1981). The content of total glutathione (GSH + GSSG) was assayed in a cuvette containing 90 µl of the supernatant, 3 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 0.115 unit/ml glutathione reductase in a final volume of 1 mL of 0.1 M sodium phosphate buffer (pH 7.5). After 5 min of incubation at room temperature, 2 mM NADPH was added and the kinetics of the reaction was monitored for 5 min. The increment in absorbance at 412 nm was converted to GSH concentration using a standard curve with known amounts of GSH. For determination of GSSG, the same DTNB recycling assay was performed after GSH derivatization by incubating at room temperature with 3% (v/v) 4-vinylpyridine for 1 h before starting DTNB assay. The concentration of GSH was calculated by subtracting the concentration of GSH + GSSG minus the concentration of GSSG.

Measurement of ROS levels ROS were estimated by measuring the oxidation rate of the fluorescent probe 2',7'-dichlorodihydrofluorescein (H_2DCF). 0.5 mg/mL intact mitochondria and 1.25 mM H_2DCF diacetate were incubated at 4 °C under constant shaking during 20 min in a buffer containing 10 mM HEPES, 100 mM KCl, 3 mM $MgCl_2$ and 3 mM KH_2PO_4 (pH 7.4). Then, the mitochondrial suspension was placed in a quartz cuvette and the assay was started by recording the basal fluorescence; 1 min later, 10 mM glutamate/malate was added and the changes in H_2DCF fluorescence were followed by 20 min. Fluorescence changes were detected in a Shimadzu RF-5301PC spectrofluorophotometer (λ_{ex} 485 nm; λ_{em} 520 nm).

Data analysis The results are expressed as the mean ± standard error from at least three independent experiments using samples from different animals. Statistical differences of the data were determined by 2-way ANOVA with Bonferroni post-hoc test. Statistically significant differences were defined as $P < 0.05$.

Results

Effects of avocado oil on plasma glucose concentrations

Glucose levels are depicted in the Fig. 1. In control rats (white circles), glucose concentrations fluctuated between 80 and 90 mg/dL throughout the experimental period. Importantly, avocado oil (black circles) did not modify this trend in control rats. As expected, diabetic rats (white triangles) exhibited higher glucose levels ranging from 120 mg/dL to 186 mg/dL during the first 9 months of the experiment, after which, a rise to 250–300 mg/dL was observed during the last three months.

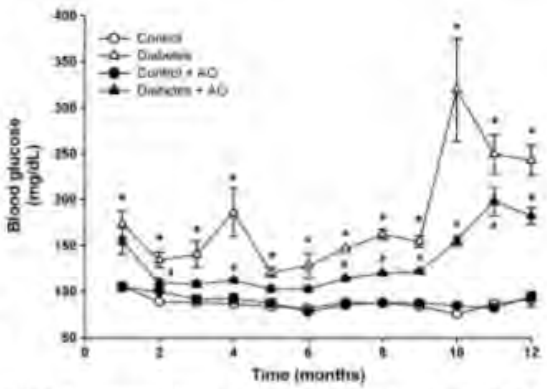

Fig. 1 Time-course of blood glucose levels in rats from control (*white circles*), diabetic (*white triangles*), control plus avocado oil (*black circles*) and diabetic plus avocado oil (*black triangles*) groups. Fasting glucose concentrations were monitored each month after the beginning of the treatment with avocado oil (i.e. month 0), which was daily administered as described in Materials and Methods. Data are presented as the mean ± standard error of $n \geq 5$. $^{*}P < 0.05$ when compared to control group; $^{\#}P < 0.05$ when compared to diabetic group (2-way ANOVA with Bonferroni post-hoc test)

Avocado oil had a hypoglycemic effect in diabetic rats (black triangles) as glucose levels were in an intermediate level between the diabetic and the control animals, although glucose concentrations also increased in the last three months of the study parallel to diabetic rats but never reached the levels observed in those animals.

Effects of diabetes and avocado oil on ROS generation and lipid peroxidation

It can be observed that ROS levels at the 3rd month were 1.6-fold higher in mitochondria from diabetic rats in comparison to control mitochondria (Fig. 2), with avocado oil fully preventing this effect without altering ROS levels in the control group. In contrast, at the 6th month, ROS levels were similar in mitochondria from control and diabetic groups, but is worth noting that ROS levels in these groups were 23- and 13-fold higher, respectively, at this time when compared to the levels of the same groups at the 3rd month. Despite this exacerbation, avocado oil still decreases ROS levels in 75% in mitochondria from the diabetic group, while a non-statistically significant diminution of 27% was observed in the control group. At the 12th month, an augment of ~90% in ROS was detected in diabetic rats with respect to control and control plus avocado oil groups. Although in a partial way, the ability of avocado oil to decrease ROS levels in mitochondria from diabetic rats remained at this stage, exhibiting these mitochondria a 21% diminution in comparison to mitochondria from diabetic rats.

The levels of lipid peroxidation are shown in the Fig. 3. Lipid peroxidation increased in mitochondria from diabetic rats in comparison to control mitochondria as the study progressed, with increments of 48.4%, 70.3% and 93.1% at 3, 6 and 12 months, respectively. Avocado oil diminished lipid peroxidation in diabetic rats at 3 and 12 months, while a non-significant trend towards decreased lipid peroxidation was detected at 6 months. Lipid peroxidation did not undergo any change in mitochondria from control rats plus avocado oil group at any stage.

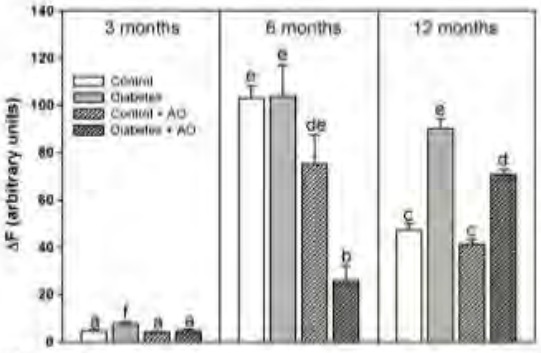

Fig. 2 ROS levels of kidney mitochondria from rats supplemented with avocado oil (AO) during 3, 6 and 12 months. The changes in the fluorescence of H_2DCF (ΔF) in response to glutamate-malate addition were quantified during 20 min and expressed as arbitrary units. Data are presented as the mean ± standard error of $n \geq 4$. Different letters indicate statistically significant differences between groups ($P < 0.05$) (2-way ANOVA with Bonferroni post-hoc test)

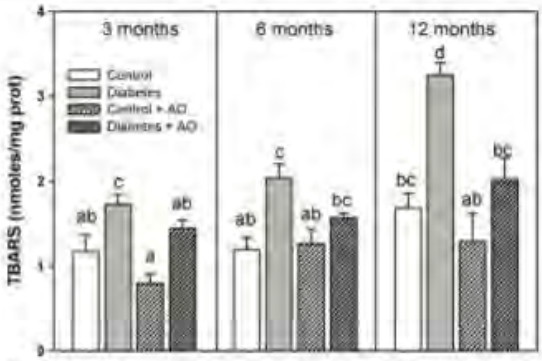

Fig. 3 Lipid peroxidation levels of kidney mitochondria from rats supplemented with avocado oil (AO) during 3, 6 and 12 months. The results are expressed as nanomoles of thiobarbituric acid reactive substances (TBARS) per milligram of mitochondrial protein. Data are presented as the mean ± standard error of $n \geq 4$. Different letters indicate statistically significant differences between groups ($P < 0.05$) (2-way ANOVA with Bonferroni post-hoc test)

Effects of avocado oil on mitochondrial glutathione

The content of glutathione and its redox status are displayed in the Fig. 4. As observed in the panel a, diabetes consistently decreased the concentration of reduced glutathione (GSH), with decrements of ~3-fold at 3 and 6 months and 4-fold at 12 months. Avocado oil fully prevented the fall in GSH at 3 and 6 months, while a partial prevention was observed at the 12th month, although GSH levels remained 2.6 fold-higher than in the diabetic group. In contrast to GSH, no significant changes in oxidized glutathione (GSSG) were observed throughout the experimental period (Fig. 4b), except by a two-fold decrease in the diabetic group at the 12th month. Regarding to total glutathione content (GSH + GSSG), diabetes induced a decay at the 6th and 12th months, with avocado oil restoring glutathione pool in a full way at the 6th month and only partially at the 12th month (Fig. 4c).

Influence of diabetes and avocado oil on mitochondrial fatty acid profile

Mitochondrial fatty acid profile was determined in order to assess probable changes induced by avocado oil contributing to a higher resistance to oxidative damage via

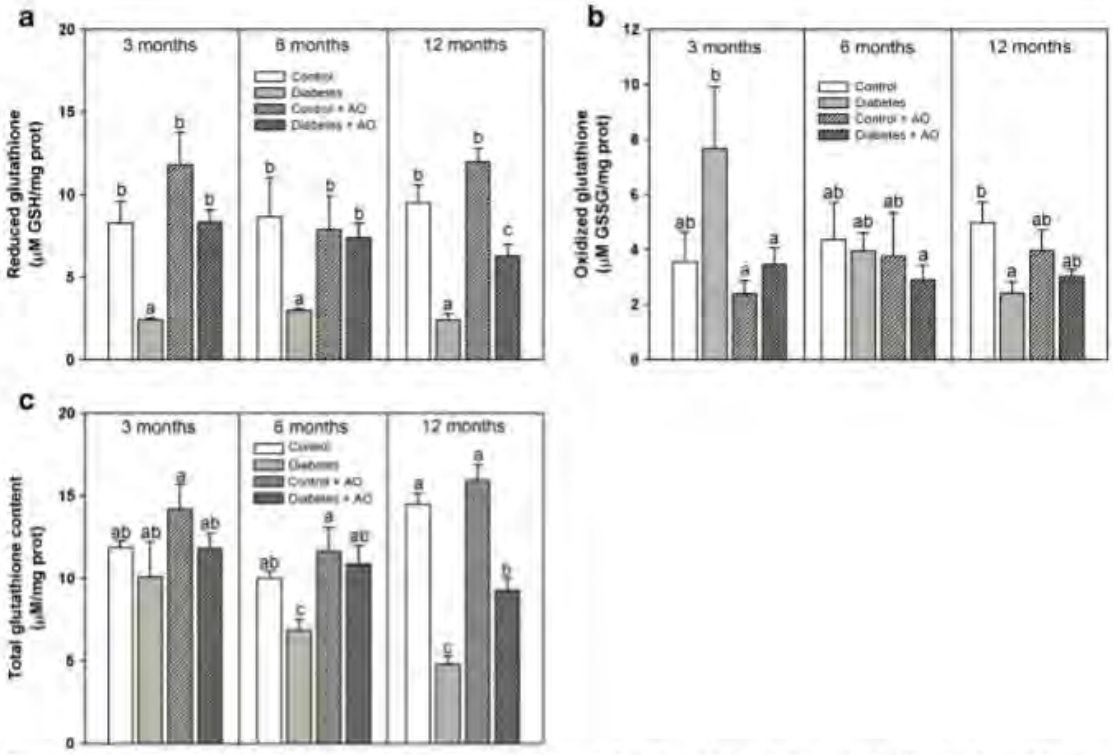

Fig. 4 Concentrations of reduced glutathione (GSH, panel a), oxidized glutathione (GSSG, panel b) and total glutathione (panel c) in kidney mitochondria from rats treated during 3, 6 and 12 months with avocado oil (AO). Data are presented as the mean ± standard error of $n \geq 3$. Different letters indicate statistically significant differences between groups ($P < 0.05$) (2-way ANOVA with Bonferroni post-hoc test)

decreased peroxidizability of mitochondrial membranes. The only change observed in the percentage of saturated fatty acids was an increase in the control plus avocado oil group at the 12th month (Fig. 5a). Regarding to monounsaturated fatty acids (MUFA), significant increments in this fatty acid were observed in mitochondria from diabetic rats supplemented with avocado oil at any stage of the protocol when compared to the diabetic group. In control rats, MUFA percentage increased only at the 12 month (Fig. 5b). A moderate diminution of this fatty acid was observed solely in diabetic rats at the 3rd month. The only effect produced by avocado oil on polyunsaturated fatty acids (PUFA) content was a decrease in control mitochondria at the 12th month, while diabetes did not induced any modification (Fig. 5c). On the other hand, the peroxidizability index (PI), which estimates the susceptibility of a membrane to undergo lipid peroxidation based on its fatty acid composition, was calculated from the data of mitochondrial fatty acids depicted in the Table S1. No significant changes among all the experimental groups were observed at the 3rd and the 6th months (Fig. 5d). Supplementation with avocado oil only decreased the PI of mitochondria from control rats at the 12th month.

Discussion

The results of the present study show that, in kidney mitochondria from diabetic rats, avocado oil decreases ROS levels at all the stages of the study (Fig. 2), diminishes lipid peroxidation at the 3rd and 12th months (Fig. 3), prevents the depletion of GSH (Fig. 4a), replenishes the pool of total glutathione (Fig. 4c) and increases the content of MUFA (Fig. 5b), although without decreasing the peroxidizability index (Fig. 5d). Importantly, avocado oil displays a partial hypoglycemic effect, which was not statistically significant at the 3rd, 5th and 6th months of the study (Fig. 1). Regarding the later effect, a glycemic control has been achieved in type 2 diabetic individuals with diets enriched with MUFA from sources of C18:1 like avocado (Lerman-Garber et al. 1994) or olive oil administered up to 1-year (Brehm et al. 2009). The benefits of

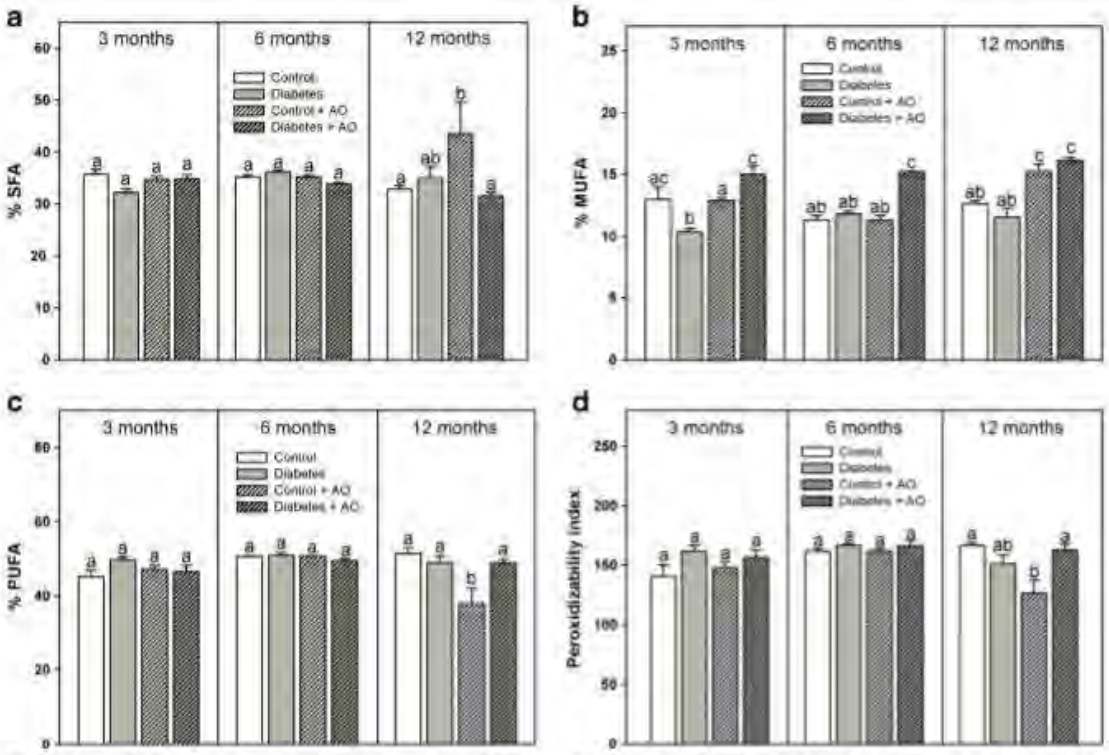

Fig. 5 Fatty acid indexes of kidney mitochondria from rats treated during 3, 6 and 12 months with avocado oil (AO). The percentages of saturated fatty acids (SFA, panel a), monounsaturated fatty acids (MUFA, panel b), polyunsaturated fatty acids (PUFA, panel c) and the peroxidizability indexes (PI, panel d) were determined from the data of mitochondrial fatty acids displayed in the Table S1. PI were calculated as reported by Pamplona et al. (1998). Data are presented as the mean ± standard error of $n \geq 4$. Different letters indicate statistically significant differences between groups ($P < 0.05$) (2-way ANOVA with Bonferroni post-hoc test)

C18:1 in glucose levels have been linked to enhanced release of glucagon-like peptide-1 (GLP-1), which improves both the secretion and sensitivity to insulin, inhibits the secretion of glucagon and delays gastric absorption of nutrients (Rocca et al. 2001). In addition, oxidative stress has been demonstrated to disrupt GLUT4 translocation from internal pools to plasma membrane via disruption of the trafficking of proteins needed for that process (Tirosh et al. 1999). Thus, the high oleic acid content in avocado oil and its antioxidant capacity demonstrated in this and other studies might be involved in the diminution of blood glucose concentrations by modulating the effects of GLP-1 and GLUT4 on glucose transport and metabolism.

Glucose absorption in the kidney is primarily mediated by non-insulin dependent transporters like SLGT-2 and GLUT-2 (Bakris et al. 2009). Thus, glucose overload occurs in renal cells during diabetes, leading to increased glycolytic flux, enhanced pyruvate formation and high $NADH/NAD^+$ ratios (Forbes et al. 2008). In turn, elevated $NADH/NAD^+$ ratios lead to augmented ROS production in the complex I in forward mode (Kareyeva et al. 2012). Alternatively, inhibition of ROS production by substrate has been observed in complex I at high NADH concentrations, leading to NADH oxidation and ROS production by soluble matrix dehydrogenases (Grivennikova and Vinogradov 2006). Accordingly, at the 3rd month of treatment, the parallel increase in blood glucose and ROS levels in diabetic rats, as well as the decrease induced by avocado oil in these parameters fits well with this picture (Figs 1 and 2), although the effect of avocado oil lacks of statistical significance. However, when diabetes progress up to 6 months, it seems that there are additional factors involved in ROS production, as there is no a clear relation among blood glucose and ROS levels at that time. Meanwhile, at 12 months, blood glucose and ROS levels shows again some degree of concordance, with diabetes increasing both glucose and ROS levels, although not in a similar proportion, and avocado oil having the contrary effect except in the control plus avocado oil group. Considering that mitochondrial ROS levels are central in the development of diabetic nephropathy (Chacko et al. 2010; Hou et al. 2016),

the lack of a clear correlation between glucose levels and ROS generation during diabetes progression is in agreement to the controversial role of hypoglycemic therapy against this complication in diabetic patients (Gross et al. 2005). Indeed, a more effective therapy against diabetic nephropathy than hypoglycemic agents is the blocking of angiotensin II actions by antagonizing the activation of AT_1 receptors (Brenner et al. 2001). It has been described that AT_1 blocking decrease mitochondrial ROS production by inhibiting NADPH oxidase, whose activity drives to oxidative damage in the electron transport chain, enhanced ROS generation, and impaired kidney function (de Cavanagh et al. 2006; Dikalov and Nazarewicz 2013). These findings supports the idea that decreasing mitochondrial ROS generation may be more beneficial for kidney disease attenuation than the decrease of blood glucose levels. In this regard, avocado oil decreased ROS levels (Fig. 2) and lipid peroxidation (Fig. 3) in mitochondria from the diabetic rats even at the 12th month of treatment, with a concomitant augment of GSH concentrations (Fig. 4a). This suggest that at long term, avocado oil decreases oxidative stress in diabetic mitochondria by boosting the antioxidant capacity of mitochondrial glutathione system rather than by improving blood glucose levels, the former occurring by augmenting the pool of total glutathione (Fig. 4c) and by enhancing its reduced state (Fig. 4a). Given the central role of mitochondrial glutathione on the defense against oxidative stress and other toxicants (Marí et al. 2009), this has led to the suggestion that modulation of the redox state of mitochondrial glutathione may be a therapeutic target to treat diabetic kidney disease (Lash 2015). Accordingly, the dietary supplementation during two months with GSH decreases renal oxidative stress and normalizes urinary markers of kidney damage in STZ-induced diabetic rats (Ueno et al. 2002). Nevertheless, it has been argued that supplementation with GSH only provokes transient increments of mitochondrial GSH concentrations, conferring only short-term improvement of mitochondrial antioxidant status (Lash 2015), in contrast to the sustained improvement of both GSH levels and total glutathione in mitochondria from diabetic rats observed up to 12 months in this study (Figs 4a and c).

A probable factor explaining the maintenance of glutathione in its reduced form by avocado oil might be the presence of β-sitosterol, the main phytosterol present in the edible portion of avocado (Dreher and Davenport 2013), which we have also detected in a preliminary characterization of the oil used in this study along with other bioactive compounds like campesterol and squalene (unpublished data). β-sitosterol increases the amount of GSH in stressed macrophages via the activation of the estrogen/phosphatidylinositol 3-kinase pathway (Vivancos and Moreno 2005), being this pathway active also in the kidney (Satake et al. 2008). The improvement on GSH by β-sitosterol was attributed to a higher activity of glutathione peroxidase (GPX); however, in our case, we thought that increased availability of GSH might be also mediated by increased GSSG reduction by glutathione reductase, being this possibility currently explored in our laboratory.

There is an apparent lack of correlation among the levels of ROS and the extent of lipid peroxidation at the 6th month of the study, as it should not be expected a rise in lipid peroxidation in mitochondria from diabetic rats (Fig. 3) because not a parallel augment was seen in the ROS levels of that group in comparison to control mitochondria (Fig. 2). This controversy can be explained considering that there was a more clear inverse relationship between the levels lipid peroxidation (Fig. 3) and GSH levels (Fig. 4a) in mitochondria from diabetic rats, in such way that lipid peroxidation may be more determined by the extent of glutathione reduction than by the levels of ROS. This makes sense considering that lipid peroxidation is counteracted in mitochondrial membranes by glutathione peroxidase 4 (Gpx4) in a process requiring the redox cycling of glutathione by glutathione reductase (Nomura et al. 1999). Therefore, the drastic drop in GSH content in the diabetic group may drive to a decreased ability of Gpx4 to repair peroxidized phospholipids, making membrane phospholipids more prone to be peroxidized even by unaltered levels of ROS as observed at 6 months in diabetic rats.

Total glutathione content was decreased in mitochondria from diabetic rats at the 6th and the 12th months (Fig. 4c), suggesting that diabetes impairs the transport of this antioxidant into mitochondria. Glutathione is transported into kidney mitochondria by the dicarboxylate and 2-oxoglutarate carriers (Lash 2012), which contain reactive –SH groups whose chemical modification inhibits its activity by interfering with substrate binding (Palmieri et al. 1974; Capobianco et al. 1996). In turn, there are proteins with exposed cysteines that react with ROS to yield oxidized thiol species like sulfenic acids, producing inactivated proteins that can be reactivated by enzymatic reduction of oxidized thiols at expense of GSH (Hurd et al. 2008). Based on these antecedents, it is feasible to hypothesize that decreased total glutathione content in mitochondria from diabetic rats might proceed by oxidation of reactive –SH groups from glutathione transporters (i.e. dicarboxylate and 2-oxoglutarate carriers) due to enhanced ROS generation and/or decreased GSH availability, inhibiting in this way the binding of glutathione and its internalization into mitochondrial matrix. Moreover, the increase in GSH concentration, the lower levels of ROS, and the higher content of total glutathione elicited by avocado oil fits well with the idea that the oil increases mitochondrial glutathione content by generating a more reduced redox status that allows better glutathione transport by maintaining the thiols from mitochondrial carriers in a more reduced form.

Membrane fatty acid composition determines the physical properties of biomembranes, as increased content of PUFA with 4, 5 and 6 double bonds augments membrane fluidity (Yang et al. 2011). It has been shown that mitochondrial

glutathione transport is affected by decreased inner membrane fluidity (Lluis et al. 2003). According to our results, it would not be expected an influence of membrane fatty acid composition over glutathione content, since not consistent changes in these fatty acids were detected throughout the study (Table S1). The more remarkable change in fatty acids was an increase in C18:1 but this fatty acid does not promote changes in membrane fluidity (Yang et al. 2011). According to several studies (Bruch and Thayer 1983; Dobretsov et al. 1977; Sevanian et al. 1988; Chen and Yu 1994), another factor decreasing membrane fluidity is lipid peroxidation. Consistently, lipid peroxidation in mitochondria from diabetic rats increased as the study progress, until reaching twice the levels observed in control rats (Fig. 3). Parallelly, total glutathione levels dropped during the progress of the study up to less than 35% of the levels of control mitochondria (Fig. 4c). Therefore, the inverse correlation between lipid peroxidation levels and total glutathione content suggest that lipid peroxidation leads to a decrease in membrane fluidity that impairs glutathione transport, resulting in lower levels of total glutathione in mitochondria from diabetic rats. Conversely, in mitochondria from diabetic rats treated with avocado oil, the decrease of lipid peroxidation levels correlates with an increase in total glutathione content that reach at 12 months twice the concentrations with respect to mitochondria from diabetic rats (Figs 4 and 5, respectively). This suggest that avocado oil improves at the long term the total content of mitochondrial glutathione also by decreasing lipid peroxidation levels, which, in turn, it might be augmenting membrane fluidity and enhancing the activity of glutathione transporters, although measurements of membrane viscosity are required to further validate this hypothesis. In summary, avocado oil might be augmenting the pool of mitochondrial glutathione by a mechanism involving attenuation of thiol oxidation in glutathione transporters and improvement of membrane fluidity by decreasing lipid peroxidation. More importantly, the augment of GSH levels by avocado oil observed in this study and the same outcome obtained in previous studies from our group in liver and brain mitochondria from type 1 diabetic rats (Ortiz-Avila et al. 2015a; Ortiz-Avila et al. 2015b), suggest that avocado oil is an efficient "enhancer" of mitochondrial GSH levels, which leads to an improvement of mitochondrial redox status that it is translated into a lower levels of ROS and attenuated oxidative damage even at long term (i.e. 1 year) as observed in the present study. Despite avocado oil increases the mitochondrial content of C18:1 and the amount of MUFA (Table S1 and Fig. 5b, respectively), this does not seems to contribute to augmented resistance to oxidative stress as peroxidizability index remained constant in the diabetic group throughout the study (Fig. 5d). This disagree with an study showing the benefits of the mitochondrial enrichment with MUFA in aged rats consuming olive oil (Quiles et al. 2002), but this discrepancy may be attributed to the differences in the type and content of antioxidants and the concentration of C18:1 among these oils.

The pathogenesis of diabetic nephropathy involves glomerular mesangial expansion and tubulointerstitital fibrosis due to remodeling of extracellular matrix, the latter event being triggered by upregulation of TGF-β1 and PAI-1. This occurs through ROS-induced activation of transcription factors like NF-κB, AP-1 and SP1 in a process mediated by PKC (Lee et al. 2003). Mitochondrial ROS produced at the electron transport chain have been identified as the main PKC activators during hyperglycemia since the inhibition of complex II, the uncoupling of respiration and MnSOD overexpression fully abolished PKC activation and other pathways of kidney damage (Nishikawa et al. 2000). Given the predominant role of excessive mitochondrial ROS generation in the etiology of diabetic renal damage, the search for strategies to scavenging ROS or enhancing mitochondrial function has been encouraged for the treatment of this diabetic complication (Che et al. 2014). On this basis, our results about the prevention of excessive ROS generation and the improvement of GSH in kidney mitochondria, provide promising evidence about the potential of avocado oil to prevent and/or delay the development of kidney nephropathy, which is intensively investigated in our laboratory to obtain further data supporting the value of avocado oil as a functional food to aid to mitigate the catastrophic consequences of diabetes, one of the main health challenges facing the mankind in the current century given the pandemic character of this disease (van Dieren et al. 2010).

In conclusion, in renal mitochondria from type 2 diabetic rats, avocado oil counteracts increased ROS levels and exacerbated lipid peroxidation in a sustained way, which is attributed rather to an increase in total glutathione pool and enhanced reduction of this molecule than to substantial changes in mitochondrial membranes or to the hypoglycemic effect of the oil.

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8. CAPÍTULO IV. EFECTO DEL ACEITE DE AGUACATE SOBRE EL PERFIL METABÓLICO Y EL DESARROLLO DE NEFROPATÍA DIABÉTICA EN RATAS GOTO-KAKIZAKI.

EFECTO DEL ACEITE DE AGUACATE SOBRE EL PERFIL METABÓLICO Y EL DESARROLLO DE NEFROPATÍA DIABÉTICA EN RATAS GOTO-KAKIZAKI.

RESUMEN

El descontrol tanto en el metabolismo de glucosa como de lípidos durante la diabetes, debido a la disminución en la síntesis de sustancias reguladoras como lo son la insulina y la adiponectina, propicia la aparición de complicaciones. Una de las complicaciones más frecuentes y devastadoras es la nefropatía diabética, la cual se caracteriza por niveles altos de proteinuria debido a una filtración glomerular inadecuada. El desarrollo de la nefropatía se ha asociado con el estrés oxidativo mitocondrial y a la activación de vías metabólicas que dan como resultado la aparición de fibrosis glomerular entre otras alteraciones. El objetivo de este trabajo fue evaluar el efecto del aceite de aguacate sobre el perfil metabólico y el desarrollo de nefropatía diabética en un modelo de diabetes tipo 2.

Se utilizaron ratas Goto-Kakizaki como modelo de diabetes tipo 2, a las cuales se les administro el aceite de aguacate vía oral por 3, 6 y 12 meses. Los resultados mostraron que el tratamiento con el aceite de aguacate mantiene por más tiempo los niveles de adiponectina, sin embargo no tuvo efecto en los niveles de insulina. El tratamiento mejoró el perfil de lípidos en las ratas al disminuir los niveles de colesterol, triglicéridos y LDL y mantener las concentraciones de HDL. Otro aspecto importante fue que el aceite no produjo un aumento de peso en las ratas diabéticas. Al evaluar la función renal, el aceite contrarrestó de manera parcial el aumento en la proteinuria observada en el grupo diabético y en la evaluación histopatológica, las ratas diabéticas mostraron signos de nefropatía diabética como acumulación de matriz extracelular en el glomérulo y engrosamiento de la membrana basal glomerular desde los 3 meses de tratamiento. El tratamiento con el aceite de aguacate retrasó la aparición de estas alteraciones hasta los 6 meses. En cuanto a la función mitocondrial, las ratas diabéticas mostraron el menor acoplamiento a lo largo del tratamiento lo cual fue prevenido por el aceite de aguacate. Estos resultados muestran que el aceite de aguacate mejoró varios parámetros del perfil metabólico y retrasó el desarrollo de la nefropatía diabética, probablemente debido a una mejoría en la función mitocondrial, esto debido a los componentes del aceite de aguacate ya que algunos pueden funcionar como antioxidantes, mientras que otros, como el ácido oleico, que tienen un efecto benéfico en el metabolismo de lípidos y carbohidratos.

Palabras clave: diabetes, riñón, adiponectina, perfil de lípidos, proteinuria.

ABSTRACT

Uncontrolled glucose and lipid metabolism during diabetes, due to the decrease in the synthesis of regulatory substances such as insulin and adiponectin, promotes the development of diabetic complications. One of the most frequent and devastating complications is diabetic nephropathy, which is characterized by high levels of proteinuria due to inadequate glomerular filtration. The aim of this study was to evaluate the effect of avocado oil on metabolic profile and the development of diabetic nephropathy in a model of type 2 diabetes.

Goto-Kakizaki rats were used as a model of type 2 diabetes, and avocado oil was administered orally for 3, 6 and 12 months. The results showed that treatment with avocado oil maintained for a longer time adiponectin levels, however had no effect on insulin levels. The treatment improved the lipid profile in rats by lowering cholesterol, triglyceride and LDL levels and maintaining HDL concentrations. Another important aspect was that avocado oil did not result in weight gain in diabetic rats. When evaluated renal function, the oil partially counteracted the increase in proteinuria observed in the diabetic group, while in the histopathological evaluation diabetic rats showed signs of diabetic nephropathy such as accumulation of extracellular matrix in the glomerulus and thickening of the basement membrane starting from 3 months of treatment. Treatment with avocado oil delayed the development of these alterations until 6 months. Regarding mitochondrial function, the diabetic rats showed mitochondrial dysfunction throughout the treatment, and this effect was prevented in some cases by the avocado oil. These results show that avocado oil improved several parameters of the metabolic profile and delayed the development of diabetic nephropathy, probably due to an improvement in mitochondrial function, due to the components of avocado oil since some may function as antioxidants, while others, such as oleic acid, have a beneficial effect on the metabolism of lipids and carbohydrates.

Keywords: diabetes, kidney, adiponectin, lipid profile, proteinuria.

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La diabetes es una enfermedad crónica que aparece cuando el páncreas no produce insulina suficiente o cuando el organismo no utiliza eficazmente la insulina que produce (OMS, 2017). La insulina es una hormona secretada por las células β de los islotes de Langerhans del páncreas (Clark *et al.*, 2001). La principal función de la insulina es contrarrestar la acción concertada de varias hormonas generadoras de hiperglicemia y para en niveles fisiológicos los niveles de glucosa en sangre. Además de su papel en la regulación metabolismo de la glucosa, la insulina estimula la lipogénesis, disminuye la lipólisis y aumenta el transporte de aminoácidos en las células (Turner *et al.*, 1979). Además de la insulina existen otras moléculas que ayudan a regular las concentraciones plasmáticas de glucosa, una de las más importantes es la adiponectina. La adiponectina es una adipocina que modula numerosos procesos metabólicos, incrementa la sensibilidad de insulina y produce un aumento en la β-oxidación de los ácidos grasos, lo que da como resultado la reducción de la cantidad de ácidos grasos circulantes y de triglicéridos intracelulares contenidos en el hígado y en los músculos (Fasshauer & Blüher, 2015). Debido a que otra de las alteraciones que se presenta con mucha frecuencia durante la diabetes es la dislipidemia, la adiponectina juega un papel importante al regular tanto el metabolismo de carbohidratos como de lípidos. Las dislipidemias son uno de los principales factores de riesgo para el desarrollo de enfermedades cardiovasculares y estas a su vez constituyen una de las principales causas de morbi-mortalidad en los pacientes diabéticos (Haffenr *et al.*, 1998). Estos pacientes presentan un patrón de alteraciones lipídicas caracterizadas por elevación moderada del colesterol LDL, incremento de triglicéridos y reducción del colesterol HDL (dislipidemia aterogénica) (Krauss & Siri, 2004). Las diversas guías de manejo de dislipidemias coinciden en que la reducción del colesterol LDL, es el principal objetivo terapéutico en los pacientes diabéticos (Mooradian, 2009).

Otra complicación muy común, que forma parte de las complicaciones microvasculares es la nefropatía diabética, la cual es la complicación más devastadora y costosa que sufren los pacientes con diabetes en todo el mundo. La hiperglucemia es la responsable del desarrollo y la progresión de la nefropatía diabética a través de diversas alteraciones metabólicas que producen un incremento en el estrés oxidativo. Uno de los principales responsables del aumento en el estrés oxidativo renal durante la diabetes es el metabolismo mitocondrial, mayormente debido a alteraciones en la cadena transportadora de electrones (Granata *et al.*, 2009). Las especies reactivas de oxígeno (ERO) producidas en la mitocondria pueden funcionar como reguladores de diversas vías metabólicas que pueden activar factores de transcripción tales como NF-κB, PKC y AP-1, los cuales pueden aumentar la expresión de TFG-β1, la citosina más importante involucrada en el desarrollo del daño renal (Lee *et al.*, 2003).

En estudios clínicos, a la pulpa del aguacate se le han atribuido efectos tales como un mejor control glucémico, el descenso del colesterol total, de las LDL y los triglicéridos, con un aumento de las HDL y por lo tanto una mejoría del índice aterogénico en pacientes con colesterol normal, con hiperlipidemia, con hipertrigliceridemia y con diabetes mellitus tipo 2 (Alvizouri *et al.*, 2009). Además, como se mostró en el capítulo III, el tratamiento con aceite de aguacate disminuyó las concentraciones plasmáticas de glucosa en las ratas Goto-Kakizaki incluso después de 12 meses de tratamiento y disminuyó el estrés oxidativo en las mitocondrias de riñón al disminuir la producción de ERO, bajar los niveles de peroxidación de lípidos y aumentar los niveles de glutatión reducido (GSH) (Ortiz Avila *et al.*, 2017).

Tomando en cuenta que el estrés oxidativo es un punto central para el desarrollo y la progresión de la nefropatía diabética y que el tratamiento con el aceite de aguacate ha probado disminuir este parámetro en el riñón, además de que el consumo de aguacate mejora el perfil de lípidos y el control de la hiperglucemia, el objetivo de este trabajo es determinar el efecto del aceite de aguacate sobre el perfil metabólico y el desarrollo de nefropatía diabética así como la función mitocondrial en el riñón de ratas con diabetes tipo 2.

MATERIALES Y MÉTODOS

Animales

Se emplearon ratas Goto-Kakizaki (GK) macho las cuales se mantuvieron en un bioterio bajo condiciones de temperatura controlada y ciclos de luz/oscuridad de 12 horas. Fueron alimentadas con una dieta especial para roedores y agua ad libitum. Para el manejo de los animales se siguieron las recomendaciones de la Norma Oficial Mexicana para el uso de animales expedida por la Secretaría de Agricultura en el párrafo de Regulaciones Federales para el Uso de Animales (NOM-062-ZOO-1999). Las ratas Goto-Kakizaki son un modelo de diabetes tipo 2 debido a que presentan un escaso número de islotes de Langerhans y por tanto, hay una baja producción de insulina. Las ratas Goto-Kakizaki provienen de la cepa Wistar y por ello se utilizaron como control.

Diseño experimental

Para el estudio las ratas fueron divididas al azar en cuatro grupos, de ocho ratas cada uno. Ratas Wistar: Grupo 1-Control y Grupo 2-Control + aceite de aguacate. Ratas Goto-Kakizaki: Grupo 3-Diabético y Grupo 4-Diabético + aceite de aguacate. Los grupos 2 y 4 recibieron una dosis de aceite de aguacate de 400µl/100g de peso corporal y los grupos 1 y 3 recibían solamente agua. La administración se realizó vía oral durante un periodo de 3, 6 y 12 meses. Se realizó un registro de los niveles de glucosa y peso de los animales a lo largo del tratamiento. Al término del tratamiento las

ratas fueron sacrificadas con un ayuno de 12 h y se obtuvo suero para la determinación de diversos parámetros bioquímicos. También se extrajeron los riñones, de los cuales se aislaron mitocondrias y también se fijó el tejido para posteriormente realizar los cortes histológicos.

Determinación de insulina y adiponectina

Para la determinación de las concentraciones plasmáticas de adiponectina se empleó el kit ELISA de Inmunoensayo Enzimático de Adiponectina (Acrp30) de Sigma-Aldrich. En la determinación sérica de insulina también se utilizó un kit ELISA inmunoabsorbente enzimático de Sigma-Aldrich, en una placa de 96 pozos para la medición cuantitativa de insulina en muestras biológicas.

Determinación del perfil de lípidos

Las determinaciones de las que consta el perfil de lípidos son: colesterol total, triglicéridos, HDL y LDL. Estas se realizaron en el suero de las ratas mediante pruebas enzimáticas acopladas a reacciones colorimétricas utilizando los kits de Spinreact.

Determinación de proteinuria

Para la determinación de proteína en orina de 24 h, cada rata se mantuvo en una jaula metabólica durante 24 h donde se colecto la orina. Una vez transcurrido este tiempo, se midió el volumen total de la orina y se realizó la determinación de proteínas totales en orina. Las proteínas presentes en la orina reaccionan en medio ácido con el complejo rojo de pirogalol-molibdato originando un complejo coloreado que se cuantifica espectrofotométricamente a 598 nm utilizando un kit de Spinreact. Se ha usado a la albuminuria, es decir, en la excreción urinaria de albúmina entre más de 300 mg/24 h, como un marcador de nefropatía diabética desde que fue descrita en 1964 por Keen y Viberti (Keen & Viberti, 1981).

Histopatología

Las muestras fueron fijadas con paraformaldehído al 4% y embebidas en parafina para así realizar cortes de tejido utilizando un micrótomo, los cuales fueron teñidos con la tinción de PAS (ácido per iódico-de Schiff) para evaluar posteriormente el nivel de daño renal. Los cortes y las tinciones fueron realizados por la Histotecnóloga María Guadalupe Hiriart Valencia en el Laboratorio de Morfología del Instituto Nacional de Enfermedades Respiratorias y la evaluación histopatológica por la Dra. Ma. del Consuelo Figueroa García.

Aislamiento de mitocondrias

Las mitocondrias de riñón se aislaron mediante centrifugación diferencial con el método descrito por Saavedra-Molina y Devlin (1997). Para obtener las mitocondrias, los riñones fueron triturados y lavados con medio 1 (220mM manitol, 70 mM sacarosa, 2 mM MOPS y 1 mM EGTA, a pH 7.4). Los fragmentos fueron homogenizados y centrifugados a 2000 rpm y el sobrenadante se centrifugó nuevamente a 7500 rpm y la pastilla resultante fue lavada con medio 2 (220mM manitol, 70 mM sacarosa y 2 mM MOPS, a pH 7.4) y centrifugada a 9000 rpm. Finalmente la pastilla se resuspendió en 500µl de medio 2. Todas las centrifugaciones se realizaron durante 10 minutos a 4°C. La concentración de proteína mitocondrial fue determinada mediante el método de Biuret.

Medición del consumo de oxígeno

Las mitocondrias recién aisladas fueron resuspendidas en un volumen final de 2ml de buffer de respiración (HEPES 10mM, KCl 100mM, KH2PO4 3mM y MgCl2 3mM a pH de 7.4) en una cámara de vidrio sellada y la velocidad de consumo de oxígeno fue determinada a temperatura ambiente usando un electrodo de oxígeno de tipo Clark acoplado a un monitor de oxigeno YSI 5300 y a un graficador. Los trazos de respiración se iniciaron inmediatamente después de inducir la respiración basal (estado 4) mediante la adición de 10mM de glutamato/malato como sustrato respiratorio. A los tres minutos se adicionaron 10µl de ADP 100mM, lo cual abate el potencial de membrana generado a través de la membrana interna mitocondrial debido al reingreso de los H+ por la F1F0 ATP sintasa hacia la matriz durante la fosforilación del ADP, dando lugar a un aumento en el flujo de electrones en la CTE y en la velocidad de consumo de O2 (estado 3) para restablecer el Δψm. La respiración fue inhibida con la adición de 1mM de KCN, lo cual permite descartar el consumo de oxigeno ajeno a la actividad de la CTE (Ortiz-Avila *et al.*, 2015).

RESULTADOS

Efecto del aceite de aguacate en los niveles de insulina y adiponectina

Debido al efecto hipoglucemiante del aceite de aguacate, anteriormente reportado (Ortiz Avila O., 2017), se determinó si el efecto es debido al aumento en las concentraciones plasmáticas, ya sea de insulina o de adiponectina. Al medir las concentraciones de insulina se observó una disminución en las ratas diabéticas (13.5%), respecto a las ratas control a los 3 meses de tratamiento, mientras que los niveles continuaron disminuyendo 33.2% a los 6 meses y hasta un 73.3% a los 12 meses. El tratamiento con el aceite de aguacate no modificó este parámetro, se observó una disminución gradual de los niveles de insulina al igual que en el grupo diabético sin tratamiento (Fig. 1). Con relación a los niveles de adiponectina, a los 3

meses de observó una disminución drástica, del 70%, en el suero de las ratas diabéticas (Fig. 2), mientras que el tratamiento con el aceite de aguacate previno la disminución en la concentración de este parámetro. A los 6 y 12 meses de tratamiento, la adiponectina disminuyó su concentración en las ratas diabéticas, hasta en un 77.2% por debajo del grupo control, y con el tratamiento de aceite de aguacate se obtuvo una disminución del efecto mostrado a los 3 meses de tratamiento, ya que a los 6 meses las concentraciones plasmáticas de adiponectina se redujeron un 55.6% y a los 12 meses la concentración fue similar a la de las ratas diabéticas sin tratamiento.

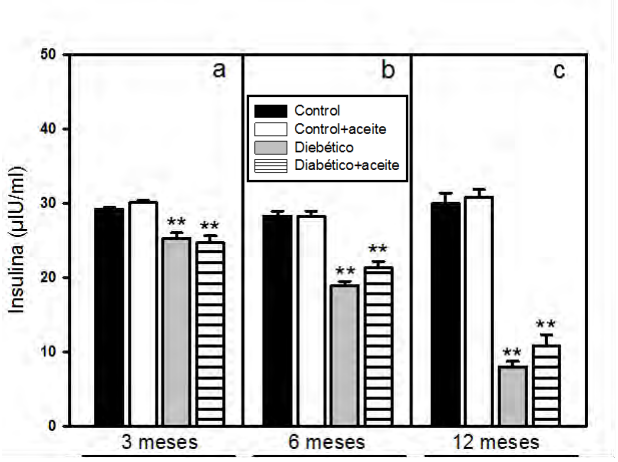

Figura 1.- Efecto del aceite de aguacate sobre los niveles de insulina. Los datos se presentan como la media ± EE de una n=4-8. **p<0.05 vs. Control; ANOVA de dos vías *(Bonferroni*).

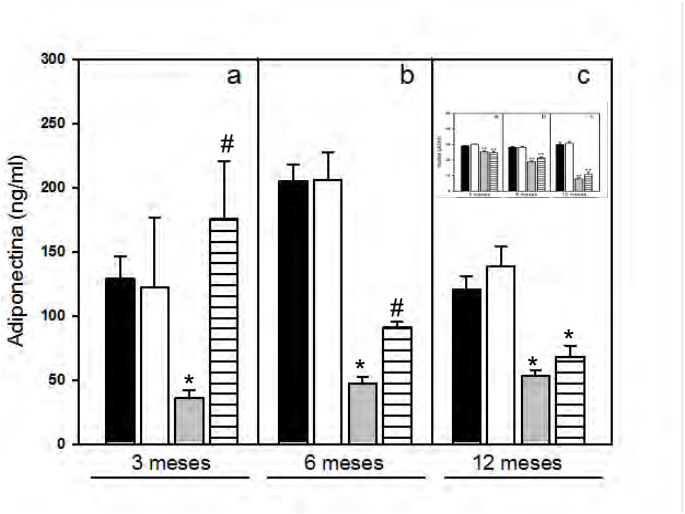

Figura 2.- Efecto del aceite de aguacate sobre los niveles de adiponectina. Los datos se presentan como la media ± EE de una n=4-8. *p<0.05 vs. Control; $^{\#}$p<0.05 vs. Diabético; ANOVA de dos vías *(Bonferroni*).

Efecto del aceite de aguacate sobre el peso y el perfil de lípidos

Debido al incremento en el contenido de lípidos en la dieta de los animales a causa del tratamiento con el aceite de aguacate, se realizó un registro del peso de los mismos para determinar si el tratamiento alteraba este parámetro. Se observó que las ratas Wistar (grupos control y control+aceite) tuvieron una mayor ganancia de peso que las ratas Goto-Kakizaki (grupos diabético y diabético+aceite) a lo largo del tratamiento (Fig. 3). En el caso de los grupos control, el tratamiento con aceite de aguacate produjo un ligero incremento del 7.7% en el peso de los animales a partir del séptimo mes de tratamiento, sin embargo, al finalizar los 12 meses de tratamiento no hubo diferencias significativas. Para las ratas diabéticas, la administración del aceite de aguacate no produjo un incremento en su peso.

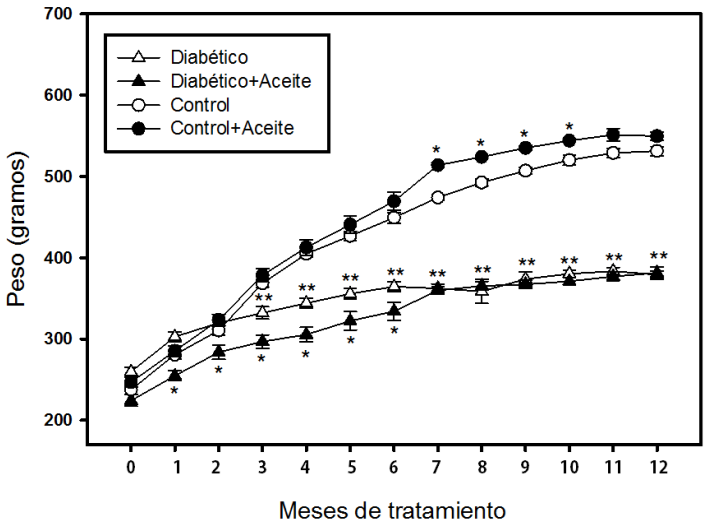

Figura 3.- Efecto del aceite de aguacate sobre el peso de las ratas. Los datos se presentan como la media ± EE de una n<4. *p<0.05 vs. Diabético; **p<0.05 vs. Control; ANOVA de dos vías *(Bonferroni*).

En relación con el perfil de lípidos, al determinar las concentraciones de colesterol total en suero a los 3 meses de tratamiento se observó que la diabetes incremento los niveles de colesterol un 34% y el tratamiento con el aceite de aguacate previno de este incremento (Fig. 4, panel I). En las determinaciones siguientes, de 6 y 12 meses, no hubo diferencias entre los distintos grupos. En cuanto a las concentraciones de triglicéridos no se observaron diferencias a los 3 y 6 meses de tratamiento entre los diferentes grupos (Fig. 4, panel II). Sin embargo, a los 12 meses se observó un aumento de 2.08 veces en la concentración de triglicéridos en el grupo diabético, y este

aumento fue prevenido con el tratamiento con el aceite de aguacate. Al evaluar las LDL se observó que la diabetes incrementó las concentraciones de esta lipoproteína ya que a lo largo del estudio el grupo diabético mostró un aumento entre el 70 y el 100% respecto al grupo control (Fig. 4, panel III) y el tratamiento con el aceite de aguacate ayudó a prevenir de este incremento en las LDL a lo largo del estudio. Las HDL por su parte, no variaron a los 3 y 6 meses entre dos diferentes grupos (Fig. 4, panel IV), pero a los 12 meses se observó una disminución del 43% en la concentración de esta lipoproteína en el grupo de ratas diabéticas, lo cual fue prevenido con el tratamiento con el aceite de aguacate.

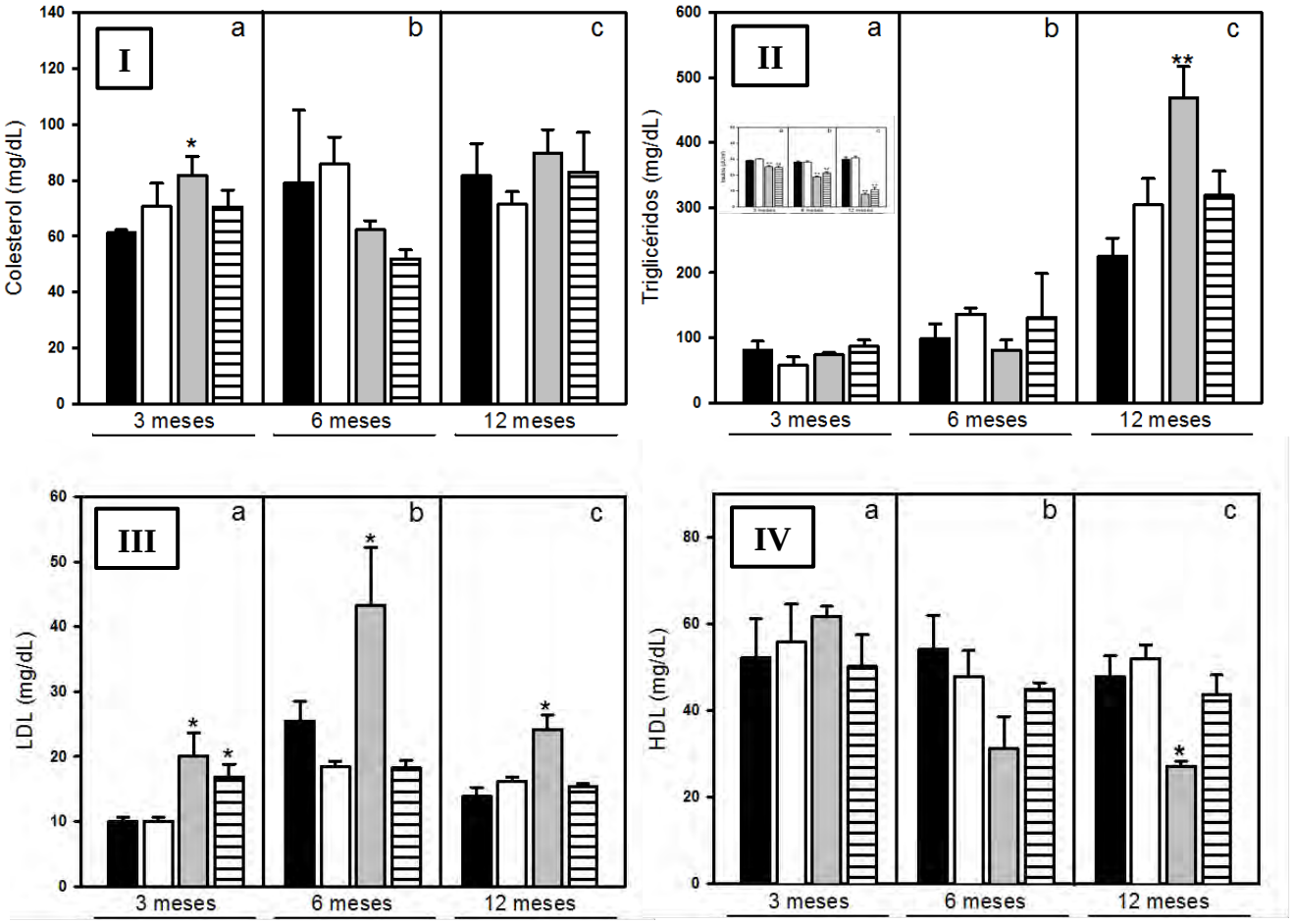

Figura 4.- Efecto del aceite de aguacate sobre el perfil de lípidos séricos de las ratas. Los datos se presentan como la media ± EE de una n>4. *p<0.05 vs. Control; **p<0.01 vs. Control; ANOVA de dos vías *(Bonferroni)*.

Efecto del aceite de aguacate en la concentración de proteína en orina

La determinación de proteína en orina de 24 h es un valor que refleja si existe una alteración en la filtración renal, debido a esto se eligió como una prueba para evaluar la función renal. En los resultados se observó que la diabetes produjo un incremento en la concentración de proteína en orina conforme transcurrió el tiempo, pasando de 358 mg de proteína en orina/24h a los 3 meses, hasta 655 mg de proteína en orina/24h a los 12 meses (Fig 5). El tratamiento con el aceite de aguacate reduce la proteinuria alrededor de un 50% respecto al grupo diabético a lo largo del estudio, sin embargo, el tratamiento no impide que el grado de proteinuria también vaya aumentando de manera gradual.

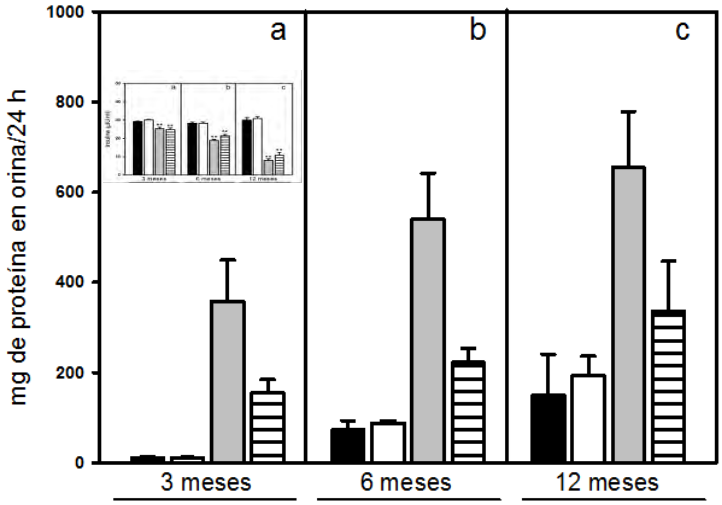

Figura 5.- Efecto del aceite de aguacate sobre la proteinuria de las ratas. Los datos se presentan como la media ± EE de una n>4. *p<0.05 vs. Control; ANOVA de dos vías *(Bonferroni*).

Efecto del aceite de aguacate en los cambios histopatológicos en el riñón

Con el fin de evaluar los cambios histológicos que se presentan durante el desarrollo de la nefropatía diabética y el efecto del tratamiento con el aceite de aguacate, se realizaron costes histológicos de riñón, los cuales fueron teñidos mediante la técnica de PAS. Las histologías de las ratas diabéticas mostraron alteraciones en el tejido características de la nefropatía diabética, desde los 3 meses se observó un engrosamiento de la lámina basal glomerular así como una ligera acumulación de fibrina y colágeno (matriz extracelular) dentro del glomérulo así como signos de inflamación (Fig. 6). A los 6 y 12 meses estas mismas alteraciones fueron más evidentes en los riñones de las ratas diabéticas y además se encontraron indicios de

fibrosis tubular, hiperplasia vascular y amiloidosis, todos ellas alteraciones histológicas relacionadas con la nefropatía diabética. Por otra parte, el tratamiento con el aceite de aguacate retrasó la aparición de estas alteraciones histopatológicas, ya que a los 3 meses no se encontraron indicios de daño renal, a los 6 meses comenzó a aparecer tanto el engrosamiento de la lámina basal glomerular como la acumulación de matriz extracelular e incluso a los 12 meses las alteraciones histológicas no son tan evidentes como en el caso de las ratas diabéticas que no recibieron tratamiento. Cabe mencionar que a los 12 meses se encontraron leves indicios de acumulación de matriz extracelular en el grupo control al que se le administró el aceite de aguacate.

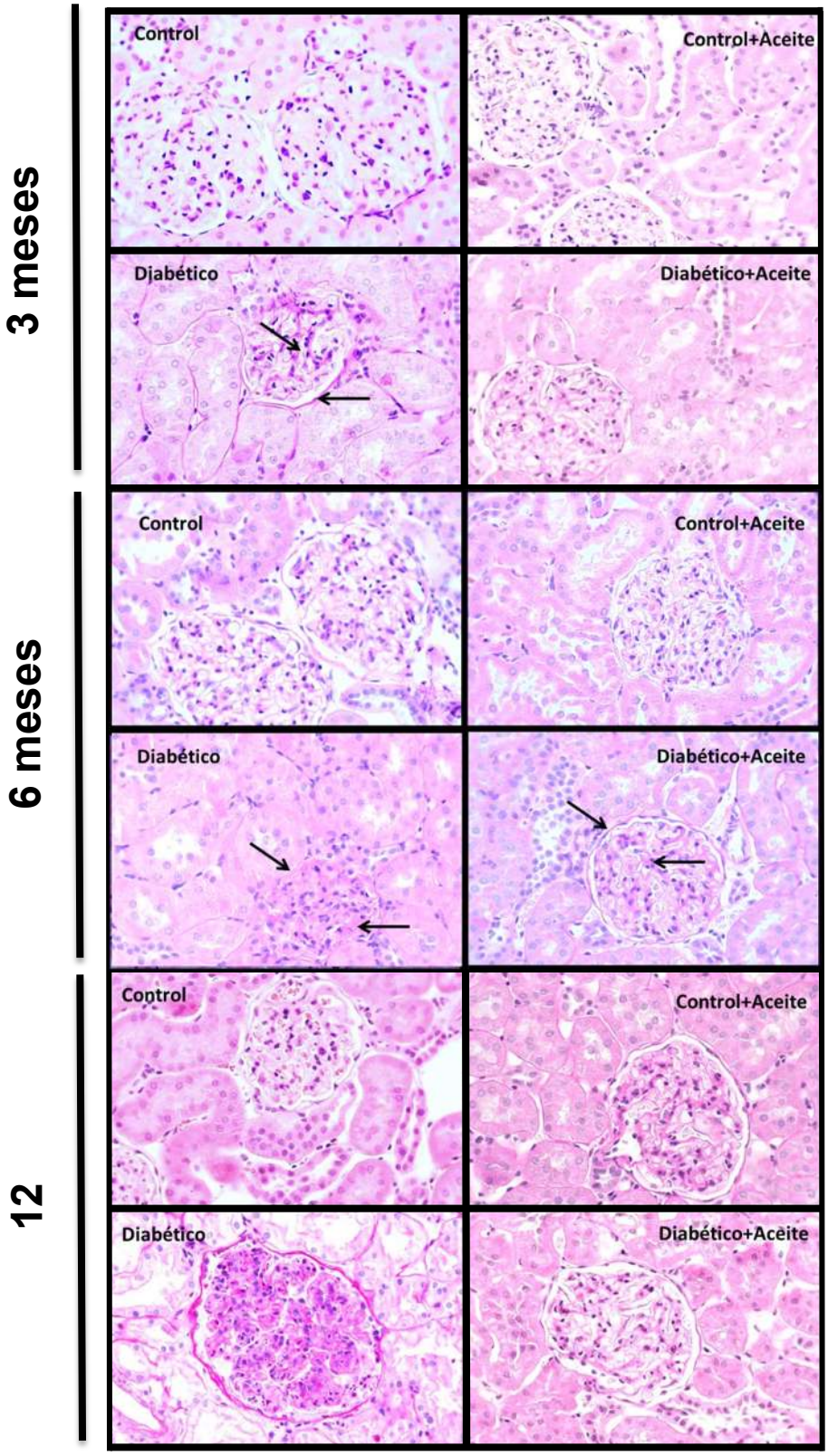

Figura 6.- Efecto del aceite de aguacate sobre los cambios histopatológicos en los riñones de las ratas diabéticas. Fotografías representativas de cada uno de los grupos a los distintos tiempos de tratamiento. Aumento 40x.

Efecto del aceite de aguacate en la función de las mitocondrias de riñón

Al evaluar la funcionalidad de la cadena de transporte de electrones de las mitocondrias de riñón mediante el consumo de oxígeno, se observó que a los 3 meses de tratamiento las ratas diabéticas y diabéticas tratadas con aceite de aguacate tuvieron un mayor consumo de oxígeno en el estado 4 comparado con las ratas control, sin embargo, las ratas diabéticas se volvieron insensibles al ADP (estado 3), esto se reflejó en un bajo cociente respiratorio (CR), mientras que las ratas diabéticas tratadas con aceite de aguacate respondieron de una mejor manera al ADP (Tabla 1). A los 6 meses de tratamiento, el aceite de aguacate disminuyó el estado 4 alrededor de 25% en las ratas controles y diabéticas; cuando se midió el estado 3 se observó una disminución en las ratas controles tratadas con aceite de aguacate del 37% y un 31% en las ratas diabéticas en comparación con las ratas control. Sin embargo, las ratas diabéticas siguieron teniendo el CR más bajo. Finalmente, después de 12 meses de tratamiento no se observaron diferencias significativas en el estado 4 entre los grupos, el aceite de aguacate produjo un aumento en la respiración en el estado 3 en ratas controles (58%) y diabéticas (5%). Cabe resaltar que se mantuvo la tendencia en el grupo de ratas diabéticas de presentar los valores de CR más bajos en comparación con los demás grupos.

Tabla 1.- Efectos del aceite de aguacate en la respiración mitocondrial en riñones de ratas. Los datos se presentan como la media ± EE de una n=4-13. *p<0.05 vs. Control; ANOVA de dos vías *(Bonferroni*).

	Grupo	Estado 4	Estado 3	CR
3 meses	Control	0.3497±0.0184	1.2630±0.2435	3.5570±0.5263
	Control+aceite	0.8637±0.2986	1.9740±0.3697	2.6040±0.5565
	Diabético	0.5814±0.0390*	0.6663±0.0951*	1.1428±0.1343*
	Diabético+aceite	0.7805±0.1059*	1.3972±0.3575	1.7160±0.2792
6 meses	Control	1.8644±0.1285	7.0607±0.1549	4.1136±0.2828
	Control+aceite	1.4106±0.1031*	4.4240±0.3805*	3.2213±0.2423
	Diabético	1.6360±0.2544	4.8841±0.2897*	2.2361±0.2040*
	Diabético+aceite	1.0934±0.1012*	6.6941±0.0971	6.3120±1.3033
12 meses	Control	0.8722±0.2184	3.8836±0.9013	5.0635±1.2501
	Control+aceite	1.5063±0.1295*	6.1409±0.4363*	4.2598±0.4010
	Diabético	0.8995±0.1826	2.9947±0.3019	3.5591±0.4801*
	Diabético+aceite	0.9833±0.0838	4.0792±0.2361	4.4855±0.3296

DISCUSIÓN

La diabetes tipo 2 es una epidemia global y una amenaza principal para la salud humana. El aumento en los casos de diabetes va acompañado con el aumento de las complicaciones relacionadas con esta enfermedad y los gastos de los sistemas de salud (FID, 2015). En vista de su gravedad, existe la necesidad de nuevos enfoques terapéuticos para manejar la diabetes tipo 2. Está bien establecido que las intervenciones en el estilo de vida, incluidos los cambios en la dieta, desempeñan un papel vital en la prevención de la progresión de la alteración en el metabolismo de la glucosa y el desarrollo de las complicaciones asociadas a la diabetes (Huo *et al.*, 2015). Sin embargo, existen estudios limitados en relación con los efectos que puede generar el empleo de ciertos alimentos sobres el control de la diabetes y sus complicaciones.

En este estudio se analizó el efecto del aceite de aguacate sobre el perfil metabólico y el desarrollo de nefropatía diabética en ratas GK, un modelo de diabetes tipo 2. El perfil metabólico analizado consta de varias determinaciones bioquímicas como glucosa, insulina, adiponectina, colesterol total, triglicéridos, LDL y HDL, así como el peso corporal. Anteriormente, nuestro grupo de trabajo reportó que el tratamiento con aceite de aguacate tuvo un efecto hipoglucemiante en las ratas GK (Ortiz Avila *et al.*, 2017) el cual se mantuvo hasta los 12 meses. Con la finalidad de elucidar el mecanismo de este efecto hipoglucémico por parte del aceite de aguacate se realizó la determinación de las concentraciones de insulina y adipinectina. Como se observa en los resultados de la determinación de insulina (Fig. 1) la diabetes disminuye su concentración y el tratamiento con el aceite de aguacate no tuvo efecto sobre este parámetro. Por otra parte, en las ratas diabéticas también disminuye la concentración de adiponectina desde los 3 meses, sin embargo, en este caso el aceite de aguacate si tuvo un efecto, al retrasar el inicio de este descenso hasta los 12 meses (Fig. 2). Estos resultados nos indican que en cierta medida, el efecto hipoglucemiante observado con el aceite de aguacate pudiera deberse a que ayuda a mantener las concentraciones de adiponectina y esto se ha asociado con un aumento en la sensibilidad a la insulina (Fasshauer & Blüher, 2015). De hecho se ha descrito que en experimentos en roedores, la administración de adiponectina mejora la sensibilidad a la insulina y el metabolismo de la glucosa, incrementa la secreción de insulina y reduce el peso corporal (Ye & Scherer, 2013).

Las ratas diabéticas mostraron también alteraciones en el metabolismo de los lípidos, el más consistente fue un aumento en las concentraciones de LDL (Fig. 4, panel III). El aceite de aguacate, en este caso mostró un efecto hipolipemiante ya que en diversos periodos durante el estudio se observó que mantuvo las concentraciones tanto de colesterol, triglicéridos, LDL y HDL similares al control. Esto disminuye el riesgo de presentar enfermedades cardiovasculares, las cuales están íntimamente asociadas con

la diabetes tipo 2 y de hecho, son una de las principales causas de muerte en pacientes diabéticos (Dambha-Miller *et al.*, 2017). Cabe resaltar que la administración del aceite de aguacate, aunque fue un aporte extra de lípidos a la dieta, no incrementó el peso corporal en las ratas diabéticas (Fig. 3). Estos efectos positivos del tratamiento con el aceite de aguacate en las determinaciones del perfil metabólico pueden ser atribuidos a diversos componentes del aceite. Uno de ellos es el ácido oleico, el cual es el componente mayoritario del aceite de aguacate. Con relación a la adiponectina, existen estudios donde se relaciona el consumir una dieta mediterránea, la cual se caracteriza por un alto consumo de granos enteros, MUFAs (como el ácido oleico), frutas y vegetales; consumo moderado de pescado y vino, con un aumento en los niveles de adiponectina (Mantzoros *et al.*, 2016). Entonces, el alto contenido de ácido oleico en el aceite de aguacate, el cual es un componente de la dieta mediterránea, podría ser el responsable de mantener las concentraciones de adiponectina durante la diabetes. La adiponectina también está implicada en la regulación del metabolismos de lípidos (Mantzoros *et al.*, 2005), por lo que seguramente el que el aceite mantenga los niveles de esta adipocina en las ratas diabéticas tiene un efecto directo sobre un adecuado control de las concentraciones de lípidos en sangre. Además de que el ácido oleico también se ha asociado con la regulación del perfil de lípidos mediante un aumento en el catabolismo de las LDL apoB100 (Labonté *et al.*, 2013), esto debido a una incremento en la actividad del receptor para LDL (Gill *et al.*, 2003). Además el ácido oleico puede aumentar la concentración de HDL apoA1, gracias a la activación de la proteína colesterol-ester-tranferasa (Abbey & Nestel, 1994), la cual, disminuye la concentración de triglicéridos e incrementa los ésteres de colesterol en las HDL disminuyendo su degradación (Lamarche *et al.*, 1999).

En relación con los resultados de la evaluación de la función renal y el desarrollo de nefropatía diabética, se puede observar que las ratas diabéticas presentaron una marcada proteinuria desde los 3 meses (Fig. 5) lo cual es un indicio de daño renal. En este caso el tratamiento con el aceite de aguacate retrasó la aparición de la proteinuria. Estos resultados concuerdan con los hallazgos histopatológicos en los riñones de las ratas, donde, desde los 3 meses se observaron alteraciones asociadas con le nefropatía diabética en las ratas con diabetes (Fig. 6), mientras que en las que recibieron el tratamiento con el aceite de aguacate estas alteraciones se presentaron hasta los 6 meses. El retraso en el desarrollo de la nefropatía diabética gracias al tratamiento con el aceite de aguacate es un resultado muy interesante en este trabajo y los posibles mecanismos implicados en este efecto son diversos. El primero de ellos es el efecto que tuvo el aceite de aguacate sobre el control de la glucosa plasmática, se ha descrito que un adecuado control glucémico puede retardar e incluso detener la progresión de la nefropatía diabética (UKPDS Group, 1998). En este caso, aunque la disminución en la concentración de glucosa no alcanzo los niveles de las ratas control (Ortiz Avila *et al.*, 2017), aun así podría tener un efecto en el desarrollo de la nefropatía

diabética. Otro mecanismo mediante el cual el aceite de aguacate podría retrasar el desarrollo de la nefropatía diabética es gracias a su potencial antioxidante, debido a que se ha descrito que el estrés oxidativo, especialmente las ERO que se producen en la mitocondria, juegan un papel muy importante en el desarrollo de esta complicación (Granata *et al.*, 2009). La disminución en el estrés oxidativo mitocondrial gracias al tratamiento con el aceite de aguacate ya ha sido reportado en nuestro grupo de trabajo en mitocondrias de hígado (Ortiz-Avila *et al.*, 2015), cerebro (Ortiz-Avila *et al.*, 2015) y riñón (Ortiz Avila *et al.*, 2017) de ratas diabéticas.

La reducción de la función renal ha sido asociada con alteraciones significativas en el metabolismo energético, inflamación y estrés oxidativo (Khoshjou *et al.*, 2014). Debido a que la mitocondria juega un papel clave en el metabolismo energético se evaluó la función mitocondrial en los riñones de las ratas obteniendo que la diabetes provocó una disfunción mitocondrial (tabla 1). En los resultados de este estudio también se observa que el consumo del aceite de aguacate mejora la función mitocondrial durante la diabetes en las mitocondrias de riñón, y esta mejoría disminuye la formación de ERO por lo que se disminuye el estrés oxidativo mitocondrial.

Por ende, al existir una mejora en el funcionamiento mitocondrial y por tanto un control del estrés oxidativo en el riñón durante la diabetes, las vías de señalización que son activadas por el aumento en las ERO y los factores que dan como resultado las alteraciones histopatológicas como nF-κB, AP-1 y TGF-β1, involucrados en el desarrollo de la fibrosis glomerular e inflamación (Lee *et al.*, 2003), se mantienen bajo control.

CONCLUSIÓN

El tratamiento con el aceite de aguacate mejoró el perfil metabólico al reducir las concentraciones de glucosa y mantener los niveles de adiponectina y de lípidos en sangre, además de no incrementar el peso en un modelo de diabetes tipo 2, esto gracias a los componentes del aceite de aguacate, en especial al ácido oleico. El aceite de aguacate también retardó la aparición de la nefropatía diabética lo cual pudiera estar relacionado con una mejoría en la función mitocondrial, una reducción en el estrés oxidativo y algunos de los cambios observados en el perfil de metabólico.

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9. DISCUSIÓN GENERAL

Dado que la diabetes se ha convertido en un problema de salud muy grave a nivel mundial y a que el desarrollo de múltiples complicaciones asociadas a esta enfermedad incrementa la taza de morbi-mortalidad en estos pacientes es importante buscar nuevas alternativas que ayuden a un mejor control de esta enfermedad y reducir la incidencias de sus complicaciones para que los pacientes puedan tener una mejor calidad de vida.

En el presente trabajo se analizó el efecto que produce el aceite de aguacate en diferentes complicaciones asociadas a la diabetes, específicamente se evaluaron cambios en diversos parámetros bioquímicos como la glucosa y el perfil de lípidos, así como alteraciones en la función mitocondrial y el estrés oxidativo en órganos blanco de la diabetes como son el hígado, el cerebro y el riñón utilizando modelos en roedores. Uno de los modelos es el de diabetes tipo 1 (ratas-STZ), el cual se genera mediante la administración de estreptozotocina, la cual tiene un efecto directo destruyendo las células-β del páncreas. El otro modelo es de diabetes tipo 2 (ratas-GK), en este caso se utilizaron ratas Goto-Kakizaki, las cuales tienen reducido el número de células-β pancreáticas por lo que hay una baja producción de insulina y el consecuente desarrollo de diabetes. Cabe mencionar que ambos modelos tienen ventajas y desventajas, en el caso de las ratas-STZ éstas desarrollan en un tiempo relativamente corto diversas complicaciones asociadas a la enfermedad, sin embargo, los niveles de glucosa que presentan estas ratas son muy elevados (400-600 mg/dL) debido a la falta de insulina, además de que este tipo de diabetes es el menos frecuente en la población. Por su parte, las ratas-GK presentaron una moderada elevación de la concentración de glucosa, debido a que la secreción de insulina, aunque es baja, aún se encuentra presente. Otra ventaja es que la diabetes tipo 2 es la más frecuente, por arriba del 90% de los casos corresponden a este tipo de diabetes. La desventaja es que como las concentraciones de glucosa en estas ratas no sobrepasan los 300 mg/dL el desarrollo de las complicaciones asociadas a la diabetes es más lento, sin embargo, esto este es un modelo más aproximado a la realidad.

Efecto sobre la glucosa

En las ratas-STZ el tratamiento durante 90 días con el aceite de aguacate no disminuyó las concentraciones de glucosa (capítulo I, Fig. 1a), sin embargo, en las ratas-GK el aceite de aguacate ayudó a un mejor control de la glucosa a lo largo del tratamiento, aunque la disminución en la concentración de glucosa no alcanzó los niveles de las ratas control (capítulo III, Fig. 1). Este efecto ya se había reportado en pacientes con diabetes tipo 2 que incluían en la dieta el consumo de aguacate (Lerman *et al.*, 1994). La diferencia en el efecto hipoglucemiante del aceite de aguacate entre los dos modelos utilizados es debido a tres factores, el primero son las concentraciones de

glucosa tan elevadas que presentaron las ratas-STZ, debido a que el efecto hipoglucémico que presentó el aceite de aguacate no fue tan marcado, a concentraciones tan elevadas pudiera ser imperceptible. Los otros dos factores son tanto la secreción como la sensibilidad a la insulina, en cuanto a la secreción de insulina en las ratas-STZ es prácticamente nula debido al modelo de inducción (Okamoto, 1970) a diferencia de las ratas-GK donde aún existe secreción de insulina (capítulo IV, Fig. 1). La falta de producción de insulina hace más difícil el poder controlar las concentraciones plasmáticas de glucosa (Goto *et al.*, 1976). En relación a la sensibilidad hacia la insulina, en las ratas-GK el tratamiento con el aceite de aguacate mantuvo las concentraciones de adiponectina (capítulo IV, Fig. 2), una adipocina que ayuda a incrementar la sensibilidad periférica a la insulina y de esta manera reducir las concentraciones de glucosa sanguínea, a diferencia de las ratas-STZ donde este mecanismo se encuentra suprimido debido a la falta de insulina. El efecto hipoglucemiante del aceite de aguacate observado en las ratas-GK podía estar asociado entonces a un aumento en la concentración de adiponectina, además del papel del ácido oleico y el efecto antioxidante, los cuales se discutieron en el capítulo III, en su conjunto incrementan la sensibilidad a la insulina y por lo tanto disminuyen la concentración de glucosa sanguínea.

Efecto en los lípidos y el peso

El tratamiento con el aceite de aguacate produjo una mejoría en el metabolismo de lípidos durante la diabetes, tanto en las ratas-STZ como en las ratas-GK. Los cambios observados en el perfil de lípidos fueron una disminución en las concentraciones de colesterol total, triglicéridos y LDL, además de mantener la concentración de HDL en las ratas-GK (capítulo I, Fig. 1b,c; capítulo IV, Fig. 4), lo cual también ha sido reportado con el consumo de aguacate en pacientes con dislipidemia (Alvizouri *et al.*, 2009). El mecanismo mediante el cual el tratamiento con el aceite de aguacate produce estos cambios en el perfil de lípidos se ha asociado principalmente a que el ácido oleico, componente mayoritario del aceite, incrementa la degradación de las LDL gracias a un aumento en la actividad de su receptor y también disminuye el catabolismo de las HDL (Labonté *et al.*, 2013). Aunado a este efecto, el aumento en las concentraciones de adiponectina debido al tratamiento con el aceite de aguacate también tiene efectos sobre el metabolismo de lípidos incrementando su catabolismo (Fasshauer & Blüher, 2015). De manera interesante, asociado con este efecto hipolipemiante, tanto las ratas-STZ como las ratas-GK no incrementaron su peso corporal al ser administradas con el aceite (capítulo I, Tabla 1; capítulo IV, Fig. 3), lo cual disminuye los riesgos crardiometabólicos asociados con la ganancia de peso durante la diabetes (UKPDS Group, 1998) este efecto podría deberse a que como se discutido anteriormente, el ácido oleico contenido en el aceite de aguacate, favorece el catabolismo de los ácidos grasos en lugar de su acumulación.

Efecto en la función mitocondrial

Alteraciones en la función mitocondrial pueden dar como resultado el incremento en la producción de ERO y generar un estado de estrés oxidativo, el cual está asociado con el desarrollo de la mayoría de las complicaciones relacionadas con la diabetes (Nishikawa T, 2000). Al evaluar la función mitocondrial tanto en hígado, cerebro y riñón, se observó que la diabetes produce una diversas alteraciones que disminuyen la función mitocondrial en los modelos de diabetes utilizados y el tratamiento con el aceite de aguacate mejoró la respiración mitocondrial (capítulo I, Fig. 8; capítulo II, Fig. 1; capítulo IV, Tabla 1). En el caso de las mitocondrias de hígado esta mejoría se asoció con una recuperación en la actividad del complejo I (capítulo I, Fig. 7) debida a una posible protección de la oxidación en los grupos tioles de este complejo como parte del efecto antioxidante del aceite de aguacate. En el caso de las mitocondrias de cerebro, el aceite de aguacate incrementó la actividad del complejo III, con lo cual se disminuye el tiempo de vida media de las semiquinonas y por tanto la generación de ERO (capítulo I, Fig. 3), también el aceite produjo una disminución en la actividad del complejo I, con lo cual se regula la tasa de oxidación de NADH durante la diabetes y también podría ayudar a disminuir la generación de ERO en las mitocondrias de cerebro. En lo que respecta a las mitocondrias de riñón, en un estudio previo en ratas-STZ administradas con el aceite de aguacate, también se evaluó la actividad de los complejos de la cadena transportadora de electrones (Ortiz-Avila *et al.*, 2013), y se observó que el aceite incrementó la actividad del complejo III, y esto se asoció con una protección en los citocromos de este complejo. Con estos resultados se puede decir que el aceite de aguacate mejoró la función mitocondrial, esto gracias a mejoras en la cadena transportadora de electrones que son tejido-específicas.

Efecto en el estrés oxidativo y la composición de ácidos grasos en las mitocondrias

El estrés oxidativo está involucrado en la mayoría de las complicaciones que se desarrollan debido a la diabetes. Se ha descrito que la sobreproducción de radical $O_2^{\bullet-}$ mitocondrial tiene un papel central en el desarrollo del estrés oxidativo durante la diabetes y esto es debido a que puede activar diversas vías que generan estrés oxidativo como la vía de los polioles, la vía de la hexosamina, un aumento en la formación de productos finales de glicosilación avanzada (AGEs), la activación de PKC, la activación de NADPH oxidasas (NOX) y óxido nítrico sintasas (NOS) (Giacco *et al.*, 2010). En este estudio se observó que la diabetes genera un incremento en la formación de ERO en las mitocondrias de hígado, cerebro y riñón, y en todos los casos el tratamiento con el aceite de aguacate produjo una disminución en la generación de estas especies. En los tres tejidos, esta disminución en la formación de ERO trajo como consecuencia una mejora en el estado redox mitocondrial (capítulo I, Fig. 4; capítulo II, Fig. 6; capítulo III, Fig. 4), al incrementar el cociente GSH/GSSG, lo contrario que se observó en las ratas diabéticas sin tratamiento debido al estado de estrés oxidativo en

el que se encuentran. Como un parámetro del efecto del estrés oxidativo sobre las biomoléculas se determinaron los niveles de peroxidación de lípidos. En las mitocondrias de hígado y riñón (capítulo I, Fig. 5; capítulo III, Fig. 3) la diabetes incrementó los niveles de peroxidación de lípidos, lo cual concuerda con el aumento en la producción de ERO. Por otra parte, en las mitocondrias de cerebro, la diabetes no incrementó la peroxidación de lípidos (capítulo II, Fig. 5). Sin embargo, un efecto que si se observó en todos los tejidos fue que el tratamiento con el aceite de aguacate disminuye los niveles de peroxidación de lípidos en las membranas mitocondriales durante la diabetes. En relación con las membranas mitocondriales, al determinar la composición de ácidos grasos en las mitocondrias de hígado la diabetes aumentó el porcentaje de SFAs y disminuyó el de MUFAs, mientras que el aceite de aguacate no tuvo efecto alguno (capítulo I, Fig. 3). En el caso de las mitocondrias de riñón el aceite incrementó el contenido de MUFAs y esto es debido al alto contenido de ácido oleico presente en el aceite de aguacate (capítulo III, Fig. 5). Todos los efectos antioxidantes producidos por el aceite de aguacate son causados por diversos compuestos presentes en el aceite que poseen una capacidad antioxidante como son los carotenos, tocoferoles o el β-sitosterol (Dreher *et al.*, 2013).

Efecto en la fisiopatología de las complicaciones

Al analizar los cambios histológicos en el hígado de las ratas-STZ asociados a la diabetes como pueden ser el desarrollo de esteatosis, los hepatocitos con forma de “balón” o signos de inflamación, no se encontraron presentes en el tejido analizado (capítulo I, Fig. 2). En el caso de la evaluación del desarrollo de la nefropatía diabética en las ratas-GK se observó que la diabetes produjo cambios asociados con la nefropatía diabética como la acumulación de matriz mesangial dentro del glomérulo y el engrosamiento de la membrana basal glomerular (capítulo IV, Fig. 6). El tratamiento con el aceite de aguacate retrasa la aparición de estas alteraciones asociadas con la nefropatía diabética así como el desarrollo de la proteinuria (capítulo IV. Fig. 5) lo cual se refleja un mejor funcionamiento renal, esto puede asociarse principalmente con que el aceite de aguacate ayuda a mantener un mejor control glucémico y a la disminución en el estrés oxidativo.

Con estos resultados y tomando en cuenta todos los beneficios que produjo a los animales el consumo del aceite de aguacate, puede ser considerado como un alimento funcional, el cual puede ser un tratamiento alternativo o un coadyuvante en el tratamiento de la diabetes y sus complicaciones.

10. CONCLUSIÓN GENERAL

El aceite de aguacate regula el perfil metabólico durante la diabetes además de mejorar la función mitocondrial y reducir el estrés oxidativo en órganos blanco de la diabetes como son el hígado, el cerebro y el riñón, además de retrasar la aparición de la nefropatía diabética.

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