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Tesis Doctoral Internacional/International Doctoral Thesis

Influencia de la Ingesta de hidroxitirosol en la expresión proteica y génica inducida por la actividad física en ratas

Influence of Hydroxytyrosol Intake on Protein and Gene Expression Induced by Physical Activity in Rats

Tesis Doctoral

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AUTORIZACIÓN DE LOS DIRECTORES DE LA TESIS PARA SU PRESENTACIÓN

El Dr. Jesús Rodríguez Huertas y el Dr. Rafael A. Casuso Pérez como Directores de la Tesis Doctoral titulada "*Influencia de la ingesta de hidroxitirosol en la expresión proteica y génica inducida por la actividad física en ratas*" realizada por D. **Saad al Fazazi** en el Instituto de Nutrición y Tecnología de los Alimentos, INYTA (Centro de Investigación Biomédica) de la Universidad de Granada **autorizan su presentación a trámite** dado que reúne las condiciones necesarias para su defensa ante el Tribunal que designe la Universidad de Granada.

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Dedicado a mis padres, mi hermana,

mi primo Anas (que su alma descanse en paz)

y mis amigos del "Consejo Superior MC"

porque son mi motivación en el día a día.

Si caminas solo, irás más rápido.
Si lo haces acompañado, llegarás más lejos.

Proverbio chino

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Publicaciones Originales

La presente tesis ha dado lugar a las siguientes publicaciones:

1. Huertas JR., S. **Al Fazazi S**., Hidalgo-Gutierrez A., López LC., Casuso RA. Antioxidants effect of exercise: Exploring the role of the mitochondrial complex I superassembly. *Redox Biology*. 2017; 13: 477-481.

2. Casuso RA., **Al Fazazi S**., Hidalgo Gutierrez A., López LC., Rueda-Robles A., Huertas JR. Hydroxytyrosol influences exercise-induced mitochondrial respiratory complex assembly into supercomplexes in rats. *Free Radical Biology and Medicine*. 2019; 134: 304-310

3. **Al Fazazi S**., Casuso RA., Aragón-Vela J. Casals C., Huertas JR. Effects of hydroxytyrosol dose on the redox status of exercised rats: the role of hydroxytyrosol in exercise performance. *Journal of the International Society of Sports Nutrition*. 2018; 15-20

Además , enviamos a revisión 2 estudios (no publicados):

1. Casuso RA., **Al Fazazi S.**, Ruíz/Ojeda FJ., Plaza-Díaz J., Rueda-Robles A., Aragón-Vela J., Huertas JR. Hydroxytyrosol modifies skeletal muscle GLUT4/AKT/RAC1 axis in trained rats.
2. Casuso RA.; **Al Fazazi S**.; Julio Plaza-Díaz J.; Francisco J Ruiz-Ojeda FJ.; Rueda-Robles A.; Aragón-Vela G.; Huertas JR. Physiological Doses of hydroxytyrosol Modulate Gene Expression in Skeletal Muscle of Exercised Rats.

Indicios de calidad, publicaciones incluidas en el documento de tesis.

- *Redox Biology*. Factor de impacto: 7,793. Citaciones recibidas: 7192. Categoría: Bioquímica y Biología Molecular. Posición Categoría Q1: 28/299.
- *Free Radical Bio. Med.* Factor de Impacto: 5,657. Citaciones recibidas: 40820. Categorías: Bioquímica y biología Molecular. Posición Categoría Q1: 43/299.
- *Journal Int. soc. Sports Nut*. Factor de Impacto: 3,841. Citaciones recibidas: 1632. Categoría: Nutrición y Dietética-Ciencias del Deporte. Posición Categoría Q1: 9/83.

Comunicaciones presentadas en congresos

Los diferentes congresos en los que hemos presentado los diferentes resultados de la tesis son:

1. I Jornadas Científicas del Centro de Investigación Biomédica. "Influencia del ejercicio físico y la ingesta de hidroxitirosol en el estado REDOX de ratas Wistar", 2017.
2. 14th International Scientific Conference of Sport Kinetics. "Effects of physical exercise and intake of hydroxytyrosol in the REDOX status of exercised Wistar Rats", 2018.
3. XVIII Reunión de la sociedad Española de Nutrición. "Hydroxytyrosol supplementation impairs skeletal muscle glucose transport in trained Rats: an analysis of the GLUT4/AKTp axis", 2019.
4. I Congreso Nacional de Investigadores del PTS. "Hydroxytyrosol supplementation impairs skeletal muscle glucose transport in trained Rats: an analysis of the GLUT4/AKTp axis", 2019.

Colaboración internacional: Universidad de Novi Sad, Serbia

Podría considerarse la experiencia más interesante de mi formación universitaria e investigadora. Mi estancia de 3 meses en la Facultad de Deporte de la Universidad de Novi Sad en Serbia me ha permitido evaluar mi capacidad de integración y trabajo en un entorno totalmente distinto al que estoy acostumbrado. Trabajando en el Laboratorio de Bioenergética Aplicada y bajo la dirección del profesor Patrik Drid, he tenido la oportunidad de conocer a todo el equipo del profesor Sergej Ostojic, toda una eminencia en la investigación a nivel internacional, y de forjar una gran amistad con algunos de los miembros de dicho equipo.

Fruto de nuestra colaboración, hemos publicado un trabajo titulado "24-hours dynamics for serum biomarkers of creatine metabolism after an acute session of exhaustive resistance exercise in active men" en la revista *Science & Sports* (Al Fazazi, S. 2018). Además, participamos en el prestigioso congreso de la asociación Americana de Medicina Deportiva ACSM, que se celebró el 28 de mayo en Orlando, Florida (EE. UU.).

Además de los resultados meramente académicos, es necesario subrayar el honor de poder visitar las instalaciones deportivas de la ciudad, así como presenciar y participar activamente en varias sesiones de entrenamiento del Club de voleibol Vojvodina de la primera división Serbia, donde algunos de sus integrantes participan en la selección nacional.

International Collaboration: University of Novi Sad, Serbia

It has been the most interesting experience of my university and research career. My 3-month stay at the Sports Faculty of the University of Novi Sad in Serbia has allowed me to evaluate my ability to integrate and develop my competences in a totally different environment from what I usually do. Working in the Laboratory of Applied Bioenergetics and under the direction of Professor Patrik Drid, I had the opportunity to meet Professor Sergej Ostojic, an international research eminence, and his whole team, which led me to build a great friendship with some of the members.

As a result of our collaboration, we have published an article "24-hours dynamics for serum biomarkers of creatine metabolism after an acute session of exhaustive resistance exercise in active men" in the journal *Science & Sports* (Al Fazazi, S. 2018). We also participated in the prestigious congress of the American Association of Sports Medicine ACSM, celebrated this year on May 28th in Orlando, Florida (USA).

In addition to the purely academic results, I have to underline the possibility of visiting the sports facilities of the city and actively participate in several training sessions of the Vojvodina Volleyball Club of the first Serbian division, with some of its members being part of the National team.

Abreviaturas (Abbreviations)

- **AKT:** Protein Kinase B
- **ANOVA:** Analysis of Variance
- **ATP:** Adenosine Triphosphate
- **BNGE:** Blue native gel electrophoresis
- **CI:** Complex I
- **CIII:** Complex III
- **CIV:** Complex IV
- **ETC:** Electron transport chain
- **EVOO:** Extra virgin olive oil
- **EXE:** Exercised
- **EXE20:** Exercised consuming 20 mg/kg/day of HT
- **EXE300:** Exercised consuming 300 mg/kg/day of HT
- **EXElow:** Exercised consuming 0,31 mg/kg/day of HT
- **EXEmild:** Exercised consuming 4,61 mg/kg/day of HT
- **GLUT4:** Glucose transporter 4
- **HCT:** Hematocrit
- **HGB:** Hemoglobin
- **HPx:** Hydroperoxides
- **HT:** Hydroxytyrosol
- **MD:** Mediterranean diet
- **mETC:** Mitochondrial electron transport chain
- **OXPHOS:** Oxidative phosphorylation
- **PC:** Protein carbonyls
- **RAC1:** Ras-related C3 botulinum toxin substrate 1
- **ROS:** Reactive oxygen species
- **SC:** Supercomplexe
- **SCAF1:** Supercomplex assembly factor I
- **SED:** Sedentary
- **SED20:** Sedentary consuming 20 mg/kg/day of HT
- **SED300:** Sedentary consuming 300 mg /kg/day of HT
- **SKM:** Skeletal muscle
- **VOO:** Virgin olive oil

Resumen General

Los beneficios de la práctica regular de ejercicio son ampliamente conocidos y existe una evidencia considerable acerca de que, durante su práctica, aumenta la producción de radicales libres. Cierto nivel de estos radicales libres, que son compuestos oxidantes, ejerce efectos positivos sobre las funciones inmunitarias, el metabolismo celular e incluso sobre la contracción muscular y la adaptación al ejercicio sistémico. Por otro lado, la práctica de ejercicio físico extenuante puede desencadenar un desequilibrio entre la producción de radicales libres y los mecanismos de defensa antioxidante del organismo, provocando daños moleculares sobre los lípidos, las proteínas y el ADN. La suplementación con antioxidantes es una práctica común en atletas y personas sedentarias, pero existe cierta controversia en relación a los beneficios de dicha suplementación, así como las dosis recomendables que potencian los beneficios de la práctica de ejercicio. En nuestro estudio evaluamos los efectos de diferentes dosis de hidroxitirosol, un componente fenólico del aceite de oliva con propiedades antioxidantes sobre el rendimiento, la función mitocondrial (organización de los complejos mitocondriales), el estrés oxidativo y la expresión génica y proteica inducida por el ejercicio. Nuestros resultados se presentan en 3 artículos publicados en revistas internacionales y 2 estudios enviados a publicación:

PRIMER PROYECTO EXPERIMENTAL

En el **primer artículo**, "Antioxidant effect of exercise: Exploring the role of the mitocondrial complex I superassembly" (Huertas, JR. 2017) comparamos dos grupos de ratas Wistar (ejercitado y sedentario) para determinar si el efecto antioxidante sistémico del ejercicio está mediado por el ensamblaje del Complejo mitocondrial I (CI) en supercomplejos SCs. Los resultados indican que el ejercicio induce el ensamblaje crónico del CIs en SCs proporcionando protección mitocondrial contra el daño oxidativo y mejorando la homeostasis REDOX.

En el **segundo artículo**, "Hydroxytyrosol influences exercise-induced mitochondrial respiratory complex assembly into supercomplexes in rats" (Casuso, RA. 2019) evaluamos los efectos de dos dosis diferentes de HT (20 y 300 mg/kg/d) en el ensamblaje de los complejos respiratorios mitocondriales inducidos por el ejercicio y la relación de los resultados con el daño oxidativo y OPA1. Nuestros resultados muestran que 20 mg/kg/d de HT ejercieron un estímulo más poderoso que el ejercicio para inducir la formación de supercomplejos (SCs). Sin embargo, 300 mg/kg/d de HT en ratas ejercitadas dificulta el ensamblaje de CI en SC inducido por el ejercicio. Este

efecto puede explicarse por una baja regulación de la expresión relativa de las isoformas de s-OPA1.

En el **tercer artículo**, "Effects of hydroxytyrosol dose on the redox status of exercised rats: the role of hydroxytyrosol in exercise performance" (Al Fazazi, S. 2018) nuestro objetivo fue describir el efecto de una dosis baja y alta de HT en el rendimiento de la carrera y el estado REDOX en ratas sedentarias y ejercitadas. Los resultados sugieren que una dosis alta de HT (300 mg/kg/d) induce un efecto prooxidante sistémico y previene la pérdida de rendimiento observada con la dosis baja (20 mg/kg/d).

SEGUNDO PROYECTO EXPERIMENTAL

En el **estudio I (resultados no publicados)**, "Hydroxytyrosol modifies skeletal muscle GLUT4/AKT/RAC1 axis in trained rats" (Casuso, RA. 2019) evaluamos si el HT modifica los reguladores moleculares de la absorción de glucosa cuando se suplementa durante el ejercicio. Las ratas se incluyeron en 2 grupos: sedentarios y ejercitados (EXE). El grupo EXE se dividió además en dos subgrupos: uno que consumía la dosis mínima de HT que inducía un efecto antioxidante (0,31 mg/kg/d; EXElow), una dosis moderada de HT (4,61 mg/kg/d; EXEmid) y un grupo de control (EXE). Nuestros resultados muestran que una dosis de baja a moderada de HT, cuando se suplementa de forma aislada, podría alterar los efectos beneficiosos del ejercicio con respecto a la señalización de insulina y la absorción de glucosa en el músculo esquelético de ratas.

En el **estudio II (resultados no publicados),** "Physiological Doses of Hydroxytyrosol Modulate Gene Expression in Skeletal Muscle of Exercised Rats" (Casuso, RA. 2019) evaluamos si las dosis fisiológicas de hidroxitirosol (HT) pueden mejorar la transcripción del ARNm de genes metabólicos claves en el músculo esquelético ejercitado. Dos grupos de ratas Wistar ejercitadas, HTlow y HTmid, fueron suplementadas durante 10 semanas con 0,31 mg/kg/d y 4,61 mg/kg/d de HT respectivamente (dosis alcanzables con la ingesta de un nutracéutico basado en el aceite de oliva virgen extra). Otros dos grupos de ratas no se suplementaron: uno sedentario y otro ejercitado. Nuestros resultados muestran que el consumo de 4,61 mg/kg/d de HT durante el ejercicio aumenta la expresión de ARNm de proteínas metabólicas importantes. Además, la ingesta de 4,61 mg/kg/d de HT puede mejorar la oxidación de ácidos grasos de cadena larga, la oxidación de lactato y glucosa, así como el ciclo de Krebs mitocondrial en el músculo esquelético entrenado.

Summary

The benefits of regular exercise practice are widely known and there is considerable evidence that exercise increases the production of free radicals. A small portion of these oxidizing compounds exerts positive effects on immune functions, cellular metabolism and even on muscle contraction and adaptation to systemic exercise. However, the practice of strenuous physical exercise can trigger an imbalance between the production of free radicals and the body's antioxidant defense mechanisms, causing molecular damage to lipids, proteins and DNA. Antioxidant supplementation is a common practice in athletes and sedentary people, but there is controversy regarding the benefits of such supplementation as well as the suitable doses that enhance the benefits of exercise. In our study, we evaluated the effects of different doses of hydroxytyrosol, a phenolic compound of olive oil with antioxidant properties, on performance, mitochondrial function (organization of mitochondrial complexes), oxidative stress and exercise-induced gene and protein expression. Our results are presented in 5 articles published in international journals:

FIRST EXPERIMENTAL PROJECT

In the **first paper** "Antioxidants effect of exercise: exploring the role of the mitochondrial complex I superassembly" we compare two groups of Wistar rats (exercised and sedentary) to determine whether the systemic antioxidant effect of exercise is mediated by the assembly of mitochondrial CIs into supercomplexes SCs. We found that the exercise induces the chronic assembly of CIs into SCs, providing mitochondrial protection against oxidative damage and improving REDOX homeostasis.

In the **second paper** "Hydroxytyrosol influences exercise-induced mitochondrial respiratory complex assembly into supercomplexes in rats" we assessed the effects of two different doses of HT (20 and 300 mg/kg/d) on exercise-induced mitochondrial respiratory complexes assembly into supercomplexes and the relation of the results with oxidative damage and OPA1. Our results show that 20 mg/kg/d of HT exerted a more powerful stimulus than exercise alone to induce SC formation. However, 300 mg/kg/d of HT during exercise hampers exercise-induced CI assembly into SCs. This effect can be explained by a down-regulation of the relative expression of s-OPA1 isoforms.

In the **third paper** "Effects of hydroxytyrosol dose on the REDOX status of exercised rats: the role of hydroxytyrosol in exercise performance" we aimed to describe the

effect of a low and high HT dose on running performance and REDOX state in sedentary and exercised rats. The results suggest that a high HT dose (300 mg/kg/d) induce a systemic pro-oxidant effect and prevent the loss of performance observed with the low dose (20 mg/kg/d).

SECOND EXPERIMENTAL PROJECT

In **study I (unpublished results)**, "Hydroxytyrosol modifies skeletal muscle GLUT4 / AKT / RAC1 axis in trained rats", we evaluated whether HT modifies the molecular regulators of glucose uptake when it is supplemented during exercise. Rats were included in 2 groups: sedentary and exercised (EXE). The EXE was further divided into: a group that consumed the minimum dose of HT that induced an antioxidant effect (0.31 mg/kg/d; EXElow), a moderate dose of HT (4.61 mg/kg/d; EXEmid) and a control group (EXE). Our results showed that a low to moderate dose of HT, when supplemented as an isolate compound, might alter the beneficial effects of exercise regarding insulin signaling and glucose uptake in rat skeletal muscle.

In **study II (unpublished results)**, "Physiological doses of hydroxytyrosol modulate gene expression in skeletal muscle of exercised rats", we evaluated whether the physiological doses of hydroxytyrosol (HT) can enhance the mRNA transcription of key metabolic genes in exercised skeletal muscle. Two groups of exercised Wistar rats, HTlow and HTmid, were supplemented for 10 weeks with 0.31 mg / kg / d and 4.61 mg / kg / d of HT respectively (doses that can be achieved by consuming a nutraceutical based on extra virgin olive oil). Two other groups of rats were not supplemented: one sedentary and the other one exercised. Our results showed that the consumption of 4.61 mg/kg/d of HT during exercise increases the mRNA expression of important metabolic proteins. In addition, the intake of 4.61 mg/kg/d of HT can upregulate the oxidation of long-chain fatty acids, lactate and glucose oxidation, as well as the mitochondrial Krebs cycle in trained skeletal muscle.

Introducción

En los últimos años, la dieta mediterránea (DM) se ha relacionado con una menor incidencia de diversas enfermedades asociadas con el estrés oxidativo como el cáncer, la diabetes, o las enfermedades neurodegenerativas y cardiovasculares, entre otras [1]. Una gran cantidad de estudios epidemiológicos muestran una fuerte evidencia que sugiere el efecto protector de la DM principalmente en el riesgo de enfermedades cardiovasculares y ciertos tipos de cáncer [2-4]. En cuanto a la condición física, se ha demostrado que la DM mejora los niveles de resistencia cardiovascular [5,6]. Estos efectos beneficiosos se han atribuido a diversos factores: el patrón dietético total, los componentes de la comida, las conductas alimentarias y las conductas de estilo de vida [7,8,1]. El consumo de aceite de oliva virgen (AOV) como grasa dietética esencial es un patrón compartido por diferentes países mediterráneos. Varios estudios han asignado al AOV un efecto claramente beneficioso sobre la salud humana atribuido a la DM [9] y se ha prestado especial atención a las propiedades de algunos de sus compuestos provistos de actividad antioxidante: la oleuropeína y el hidroxitirosol [10].

En este contexto, la suplementación con antioxidantes se ha convertido en una práctica habitual para atletas como estrategia de apoyo a los sistemas de defensa endógenos. Sin embargo, los diferentes estudios evidencian una falta de consistencia en los resultados obtenidos. Más de 150 publicaciones defienden que la ingesta de antioxidantes atenúa el estrés oxidativo producido por la práctica de ejercicio, sin mostrar una clara implicación sobre el daño muscular o la mejora de rendimiento [11, 12].

El hidroxitirosol (HT), un compuesto fenólico que se encuentra en el VOO es parcialmente responsable de los efectos relacionados con la salud de la DM [13]. Además de otras propiedades, el hidroxitirosol muestra una poderosa capacidad antioxidante in vivo [14,15] trabajando como un eficiente eliminador de especies reactivas de oxígeno (ROS), mejorando los sistemas antioxidantes endógenos [16], rompiendo las reacciones en cadena peroxidativas y previniendo la peroxidación lipídica, entre otros efectos [14].

Las especies reactivas de oxígeno (ROS) son productos del metabolismo celular. A niveles fisiológicos bajos, las ROS funcionan como un "mensajero redox" intracelular y desempeñan un papel clave en muchos procesos fisiológicos, [17] mientras el exceso de ROS induce una modificación oxidativa celular, inhibe la función de la proteína y estimula la muerte celular. Sabemos que la señalización ROS regula numerosas adaptaciones mitocondriales [18], sin embargo, Zhen (2015) muestra el potencial antioxidante del HT (aumenta las enzimas antioxidantes de fase II y disminuye significativamente la oxidación de proteínas) y su capacidad para estimular las adaptaciones mitocondriales a través de otras vías, como la activación de la proteína

quinasa activada por AMP (AMP-K), la sirtuina-1 y el coactivador PPARγ 1- α, que constituye una proteína sensible a la energía conocida por regular la biogénesis mitocondrial y las respuestas al estrés oxidativo [19,20].

Fosforilación oxidativa (OXPHOS)

La función más importante de las mitocondrias es la producción de adenosín trifosfato (ATP) a través del sistema de fosforilación oxidativa (OXPHOS) y la generación de ROS [21]. Además, las mitocondrias son elementos clave en el metabolismo celular, la conversión de energía y la homeostasis del calcio [22]. La respiración mitocondrial (OXPHOS) se produce en la membrana interna, que se puede dividir en 2 subcompartimentos: el "borde de membrana" y la cresta [23]; que se compone de cuatro complejos respiratorios, portadores de electrones y la H^+ ATP sintasa (conocidos como cadena de transporte de electrones, ETC).

Los complejos respiratorios mitocondriales (CI-CIV) son responsables de la oxidación de NADH o $FADH_2$, que se originan en diferentes vías metabólicas (ciclo de Krebs, glucólisis u oxidación de ácidos grasos). El ATP sintasa (complejo V) utiliza el gradiente de protones producido por la oxidación de NADH y $FADH_2$ para generar ATP. El complejo I o NADH deshidrogenasa es el principal punto de entrada de electrones (NADH) a la cadena respiratoria, el succinato deshidrogenasa o complejo II representa un punto alternativo de entrada de electrones ($FADH_2$) que conecta directamente el ciclo de Krebs con la cadena respiratoria. El componente central del sistema OXPHOS es el dímero funcional citocromo c reductasa, también llamado complejo III. El citocromo C oxidasa o complejo IV representa el complejo terminal de la cadena respiratoria [24,25].

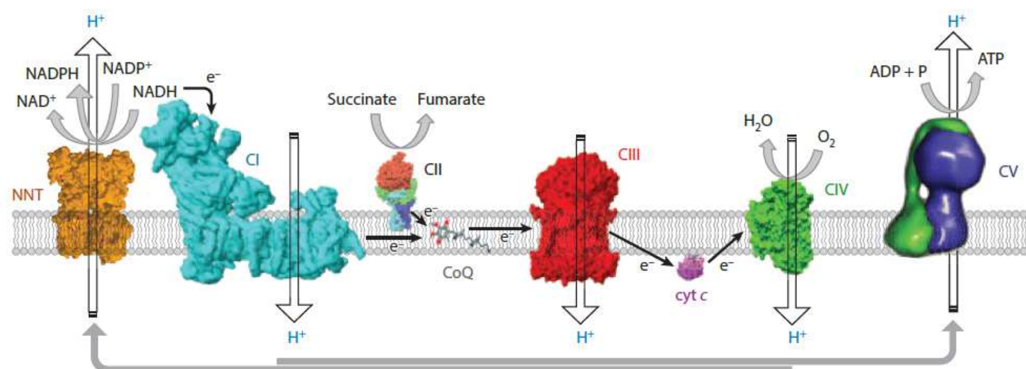

Ilustración 1: Sistema OXPHOS. Enríquez et al. 2016 (Annu. Rev. Physiol)

Las primeras demostraciones experimentales de supercomplejos por BN-PAGE señalaron que existen diferentes tipos de asociación entre los complejos respiratorios I, III y IV [26]. La combinación $I_1III_2IV_1$ se denominó *respirasoma*, siendo la unidad mínima para realizar el proceso de respiración completa de NADH a oxígeno [24].

Se han favorecido dos modelos alternativos de organización de la cadena de transporte de electrones mitocondriales (mETC), el modelo "sólido" [27] y el "de colisión aleatoria/fluido" [28]. Ambos coinciden en el número de complejos respiratorios (I-IV), pero mientras el modelo sólido propone que todos los complejos están estrechamente empaquetados para garantizar la accesibilidad y la alta eficiencia en el transporte de electrones, en el modelo fluido, los complejos se consideran entidades independientes con la CoQ y el citocromo C que actúan como portadores móviles en la membrana lipídica. En 2008, Enríquez y colaboradores desarrollaron la propuesta de Shagger H. (2000) y presentaron el "modelo de plasticidad" que acomoda los modelos sólidos y fluidos al considerar la organización de los complejos respiratorios como una estructura de diferentes asociaciones así como complejos individuales [29]. Por lo tanto, los supercomplejos y los complejos individuales coexisten dentro de la membrana mitocondrial interna; además, la asociación de complejos en supercomplejos (y viceversa) se considera un proceso dinámico que depende del estado fisiológico de la célula [30] y de las condiciones ambientales, tales como el estrés o la alta demanda de energía [31,25]. La evidencia de que la estructura de la mETC es variable y compleja, y que las diferencias en la organización están relacionadas con diferencias en la función, presenta implicaciones importantes para nuestra comprensión de la regulación en este sistema.

Estrés oxidativo

Los radicales libres se generan durante la función normal del metabolismo celular y son parte del proceso fisiológico natural de los seres vivos [32-34]. Las especies reactivas de oxígeno (ROS), las especies reactivas de nitrógeno (RNS) y otras especies reactivas (especies reactivas de cloro, especies reactivas de azufre, especies reactivas de carbono y especies reactivas de selenio) son los términos que describen radicales libres y otros derivados reactivos no radicales [35]. La idea de que las ROS funcionan de una manera únicamente nociva ha dado paso a una visión más matizada de que estas moléculas pueden modular específicamente varias vías biológicamente relevantes [36]. Hay una creciente evidencia sobre la naturaleza dual de las ROS que defiende que bajos niveles de especies reactivas pueden ser funcionales dentro de las vías de señalización. El superóxido (O_2•-) y el peróxido de hidrógeno (H_2O_2) (especies oxidantes) han sido implicados como mensajeros, aunque la mayor estabilidad del

peróxido de hidrógeno le confiere mayor preparación para funcionar como un intermediario de señalización [37].

El estrés oxidativo supone un desequilibrio entre la producción sistémica de radicales libres y la capacidad del sistema para eliminar los intermedios reactivos, lo que lleva a una interrupción de la señalización y control "redox" (daño oxidativo) [38]. La protección endógena contra el estrés oxidativo se produce gracias a algunas moléculas enzimáticas y no enzimáticas fundamentales como: glutatión (GSH), tiorredoxina (TRX), superóxido dismutasa (SOD), catalasa (CAT), entre otras. Estas desempeñan un papel fundamental como sustancias protectoras intracelulares y sirven como eficaces supresores de las ROS [39].

Las mitocondrias son los principales contribuyentes a la producción de oxidante intracelular, generando ATP de forma dependiente del oxígeno, durante el cual, el flujo de electrones por la cadena respiratoria culmina en el complejo IV [37]. Generalmente se cree que los sitios principales de producción de superóxido están dentro de los complejos I y III de la cadena de transporte de electrones [40,41].

Ejercicio y estrés oxidativo

La actividad física se considera hoy en día un elemento indispensable para la buena salud, capaz de reducir el riesgo de trastornos cardiovasculares, endocrinos, osteomusculares y del sistema inmunitario [35]. Se ha demostrado que la actividad contráctil aumenta el contenido intracelular y la actividad de superóxido, peróxido de hidrógeno y NO [42,43]. Los primeros estudios en este tema se basaron en el supuesto de que las mitocondrias eran la principal fuente de ROS durante la actividad contráctil en el músculo, pero varios estudios recientes difieren de esta perspectiva [44,45].

Pearson T. y colaboradores (2014) examinaron la contribución de las fuentes mitocondriales y no mitocondriales al aumento agudo de superóxido observado durante la contracción muscular, y concluyeron que los efectos de la NADPH oxidasa predominaban sobre las mitocondrias durante los cortos períodos de contracción (10-15 min); mostrando que la principal fuente de generación de superóxido durante una actividad contráctil a corto plazo es la NADPH oxidasa no mitocondrial [46]. Estos datos parecen indicar que las fibras musculares pueden tener sistemas generadores de superóxido en el espacio extracelular [47].

Aunque el exceso de ROS puede ser nocivo para las células, causando daño oxidativo a los lípidos, las proteínas y el ADN, estas especies también parecen actuar como mediadores de algunos procesos adaptativos después del estrés celular en condiciones fisiológicas normales. Las ROS regulan las funciones que conducen a cambios en la homeostasis celular y tisular a través de la modificación de la expresión génica [48,49].

En este sentido, la generación de ROS inducida por el ejercicio estimula una mayor actividad de los antioxidantes no enzimáticos, lo que conduce a una resistencia más eficiente a los desafíos oxidativos [35] e interviene en la activación de una serie de vías de señalización reguladas por redox [50]. Sin embargo, numerosos estudios confirman que el ejercicio extenuante aumenta la producción de oxidación excesiva en el músculo, lo que limita el rendimiento [51].

Un estudio reciente de Greggio C. y colaboradores en 2017 informaron que la cantidad de complejos ensamblados aumenta en respuesta al ejercicio, promoviendo la redistribución del CI de SC I+III2 a SC I+III2+IVn. Además, el ejercicio favoreció la redistribución de los C III y C IV a SCs funcionales, como el SC I + III2 + IVn completamente ensamblado, a expensas de SC libres [52]. Estos resultados refuerzan el modelo de "plasticidad", que sugiere que los complejos libres y ensamblados coexisten y se reclutan en respuesta a la demanda de energía.

Efectos del HT sobre el rendimiento

Ahora se sabe que el ejercicio regular y moderado representa una leve fuente de estrés capaz de inducir una respuesta adaptativa. Más importante aún, parece que esta respuesta adaptativa brinda protección contra otros factores estresantes, explicando cómo la práctica de ejercicio regular y moderado juega un papel clave en la prevención de enfermedades crónicas y degenerativas [53]. Esto se conoce con el concepto de hormesis, desarrollado por Radak y colaboradores en 2005, que explican que la adaptación ocurre solo cuando la dosis de estresante (en este caso la práctica de ejercicio) está dentro de un rango específico y seguida por un período de descanso. Cuando hay una ausencia de estrés no puede ocurrir ninguna adaptación. Por otro lado, cuando el ejercicio es demasiado pesado (sobreentrenamiento), también puede producirse daño muscular, estrés oxidativo o inflamación [54].

Varios estudios informaron que las altas dosis de antioxidantes podrían reducir los efectos del ejercicio sobre la biogénesis mitocondrial muscular, la absorción máxima de O_2 y las mejoras en la sensibilidad a la insulina [55,56]. La implicación de tales estudios es que las ROS o RNS juegan un papel clave en la regulación de múltiples adaptaciones inducidas por el entrenamiento al músculo en humanos y animales. Sin embargo, dichos hallazgos no pudieron ser repetidos por otros científicos, quienes observaron adaptaciones normales, incluso de mejoría al entrenamiento físico, a pesar de la administración de altas dosis de antioxidantes [57]. Existen algunas diferencias en el diseño experimental, que pueden explicar la variabilidad en los resultados obtenidos: las duraciones y los protocolos para el entrenamiento, la elección de marcadores de estrés oxidativo, el uso de marcadores musculares versus sanguíneos,

son algunos de ellos. Paulsen G. y colaboradores (2014) ilustraron la complejidad de relacionar los procesos de señalización con la verdadera función fisiológica [58].

Estudios anteriores sugieren que la ingesta de HT mejora la función mitocondrial, clave en la producción de energía durante el ejercicio [59]. Este efecto también se describió en otros polifenoles [60-62], sin embargo, no hay evidencia suficiente de sus efectos ergogénicos. Por otro lado, se ha informado que la suplementación con polifenoles obstaculiza las adaptaciones del músculo esquelético inducidas por el ejercicio [63,64]. Una posible explicación es que las adaptaciones al ejercicio están precedidas por explosiones transitorias de producción de ROS dentro del músculo. Por lo tanto, reducir la producción aguda de ROS dentro de cada sesión de entrenamiento con antioxidantes puede prevenir algunas adaptaciones inducidas por el ejercicio a largo plazo.

Para contextualizar la diversidad de resultados obtenidos en relación a la ingesta de HT, y profundizar en resultados anteriores de nuestro grupo de investigación, donde se demuestra que atletas de élite producen menos marcadores de peroxidación lipídica que personas sedentarias [65]; hemos evaluado los efectos fisiológicos de una práctica regular de ejercicio, así como la ingesta de diferentes dosis de HT sobre ratas entrenadas y sedentarias. Para ello, hemos dividido nuestro proyecto en dos estudios experimentales: en el primero se administraron dosis muy altas de este componente (20 y 300 mg/kg/d, cerca de la toxicidad), mientras que en el segundo se administraron dosis fisiológicas cercanas a la ingesta poblacional media (0,36 y 4,61; dosis que pueden alcanzarse con una suplementación con aceite de oliva virgen extra EVOO).

Introduction

Over the past years, the Mediterranean diet (MD) has been linked with a lower incidence of various diseases associated with oxidative stress such as cancer, diabetes, or neurodegenerative and cardiovascular diseases, among others [1]. A large number of epidemiological studies show a strong evidence suggesting the protective effect of MD mainly on the risk of cardiovascular diseases and certain types of cancer [2-4]. According to the physical condition variables, it has been shown that MD improves levels of cardiovascular endurance [5], and other quality levels of live markers [6]. These beneficial effects have been attributed to various factors: total dietary pattern, components in the food, eating behaviors and lifestyle behaviors [7,8,1]. The consumption of virgin olive oil (VOO) as the essential dietary fat is a shared pattern by different Mediterranean countries. Several studies have assigned the VOO the beneficial effect on human health attributed to the MD [9] and paying special attention to the properties of some of its compounds provided with antioxidant activity, namely oleuropein and hydroxytyrosol [10].

In this context, antioxidant supplementation has become a common practice for athletes as a support strategy for endogenous defense systems, however, the different studies show a lack of consistency in the results obtained. More than 150 publications argue that antioxidant intake attenuates the oxidative stress produced by the practice of exercise, without showing a clear implication on muscle damage or performance improvement [11, 12].

Hydroxytyrosol (HT), a polyphenolic compound found in virgin olive oil, is partially responsible for the health-related effects of the MD [13]. In addition to other properties, hydroxytyrosol shows a powerful in vivo antioxidant capacity [14,15], working as an efficient reactive oxygen species (ROS) scavenger, enhancing the endogenous antioxidant systems [16], breaking peroxidative chain reactions and preventing lipid peroxidation, among other effects [14].

Reactive oxygen species (ROS) are products of a normal metabolism. At physiological low levels, ROS functions as an intracellular redox "messenger" and plays a key role in many physiological and pathogenic processes [17], whereas an excess of ROS induce cellular oxidative modification, inhibit protein function, and stimulates cell death. We know that ROS signaling regulates numerous mitochondrial adaptations [18]. However Zhen (2015) shows the antioxidant potential of HT (increases phase II antioxidant enzymes and significantly decreases protein oxidation) and its ability to stimulate mitochondrial adaptations through the activation of AMP-activated protein kinase (AMP-K), sirtuin-1 and PPARγ coactivator 1-α, which constitute an energy-sensing protein known to regulate mitochondrial biogenesis and oxidative stress responses [19,20].

Oxidative phosphorylation (OXPHOS)

The most important function of mitochondria is the production of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) system and generation of ROS [21]. Furthermore, mitochondria are key elements in intermediate cellular metabolism, energy conversion and calcium homeostasis [22]. The mitochondrial respiration (OXPHOS) occurs in the inner membrane, that can be divided into 2 subcompartments: the "border membrane" and cristae [23]; which is composed of four respiratory complexes, electron carriers and the H^+ ATP synthase (known as electron transport chain, ETC).

Mitochondrial respiratory complexes (CI-CIV) are responsible for the oxidation of NADH or $FADH_2$, originating in different metabolic pathways (Krebs cycle, glycolysis or fatty acid oxidation). The proton gradient produced by the oxidation of NADH and $FADH_2$ is used by ATPase (complex V) to generate ATP. Complex I or NADH dehydrogenase is the main entrance point of electrons (NADH) to the respiratory chain. Succinate dehydrogenase or complex II represents an alternative point of electrons entrance ($FADH_2$) which directly connects the Krebs cycle to the respiratory chain. The central component of the OXPHOS system is the functional dimer cytochrome C reductase, also called complex III. Cytochrome C oxidase or complex IV represents the terminal complex of the respiratory chain. [24,25].

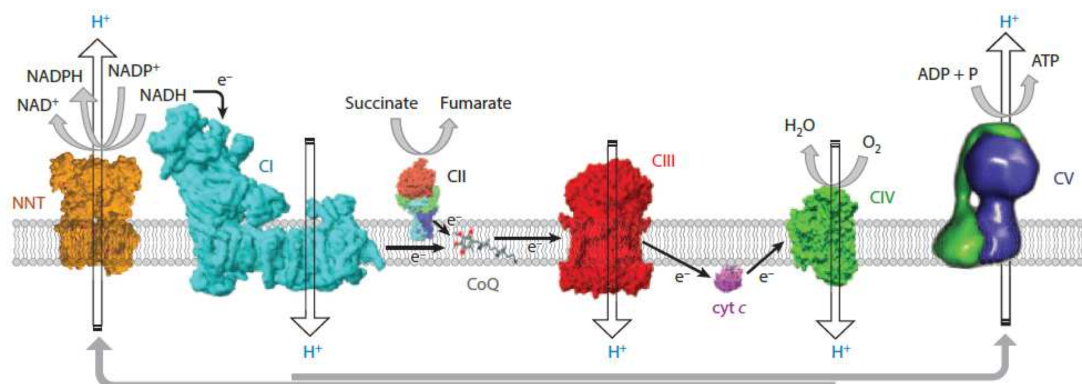

Figure 1: OXPHOS system. Enríquez et al. 2016 (Annu. Rev. Physiol)

The first experimental demonstrations of supercomplexes by BN-PAGE pointed out that different types of association are present between respiratory complexes I, III and IV [26]. The combination $I_1III_2IV_1$ was denominated *respirasome*, being the minimal unit to perform complete respiration from NADH to oxygen [24].

Two alternative organization models of the mitochondrial electron transport chain (mETC) have been favored, the solid [27] or fluid/random collision model [28]. Both agree in the number of respiratory complexes (I-IV), but while the solid model proposed that all complexes were closely packed to guarantee accessibility and high efficiency in electron transport, in the fluid model, complexes are viewed as independent entities with CoQ and cytochrome C acting as mobile carriers freely diffused in the lipid membrane. In 2008, Enríquez *et al.* developed the proposal of Shagger H. 2000 and presented the "plasticity model" which accommodate the solid and fluid models by regarding the organization of the respiratory complexes as a structure of different associations as well as individual complexes [29]. Therefore, OXPHOS supercomplexes and single OXPHOS complexes co-exist within inner mitochondrial membrane; furthermore, association of complexes into supercomplexes (and vice versa) is considered a dynamic process, which depends on the physiological state of cell [30] and environmental conditions, such as stress or high energy demand [31,25]. The demonstration that the structure of mETC is variable and complex, and that differences in the organization are related to differences in function that have important implications for our understanding of the regulation of this system.

Oxidative stress

Free radicals are generated during normal cellular metabolism function and are part of the natural physiological process of living beings [32-34]. Reactive oxygen species (ROS), reactive nitrogen species (RNS) and other reactive species (reactive chlorine species, reactive sulfur species, reactive carbonyl species and reactive selenium species) are the terms describing free radicals and other non-radical reactive derivatives [35]. The idea that ROS function purely in a harmful and direct manner has given way to a more nuanced view that these molecules can specifically modulate various biologically relevant pathways [36]. There is a growing evidence about the dual nature of ROS, showing that low levels of reactive species can be functional within signaling pathways. Superoxide ($O_2\bullet-$) and hydrogen peroxide (H_2O_2) (oxidant species) have been implicated as messengers, although the greater stability of hydrogen peroxide seems to make it better equipped to function as a signaling intermediate [37].

Oxidative stress is an imbalance between the systemic production of free radicals and system´s ability to scavenge the reactive intermediates, leading to a disruption of redox signaling and control and/or molecular damage (oxidative damage) [38]. The endogenous protection from oxidative stress occurs thanks to some pivotal enzymatic and non-enzymatic molecules as: glutathione (GSH), thioredoxin (TRX), superoxide

dismutase (SOD), catalase (CAT), among others. They play a role as an intracellular protective substances and serves as an effective ROS scavengers [39].

Mitochondria are the largest contributors to intracellular oxidant production, generating ATP in an oxygen-dependent manner, during which the flow of electrons down the respiratory chain eventually culminates at complex IV [37]. The major sites of superoxide production are generally believed to be within complex I and complex III of the electron transport chain [40,41].

Exercise and oxidative stress

Physical activity is nowadays considered as an indispensable element for good health, able to lower the risk of cardiovascular, endocrine, osteomuscular, and immune system disorders [35]. Contractile activity has been shown to increase the intracellular content and activity of superoxide, hydrogen peroxide, and NO [42,43]. The first studies in this subject were based on the assumption that mitochondria were the main source of the ROS generated during contractile activity in muscle, but several recent studies disagree with this perspective [44,45].

Pearson T. *et al.* (2014) examined the contribution of mitochondrial and non-mitochondrial sources to the acute increase in superoxide seen during muscle contraction, and concluded that NADPH oxidase effects predominated over mitochondria during the short contraction periods (10-15 min); showing that the major source of generation of superoxide during a short term contractile activity is the non-mitochondrial NADPH oxidase [46]. These data appear to indicate that muscle fibers may have been generating systems for superoxide in the extracellular space [47].

Although excess ROS can be deleterious to cells, causing oxidative damage to lipids, DNA and proteins, these species also appear to act as mediators of some adaptive processes following cellular stresses under normal physiological conditions. ROS mediate regulatory functions that lead to changes in cell and tissue homeostasis through modification of gene expression [48,49]. In that sense, exercise-induced ROS generation results in increased activity of non-enzymatic antioxidants, leading to a more efficient resistance to oxidative challenges [35] and mediating the activation of a number of redox-regulated signaling pathways [50]. Nevertheless, numerous studies confirm that strenuous exercise increases excessively oxidant production in muscle, limiting performance [51].

Recent study by Greggio *et al.*(2017) reported that the amount of superassembled complexes increases in response to exercise, promoting the redistribution of CI from SC I+III2 to SC I+III2+IVn. Furthermore, exercise preferentially favored the re-

distribution of C III and C IV to functional SC species, such as the fully assembled SC I+III2+IVn, at the expenses of free or other SCs, such as SC I+III2 [52]. These results reinforce the "plasticity" model, which suggests that free and superassembled complexes co-exist and are recruited in response to energy demand.

HT effects on performance

It is now well known that regular and moderate exercise represents a mild source of stress able to induce an adaptive response. More importantly it seems that this adaptive response provides protection against other stressors, explaining how practicing regular and moderate exercise plays a key role in the prevention of chronic and degenerative diseases [53]. This adaptive response is known with the concept of hormesis, developed by Radak *et al.* (2005) explaining that adaptation occurs only when the stressor dose (exercise bout) is within a specific range and followed by rest period. When the stressor is absent no adaptation can occur [54], on the other hand, when exercise is too heavy (overtraining), pathological conditions as muscle damage, oxidative stress, and inflammation can occur as well.

Several studies reported that high doses of antioxidants could reduce the training effects of exercise on muscle mitochondrial biogenesis, maximal O_2 uptake, and improvements in insulin sensitivity [55,56]. The implication of such studies is that ROS or RNS play a key role in regulating multiple training-induced adaptations to muscle in humans and animals. Nevertheless, such findings could not be repeated by other scientists, who reported normal, even improvement adaptations to exercise training, despite administration of high-dose antioxidants [57]. There are some differences in experimental design, which can explain the variability in the results obtained; the durations and protocols for the training, the choice of markers of oxidative stress, the use of muscle vs. blood markers, are some of them. Paulsen *et al.* (2014) illustrated the complexity of relating signaling processes to the physiological function [58].

Previous studies suggest that HT intake improves mitochondrial function, a master key in energy production during exercise [59]. This effect was also described in other polyphenols [60-62]. However, there is insufficient evidence of its ergogenic effects. On the other hand, it has been reported that supplementation with polyphenols hinders skeletal muscle adaptations induced by exercise [63,64]. A plausible explanation is that adaptations to exercise are preceded by transient bursts of ROS production within the muscle. Therefore, reducing the acute production of ROS within each training session with antioxidants can prevent some long-term exercise-induced adaptations.

To contextualize the diversity of results obtained, and to deepen within the previous results of our research group, where they showed that elite athletes produce fewer markers of lipid peroxidation than sedentary people [65]; we have evaluated the physiological effects of a regular exercise practice, as well as the intake of different doses of HT on trained and sedentary rats. We have divided our project into two experimental studies; administering in the first group very high doses of this compound (20 and 300 mg/kg/d, close to toxicity), and using physiological doses, close to the average population intake (0.36 and 4.61 mg/kg/d, doses that can be achieved with supplementation with EVOO), in the second.

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Objetivos

Objetivo general:

El objetivo general de esta tesis doctoral es analizar las diferentes adaptaciones biológicas inducidas por 10 semanas de entrenamiento en el músculo esquelético de ratas, así como la modulación que ejerce la ingesta de diferentes dosis de hidroxitirosol (HT) en dichas adaptaciones.

Objetivos específicos:

1. Comprobar si la formación de supercomplejos mitocondriales en el músculo esquelético de ratas entrenadas durante un largo periodo (10 semanas) está asociado a un menor estrés oxidativo muscular (artículo I).
2. Comprobar si distintas dosis de HT interfieren en el ensamblaje de supercomplejos mitocondriales inducido por un protocolo de entrenamiento de 10 semanas (artículo II).
3. Evaluar la participación de OPA1 en la formación de supercomplejos mitocondriales y la posible modulación por HT a distintas dosis (20 y 300 mg/kg/d), en ratas sedentarias y entrenadas (artículo II).
4. Describir los efectos de la suplementación con dos dosis diferentes de HT (20 y 300 mg/kg/d), en la capacidad física y el estado redox de ratas sedentarias y entrenadas (artículo III).
5. Evaluar los efectos de dosis fisiológicas de HT (0,31 y 4,61 mg/kg/d) en la expresión ARNm de proteínas claves inducidas por el ejercicio y relacionadas con la mejora de enfermedades metabólicas no hereditarias (estudios I y II).

Objectives

Overall objective:

The overall objective of this doctoral thesis is to analyze the different biological adaptations induced by 10 weeks of training in the skeletal muscle of rats, and the modulation produced by the intake of different doses of hydroxytyrosol (HT) in those adaptations.

Specific objectives:

1. To assess whether the formation of mitochondrial supercomplexes in skeletal muscle of trained rats over a long period (10 weeks) is associated with a reduction in muscular oxidative stress (paper I).
2. To assess whether different doses of HT interfere with the assembly of mitochondrial supercomplexes induced by a 10-week training protocol (paper II).
3. To assess the participation of OPA1 in the mitochondrial supercomplexes formation and the possible modulation by HT at different doses (20 and 300 mg/Kg/d) in sedentary and trained rats (paper II).
4. Describe the effects of supplementation with two different doses of hydroxytyrosol (20 and 300 mg/kg/d), on the physical capacity and redox status of trained and sedentary rats (paper III).
5. To assess the effects of physiological doses of hydroxytyrosol (0.31 and 4.61 mg/kg/d) on the expression mRNA of key proteins induced by exercise and related to metabolic diseases (studies I and II).

Material y metodología

La metodología y el diseño de esta tesis se describen en el presente apartado. Los análisis realizados en cada uno de los tejidos se desarrollan en los diferentes artículos publicados.

1. Animales

Todos los experimentos se realizaron de acuerdo con un protocolo aprobado por el Comité Institucional de Cuidado y Uso de Animales de la Universidad de Granada (procedimientos Granada, España. N°: 28/06/2016/116) y de conformidad con el Convenio Europeo para la Protección de Animales vertebrados utilizados con fines experimentales y otros fines científicos (CETS # 123), directiva 2010/63 / UE sobre la protección de animales utilizados con fines científicos y la ley española (RD 53/2013). Se compraron ratas Wistar macho de Charles River (EE. UU.) con seis semanas de edad. Las ratas inicialmente pesaron 212 ± 13,5 g y se mantuvieron en una habitación bien ventilada. Esta sala se conservó en condiciones estándar de temperatura (21 ± 2 °C) y con una humedad relativa entre el 40 % y el 60 %, y bajo un ciclo inverso de 12 h de luz y 12 h de oscuridad. Durante el período experimental, todas las ratas consumieron agua y comida estándar ad libitum (2,9 kcal/g). Se controlaron las ingestas diarias de alimentos y agua. Todas las intervenciones duraron 10 semanas. Las ratas se pesaron semanalmente. 72 horas después de realizar el último ejercicio, las ratas fueron ayunadas durante la noche, anestesiadas con pentobarbital y sacrificadas por sangrado.

En nuestro primer estudio, los grupos experimentales asignados de forma aleatoria fueron:

Ratas sedentarias: se asignaron 24 ratas a 3 grupos: sedentario (SED; n = 10), SED que consume 20 mg/kg/d de HT (SED20; n = 7) y SED que consume 300 mg/kg/d HT (SED300; n = 7).

Ratas ejercitadas: se asignaron 30 ratas a 3 grupos: ejercitadas (EXE; n = 10), EXE consumiendo 20 mg/kg/d de HT (EXE20; n = 10), y EXE consumiendo 300 mg/kg/d de HT (EXE300; n = 10).

En nuestro segundo estudio, los grupos experimentales asignados de forma aleatoria fueron:

Ratas sedentarias: un único grupo sedentario, (SED; n = 6)

Ratas ejercitadas: 18 ratas se asignaron a 3 grupos: ejercitadas (EXE; n = 6), EXE consumiendo 0,31 mg/kg/d de HT (HTlow; n = 6), y EXE consumiendo 4,61 mg/kg/d de HT (HTmid; n = 6).

Todas las ratas realizaron una prueba de rendimiento de velocidad máxima. Los grupos ejercitados realizaron 3 tests; antes del comienzo del estudio (prueba 1), después de 5 semanas (prueba 2) y después de 10 semanas de tratamiento (prueba 3). Los grupos sedentarios realizaron los tests 1 y 3. La prueba de velocidad máxima fue una prueba de carrera de intensidad progresiva (cinta de correr PanLab para cinco ratas, modelo LE 8710R) comenzando a una velocidad de 22 cm/s y aumentando en 5 cm/s cada minuto. (Huertas, J.R. *Redox Biology*, 2017).

2. Tratamiento con hidroxitirosol

En nuestro primer estudio, las ratas que recibieron HT (Biomaslinic, S.L., Granada, España) fueron suplementadas con una dosis baja (20 mg/kg/d) o una dosis alta (300 mg/kg/d) de HT. La suplementación comenzó una vez que las ratas se asignaron a los grupos experimentales y se detuvieron 12 h antes de que las ratas fueran sacrificadas. El HT se diluyó en agua en una botella opaca y se reemplazó todos los días para evitar su oxidación. La dilución se ajustó semanalmente de acuerdo con el peso de cada rata y su consumo promedio de agua.

En nuestro segundo proyecto, mantuvimos el mismo protocolo de entrenamiento, pero modificamos la ingesta de HT. Calculamos la dosis baja de HT como 2,5 veces la cantidad diaria consumida por la población española (es decir: 0,31 mg/kg/d: grupo HTlow). También probamos una dosis de HT que se puede lograr mediante el enriquecimiento nutracéutico de AOVE. Esta dosis de nivel medio se calculó en 35 veces la cantidad diaria consumida por la población española (es decir: 4,61 mg/kg/d: grupo HTmid). Hay que tener en cuenta que son dosis más bajas que las habitualmente probadas in vivo. Las ratas se asignaron aleatoriamente a un grupo sedentario (SED, n=6) o uno de los grupos ejercitados durante 10 semanas. Los grupos ejercitados fueron tres: EXE (n=6), HTlow (n=6) y HTmid (n=6).

3. Protocolo de ejercicio

El programa de ejercicios se dividió en dos mesociclos similares de 5 semanas; las ratas corrían 5 días a la semana y descansaban los fines de semana. Durante todo el protocolo, los animales corrieron al 75 % de su velocidad máxima calculada a partir de la prueba de velocidad máxima realizada al comienzo de cada mesociclo. Cada mesociclo aumentó progresivamente el volumen de carrera, las ratas comenzaron a correr durante 20 minutos, y este período se incrementó en 5 minutos cada dos días. Cuando se alcanzaron los 65 minutos, esta duración se mantuvo hasta el final del mesociclo. Los animales del grupo sedentario fueron atendidos diariamente y corrieron durante 10 minutos cada dos semanas para mantener su aclimatación a la cinta.

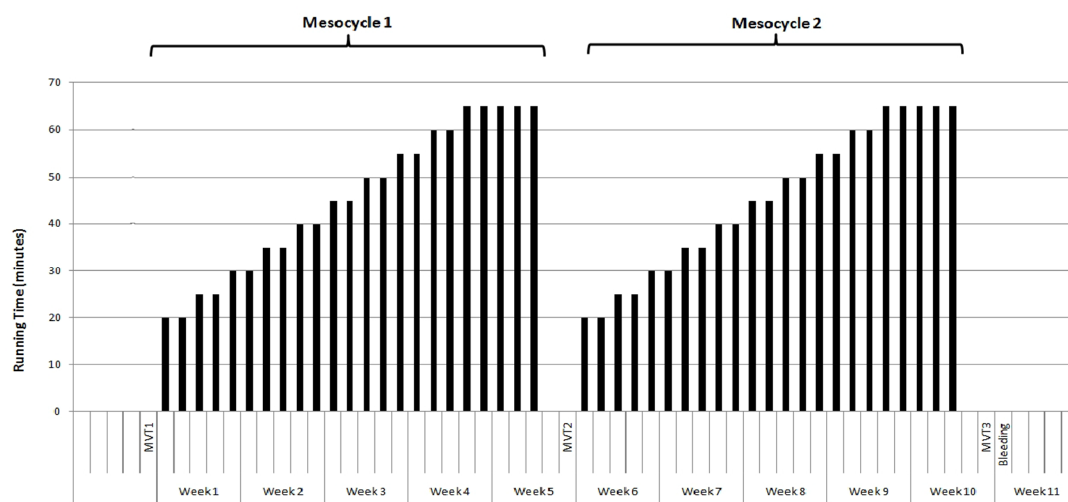

Imagen 2: organización sistemática del entrenamiento

Realizamos una prueba de carrera de intensidad progresiva (cinta de correr PanLab para 5 ratas, modelo LE 8710R) que comienza a una velocidad de 22 cm/s y aumenta en 5 cm/s cada minuto (se explica en el siguiente apartado).

Calculamos el trabajo físico realizado aplicando la fórmula: trabajo (J) = fuerza × distancia vertical, donde la fuerza = peso corporal (kg) × 9,8 m/s^2 y la distancia vertical = velocidad (m/min) × tiempo (min). Con ello, las ratas fatigadas antes de finalizar el tiempo determinado se extraen de la cinta y registramos su tiempo.

4. Test de velocidad máxima

Las ratas asignadas a grupos ejercitados realizaron tres pruebas de velocidad máxima (MVT). Una prueba de carrera de intensidad progresiva (cinta de correr PanLab para 5 ratas, modelo LE 8710R) que comienza a una velocidad de 22 cm/s y aumenta en 5 cm/s cada minuto. La prueba 1 fue para establecer su velocidad durante el primer mesociclo; la prueba 2 para ajustar la velocidad para el segundo mesociclo; y la prueba 3 se realizó 72 h después de la última sesión de entrenamiento para evaluar la capacidad de velocidad máxima. Las ratas sedentarias realizaron las pruebas 1 y 3. Después de la prueba 1, las ratas se agruparon de manera aleatoria y durante todo el protocolo, la fatiga se definió como el punto en el que las ratas permanecieron en la parte posterior de la cinta de correr en una almohadilla de choque eléctrico durante 5 s.

5. Evaluación de la formación de supercomplejos

La electroforesis (BNGE) se realizó en fracciones mitocondriales crudas de los músculos gastrocnemios de ratas. el aislamiento mitocondrial se realizó tal y como se describió anteriormente por Luna-Sánchez, M. *et al*. en 2017.

6. Obtención de tejidos

72 horas después de realizarse la última prueba de velocidad máxima, las ratas ayunaron durante la noche, se anestesiaron con pentobarbital y se sacrificaron por desangrado. Gastrocnemios y Sóleo se recogieron inmediatamente, se enjuagaron en solución salina, se congelaron en nitrógeno líquido y se almacenaron a -80 °C hasta su posterior análisis.

7. Mediciones de sangre

La sangre obtenida durante el sangrado se recogió en tubos de heparina para la medición de hemoglobina (HGB) y hematocrito (HTC) mediante un analizador de hematología automatizado KX-21 (Sysmex Corporation, Kobe, (Japón)). La sangre restante se centrifugó durante 10 min a 3000 rpm para aislar el plasma. Luego, se usaron 40 µl de plasma para la cuantificación de la concentración de HPx en plasma.

Material and methodology

The methodology and design of this thesis is described in this section. The analysis performed on each of the tissues are described in the different published articles.

1. Animals

All experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Granada (procedures Granada, Spain. N°: 28/06/2016/116) and in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (CETS # 123), directive 2010/63/EU on the protection of animals used for scientific purposes and the Spanish law (R.D. 53/2013). Male Wistar rats were purchased from Charles River (USA) at 6 weeks-old. The rats initially weighed 212 ± 13.5 g and were maintained in a well-ventilated room. This room was preserved under standard conditions of temperature (21 ± 2 °C) and relative humidity (40 % to 60 %) and under a reverse 12-hour light/dark cycle. Throughout the experimental period, all rats consumed water and standard chow ad libitum (2.9 kcal/g). Daily food and water intakes were monitored. All interventions lasted for 10 weeks. Rats were weighed weekly. 72 hours after performing the last exercise, rats were fasted overnight, anesthetized with pentobarbital and sacrificed by exsanguination.

In our first study, the randomized experimental groups were:

Sedentary rats: 24 rats were allocated into 3 groups: sedentary (SED; n = 10), SED consuming 20 mg/kg/d HT (SED20; n = 7), and SED consuming 300 mg/kg/d HT (SED300; n = 7).

Exercised rats: 30 rats were allocated into 3 groups: exercised (EXE; n = 10), EXE consuming 20 mg/kg/d HT (EXE20; n = 10), and EXE consuming 300 mg/kg/d HT (EXE300; n = 10).

In our second study, the randomized experimental groups were:

Sedentary rats: A single sedentary group (SED, n = 6)

Rats exercised: 18 rats were assigned to 3 groups: exercised (EXE; n = 6), EXE consuming 0.31 mg/kg/d of HT (HTlow; n = 6), and EXE consuming 4.61 mg/kg/d of HT (HTmid; n = 6).

All the rats performed a maximal velocity performance test. Exercised groups performed 3 tests: prior to the beginning of the study (test 1), after 5 weeks (test 2) and after 10 weeks of treatment (test 3). Sedentary groups performed tests 1 and 3.

The maximal velocity test was a progressive intensity running test (PanLab treadmill for 5 rats, model LE 8710R) starting at a velocity of 22 cm/s and increasing by 5 cm/s every minute. (Huertas, J.R. *Redox Biology* 2017).

2. Hydroxytyrosol treatment

In our first study, rats receiving HT (Biomaslinic, S.L., Granada, Spain) were supplemented with a low dosage (20 mg/kg/d) or a high dosage (300 mg/kg/d) of HT. Supplementation began once the rats were allocated into the experimental groups and stopped 12 hours before rats were euthanized. HT was diluted in water in an opaque drinking bottle, and was replaced every day in order to prevent HT oxidation. The dilution was adjusted weekly according to the weight of each rat and its average water intake.

In our second project, we have maintained the same training protocol but modified the intake of HT. We calculated the low HT dose as 2.5 times the daily amount consumed by the Spanish population (i.e. 0.31 mg/kg/d: HTlow group). We also tested a HT dose that can be achieved by nutraceutical enrichment of EVOO, this mid-level dose was calculated as 35 times the daily amount consumed by the Spanish population (i.e. 4.61 mg/kg/d: HTmid group). Note that these are lower doses than usually tested in vivo. Rats were randomly allocated into a sedentary group (SED, n=6) or one of the exercised groups for 10 weeks. The exercised groups were three: EXE (n=6), HTlow (n=6) and HTmid (n=6).

3. Exercise procedure

The exercise program was divided into two similar 5 week mesocycles; the rats ran 5 days per week and rested during the weekends. During the entire protocol, the animals ran at 75 % of their maximal velocity as calculated from the maximal velocity test performed at the beginning of each mesocycle. Each mesocycle progressively increased the running volume; the rats began running for 20 min, and this period was increased by 5 min every 2 days, and when 65 min was achieved, this duration was maintained until the end of the mesocycle. The animals of the sedentary group were daily handled and run for 10 min every 2 weeks to maintain their acclimation to the treadmill.

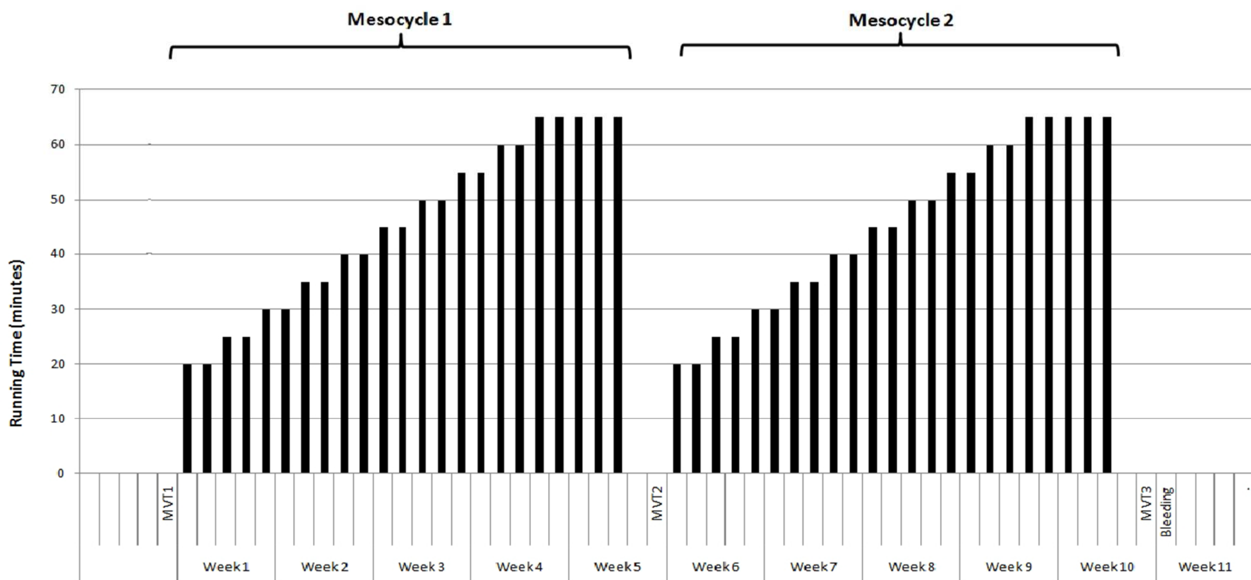

Figure 2: Schematic organization of the training protocol

We performed a progressive intensity race test (PanLab treadmill for 5 rats, model LE 8710R) that started at a speed of 22 cm/s and increases by 5 cm/s every minute (explained in the next section).

We calculated the physical work performed by applying the formula: work (J) = force × vertical distance, where force = body weight (kg) × 9.8 m/s², and vertical distance = speed (m/min) × time (min). Therefore, rats becoming fatigued before finishing the target time of a certain training session were removed from the treadmill and the time was recorded.

4. Maximal velocity test (MVT)

Rats allocated into exercised groups performed 3 maximal velocity tests (MVTs). A progressive intensity running test (PanLab treadmill for 5 rats, model LE 8710R) starting at a velocity of 22 cm/s and increasing by 5 cm/s every minute. Test 1 was to establish their velocity during the first mesocycle; test 2 to adjust the velocity for the second mesocycle; and test 3 was performed 72 h after the last training session to evaluate maximal velocity capacity. Sedentary rats performed tests 1 and 3. After test 1, rats were grouped randomly and through the entire protocol, fatigue was defined as the point at which rats remained at the back of the treadmill on an electric shock pad for 5 s.

5. Evaluation of supercomplex formation

Blue native gel electrophoresis (BNGE) was performed on crude mitochondrial fractions from the gastrocnemius muscles of rats. Mitochondrial isolation was performed as previously described (Luna-Sánchez, M. *et al.* 2017).

6. Tissue collection

72 hours after the last maximal velocity test was performed, rats were fasted overnight, anesthetized with pentobarbital and sacrificed by exsanguination. Gastrocnemius and Soleus were immediately collected, rinsed in saline solution, frozen in liquid nitrogen, and stored at -80 °C until their further analysis.

7. Blood measurements

A portion of the blood obtained during the exsanguination procedure was collected into heparin tubes for the measurement of hemoglobin (HGB) and hematocrit (HCT) using a KX-21 Automated Hematology Analyzer (Sysmex Corporation, Kobe (Japan)). The remaining blood was centrifuged for 10 min at 3000 rpm in order to isolate plasma. Then, 40 µl of plasma was used for the quantification of HPx concentration in plasma.

Resultados y discusión

La sección de resultados y discusión se presenta en los artículos originalmente publicados.

Results and discussion

The results and discussion section are presented in the originally published articles.

Artículos (publicaciones originales) I-III

Artículo I

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Short Communication

Antioxidant effect of exercise: Exploring the role of the mitochondrial complex I superassembly

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ARTICLE INFO

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Complex I
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ABSTRACT

Mitochondrial respiratory complexes become assembled into supercomplexes (SC) under physiological conditions. One of the functional roles of these entities is the limitation of reactive oxygen species (ROS) produced by complex I (CI) of the respiratory chain. We sought to determine whether the systemic antioxidant effect of exercise is mediated by the assembly of mitochondrial CIs into SCs in rats. Male Wistar rats were exercise trained or remained sedentary for ten weeks; then, blood samples were collected, and the gastrocnemius muscle was isolated. The assembly of mitochondrial SCs and the lipid peroxidation of the mitochondrial and plasmatic fractions were assessed. Our results demonstrate that exercise induced the assembly of CI into SCs in the gastrocnemius and induced a systemic decrease in lipid peroxidation. We also found an inverse association between the superassembly of CIs and mitochondrial lipid peroxidation ($p < 0.01$) and protein carbonyls ($p < 0.05$). We conclude that exercise induces the chronic assembly of CIs into SCs, which provide mitochondrial protection against oxidative damage, at least in the studied muscle. Given the relevant role that mitochondria play in health and disease, these findings should help to elucidate the role of exercise as a therapeutic approach for metabolic diseases.

Antioxidant effect of exercise: Exploring the role of the mitochondrial complex I super assembly

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A R T I C L E I N F O

Keywords: Exercise Supercomplexes Complex I Reactive oxygen species Antioxidants

A B S T R A C T

Mitochondrial respiratory complexes become assembled into supercomplexes (SC) under physiological conditions. One of the functional roles of these entities is the limitation of reactive oxygen species (ROS) produced by complex I (CI) of the respiratory chain. We sought to determine whether the systemic antioxidant effect of exercise is mediated by the assembly of mitochondrial CIs into SCs in rats. Male Wistar rats were exercise trained or remained sedentary for ten weeks; then, blood samples were collected, and the gastrocnemius muscle was isolated. The assembly of mitochondrial SCs and the lipid peroxidation of the mitochondrial and plasmatic fractions were assessed. Our results demonstrate that exercise induced the assembly of CI into SCs in the gastrocnemius and induced a systemic decrease in lipid peroxidation. We also found an inverse association between the super assembly of CIs and mitochondrial lipid peroxidation ($p < 0.01$) and protein carbonyls ($p < 0.05$). We conclude that exercise induces the chronic assembly of CIs into SCs, which provide mitochondrial protection against oxidative damage, at least in the studied muscle. Given the relevant role that mitochondria play in health and disease, these findings should help to elucidate the role of exercise as a therapeutic approach for metabolic diseases.

1. Introduction

Mitochondria are critical organelles that are involved in many aspects of cell activity. The best characterized function of mitochondria is ATP production through the oxidative phosphorylation system and the generation of reactive oxygen species (ROS), mainly by complexes I (CI) and III (CIII) [7,12]. Respiratory complexes have received increased attention because they can undergo superassemblies into super-complexes (SC) [22]. Rather than mere structural entities, these SCs also have functional roles in the respiratory chain [6], such as the prevention of excessive ROS production; this prevention is conducted by CIs when they are assembled into SCs [20].

Exercise is one of the best-known stimuli for mitochondrial function in skeletal muscle [11]. Accordingly, a recent study reported that exercise induces chronic SC assembly, which is related to mitochondrial respiration and whole-body oxygen consumption [10]. However, an association between SC assembly and mitochondrial redox homeostasis induced by exercise has not yet been tested.

Recent findings from our research group demonstrate that lifelong endurance exercise training induces a systemic decrease in lipid per- oxidation [2], as does moderate hypoxic training [4]. Frequently, the antioxidant effect of exercise has been explained by an increased con- tent and activity of antioxidant enzymes [3,8]; however, this is not the case under our experimental conditions [2,4]. Because ex vivo studies demonstrate that CI is the main source of mitochondrial ROS production under aerobic exercise conditions [9], our hypothesis states that the CI assembly into an SC may have a primary role in the antioxidant effects of endurance exercise.

We aimed to identify SC formation within the skeletal muscle of chronically exercised rats and evaluate the association between the formation of SC and the oxidative status of the muscle.

2. Materials and methods

2.1. ANIMALS

All experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Granada (procedures Granada, Spain. N°: 28/06/2016/116) and in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (CETS # 123), directive 2010/63/EU on the protection of animals use for scientific purposes and the Spanish law (R.D. 53/2013). Male Wistar rats were purchased from Charles River (USA) at six weeks of age. The rats were acclimated to the experimental conditions for two weeks. Next, the rats were randomly allocated into a sedentary (n = 6) or an exercised (n = 6) group for ten weeks, were weighed weekly, and their food and water intakes were also recorded. Seventy-two hours after the last exercise was performed, the rats were fasted overnight, anaesthetized with pentobarbital and euthanized by bleeding.

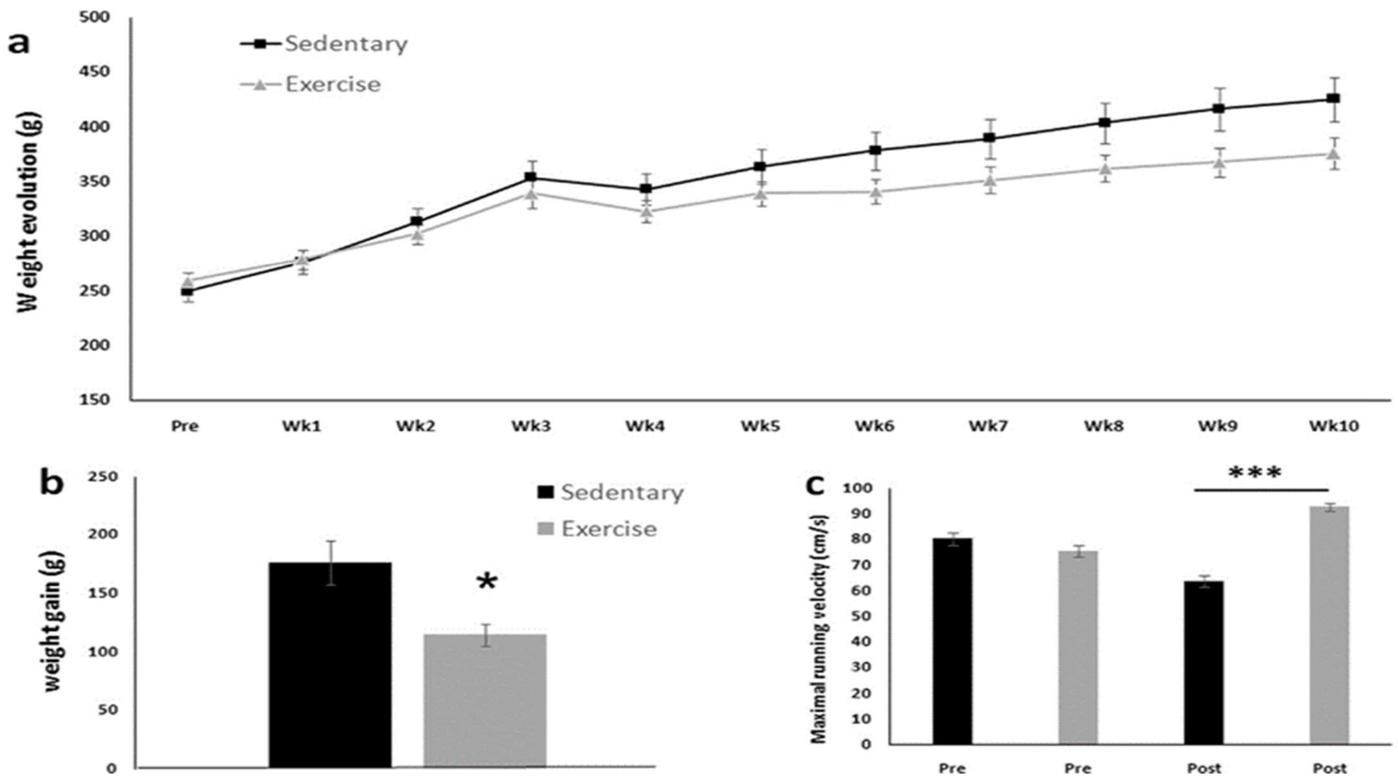

Fig. 1. Weight responses and running performances. a) Weekly evolution of the animal's weights. b) Weight gain was lower in the exercised rats than in the sedentary rats. c) The maximal running velocity was significantly higher in the exercised animals after the 10-week running protocol. *p < 0.05 and ***p < 0.001 vs. sedentary rats.

2.2. Exercise procedures

The animals were acclimated to running on a motorized treadmill (Rat 5-lanes Touchcreen Treadmill for rats, LE8710RTS, PanLab, Spain) for 5 sessions throughout the two weeks of the acclimation period. The exercise program was divided into two similar 5-wk mesocycles; the rats ran 5 days per week and rested during the weekends (Fig. S1). During the entire protocol, the animals ran at 75% of their maximal velocity as calculated from the maximal velocity test performed at the beginning of each mesocycle. Each mesocycle progressively increased the running volume, the rats began running for 20 min, and this period was increased by 5 min every two days, and when 65 min was achieved, this duration was maintained until the end of the mesocycle. The ani- mals of the sedentary group were daily handled and run for 10 min every two weeks to maintain their acclimation to the treadmill.

The maximal velocity tests were performed before the training period and at week 10 of the training period. These tests started at a velocity of 22 cm/s, and the velocity was increased by 5 cm/s every minute until fatigue was achieved. Fatigue was defined as the point at which the rats remained at the back of the treadmill on an electric shock pad for 5 s.

2.3. *Evaluation of supercomplex formation in the skeletal muscle by BNGE*

Blue native gel electrophoresis (BNGE) was performed on crude mitochondrial fractions from the gastrocnemius muscles of rats. Mitochondrial isolation was performed as previously described [17]. One aliquot of the crude mitochondrial fraction was used for protein determination. The remaining samples were then centrifuged at 13,000 × g for 3 min at 4 °C. The mitochondrial pellets were suspended in an appropriate volume of medium C (1 M aminocaproic acid, 50 mM Bis-Tris-HCl [pH 7.0]) to create a protein concentration of 10 mg/ml, and the membrane proteins were solubilized with digitonin (4 g/g) and incubated for 10 min in ice. After 30 min of centrifugation at 13,000 × g (4 °C), the supernatants were collected, and 3 µL of 5% Brilliant Blue G dye prepared in 1 M aminocaproic acid was added. Mitochondrial proteins (100 µg) were then loaded and run on a 3–13% gradient native gel as previously described [18]. After electrophoresis, the complexes were electroblotted onto PVDF membranes and sequentially tested with specific antibodies against CI, anti-NDUFA9 (Abcam, ab14713), CIII, anti-ubiquinol-cytochrome c reductase core protein I (Abcam, ab110252) and Vdac1 (Abcam, ab14734).

2.4. *Lipid peroxidation in the plasma and mitochondria*

The concentration of hydroperoxides (HPx), a specific and direct biomarker of lipid peroxidation, was determined with a Sigma PD1 kit (St Louis, MO, USA). The absorbance changes at 560 nm were monitored by spectrophotometry. Blood obtained from the bleeding procedure was centrifuged for 10 min at 3000 rpm to isolate the plasma. Then, 40 µL of plasma was used for the quantification of the HPx concentration in plasma. A total of 100 µg of protein from the mitochondrial fraction was used to determine the mitochondrial concentration of HPx.

2.5. *Protein carbonyls in the plasma and mitochondria*

The formation of protein carbonyl adducts, a marker of oxidative stress [24], was used as an index of oxidative modifications of mitochondrial and plasma proteins. Protein carbonyls were measured in duplicate using an ELISA-based assay according to manufacturer's instructions (OxiSelect Protein Carbonyl ELISA Kit; Cell Biolabs Inc., San Diego, USA).

2.6. *Statistics*

The statistical analyses were performed using SPSS (version 22 for Windows; IBM Corp., Armonk, NY, USA). The data are presented as the means ± the standard errors of the means (SEM), and $p < 0.05$ was considered significant. Unpaired t-tests were used to analyze differences between the exercised and sedentary rats. Pearson

correlation analyses were used to elucidate the relationships of the concentrations of HPx and PC with the super assembly of CI.

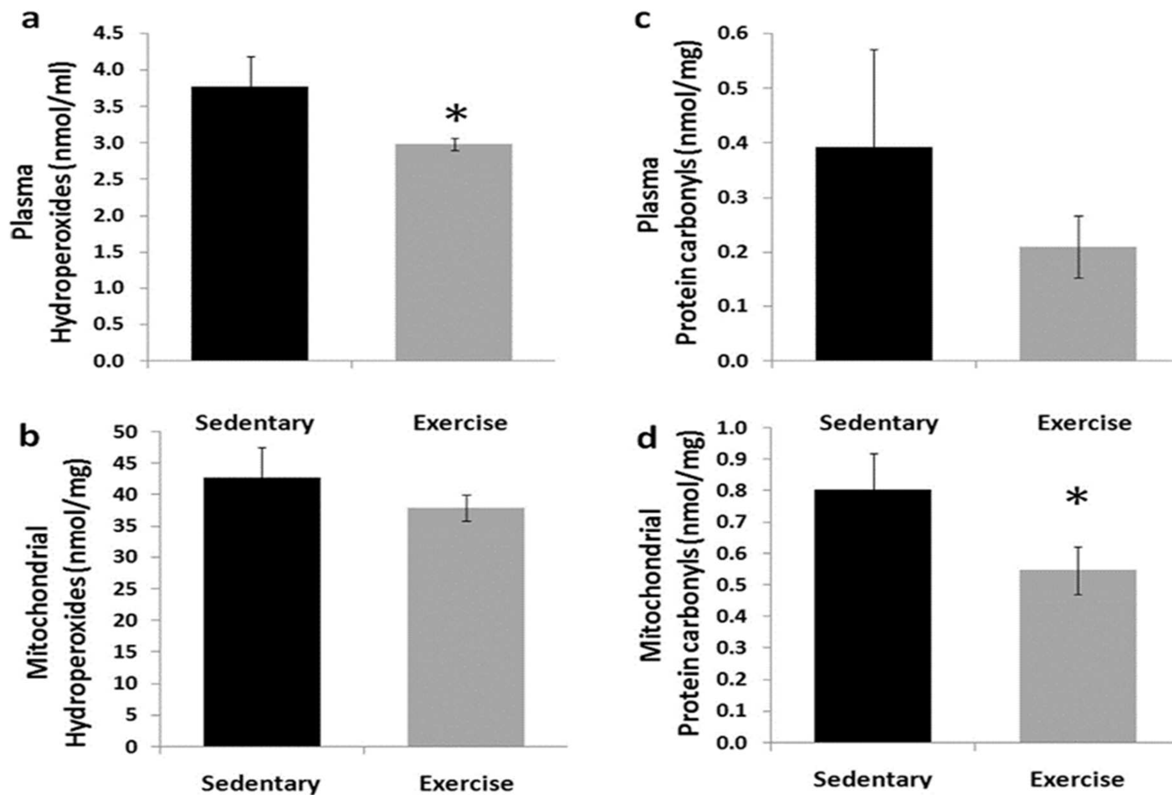

Fig. 2. Antioxidant responses to exercise. a) Endurance exercise decreased the circulating concentration of hydroperoxides. b) Mitochondrial hydroperoxide concentration. c) Circulating protein carbonyls. d) Exercise reduced mitochondrial protein carbonyls. * $p < 0.05$ and ** $p = 0.01$ vs. sedentary rats.

3. Results

Fig. 1a illustrates the weekly weight changes of the sedentary rats and the exercised rats over the 10 weeks. The exercised rats exhibited less weight gain ($p = 0.015$) than the sedentary rats (Fig. 1b). A maximal velocity test (Fig. 1c) revealed that the sedentary rats de- creased their performances by 20% from Test 1 (80 ± 6.1 cm/s) to Test 2 (64 ± 5.2 cm/s), while the exercised rats increased their performance by 24% from Test 1 (75 ± 5.2 cm/s) to Test 2 (93 ± 3.8 cm/s). The performance at Test 2 was significantly higher for the exercised animals ($p < 0.001$).

Fig. 2a shows that exercised rats (2.9 ± 0.19 nmol/ml) exhibited lower plasma concentrations of HPx ($p < 0.05$) than the sedentary animals (3.8 ± 0.90 nmol/ml). The mitochondrial concentration of HPx (Fig. 2b) decreased by 12% in the exercised (38 ± 5.0 nmol/mg) rats compared with the sedentary rats (43 ± 11.5 nmol/mg), but the difference was not statistically significant. PC analysis revealed no difference in the plasma concentration of HPx between the sedentary (0.39 ± 0.18 nmol/ml) and exercised rats (0.21 ± 0.06 nmol/ml). However, the mitochondrial PC (Fig. 2d) decreased ($p < 0.05$) in the exercised rats (0.80 ± 0.10 nmol/mg) compared with the sedentary animals (0.54 ± 0.07 nmol/mg).

We next performed a BNGE analysis on the crude mitochondrial fraction of the gastrocnemius muscle. We found that the SC/free CI ratio was 26% higher ($p = 0.01$) in the exercised animals than in the sedentary animals (Fig. 3a). However, the SC/free CIII ratio was almost identical between the exercised and sedentary animals (Fig. 3b). Pearson correlation analysis (Fig. 3c) revealed a significant inverse relationship ($p < 0.01$) between the CI super assembly and the mitochondrial production of HPx. Additionally, we found a significant inverse relationship ($p < 0.05$) between CI super assembly and the mitochondrial protein carbonyls (Fig. 3d). No significant relationship was found between CI and plasma markers.

4. Discussion

Endurance exercise as an antioxidant is extensively recognized. The mechanisms triggered by exercise induce effects that include the in- creased expressions and activities of key antioxidant enzymes [3,8]. Moreover, exercise induces the mobilization of non-enzymatic anti- oxidants to mitochondrial membranes to prevent their oxidative da- mage [21]. However, subjects performing lifelong endurance exercise exhibit a systemic decrease in lipid peroxidation that is not related to the antioxidant system [2]. In the present study, we demonstrate that exercise-induced chronic superassemblies of CI protect against mi- tochondrial oxidative damage.

The antioxidant effects of the super assembly of mitochondrial complexes have been previously assessed in vitro. Maranzana *et al.* [20] demonstrated that SC assembly prevents excessive ROS generation by CI. More recently, observations based on cryo-electron microscopy suggest that the SC architecture might prevent excessive ROS production by limiting the maximal activity of the mitochondrial complexes [13]. Although CIs are fully functional both as free complexes and when assembled into SCs [19], the supramolecular organization of the CIs that is induced by exercise can exert a fine-tuned control of mitochondrial ROS production. This notion is consistent with the hypothesis that states that CI-containing SCs may physiologically occur with the aim of

preventing excessive ROS production within living cells [16]. Additionally, under conditions of oxidative stress, the SC I1III2 is dissociated, which leads to a less efficient electron transfer [15]. Therefore, SCs can be functional entities that are optimized for pre- venting excessive ROS production and allow proper mitochondrial function during stressful stimuli such as exercise.

Nevertheless, our data revealed that only the CI is assembled into the SC. A plausible explanation for the absence of the super assembly of CIII comes from the stoichiometry of the mitochondrial respiratory complexes. Indeed, the ratio for complexes I:III:IV is 1.1:3.0:6.7 in mammals [23]. However, the main SC association formed in mammals, the so-called respirasome, is comprised of one copy of CI, two copies of CIII and several copies of CIV (I1III2IVn). Therefore, as previously suggested [14], one-third of the total CIII may not be bound to monomeric CI, which strengthens the observation that the CI assembly into the SC may be crucial for the control of mitochondrial ROS production. In contrast, Greggio and colleagues [10] demonstrated that endurance exercise induces SC assembly of both CI and III in older sub- jects. There are several potential reasons for this inconsistency. First, the species or model-specific differences could underlie this difference. For example, supercomplex assembly factor I (SCAF1) is the protein responsible for CIII and IV super assembly; however, some murine models, such as C57BL/6 mice, only express a short isoform of this protein, which hampers the super assembly of CIII and IV [5]. Although no evidence has been reported for Wistar rats, the mechanisms and proteins involved in SC assembly are still poorly understood, and this possibility cannot be ruled out. Second, the study by Greggio [10] was conducted in older adults ranging from 60 to 80 years old. Because mitochondrial metabolism is impaired by the ageing of the skeletal muscle, exercise might lead to stronger stimuli for SC assembly within elderly skeletal muscle. Indeed, some age-related metabolic diseases are triggered by increased mitochondrial oxidative damage [1]. Thus, understanding the cellular mechanisms that regulate ROS production in health and disease is critical to improving our knowledge about the ageing process.

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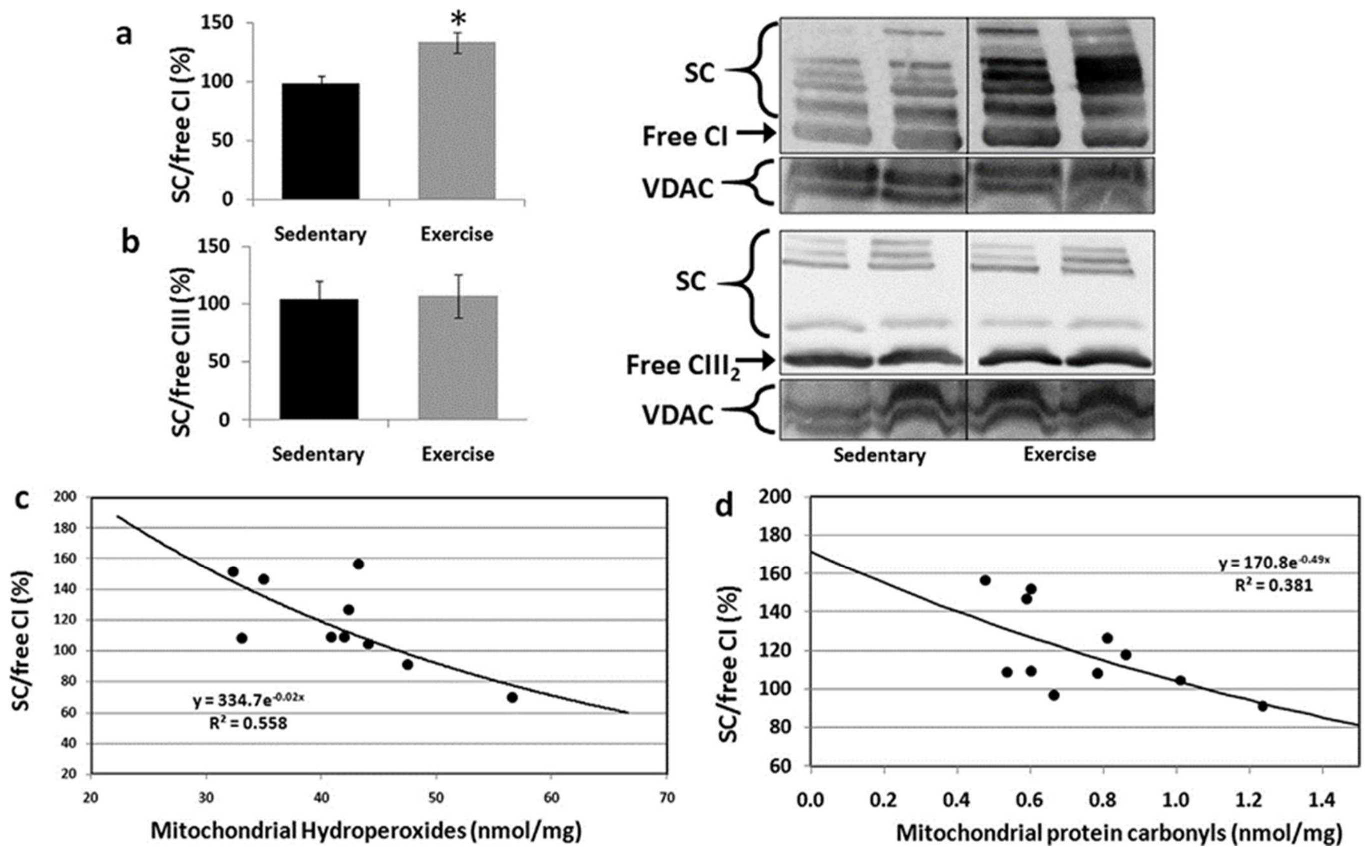

Fig. 3. Exercise-induced complex I super assembly is related to mitochondrial oxidative damage. a) Exercise induced mitochondrial complex I assembly into SCs. b) Mitochondrial complex III assembly into SCs. c) The percentage of SCs containing complex I was related ($p < 0.01$) to mitochondrial hydroperoxide production. d) The percentage of SCs containing complex I was related ($p < 0.05$) to mitochondrial protein carbonyls. *p = 0.01 vs. sedentary rats.

The specific contribution of SCs to the systemic antioxidant effects of exercise is unknown; this constitutes the main limitation of the present study. Indeed, the large number of antioxidant mechanisms triggered by exercise [3,8,12] may explain the lack of association found between the super assembly of CI and the systemic antioxidant effects of exercise. Notably, we did not find a significant decrease in plasma PC in the exercised animals due to the high variability. However, our results are within the range that has been previously described [24].

5. Conclusions

Exercise induces the chronic super assembly of mitochondrial CI to improve mitochondrial redox homeostasis. However, the contribution of SC to the antioxidant effects of exercise remains unknown. Further research to elucidate whether exercise-induced SC assembly can improve substrate channelling or whether it might prevent mitochondrial dysfunction is needed, given that this type of information would have clear implications in mitochondrial medicine and sport science.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.07.009.

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Artículo II

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Original article

Hydroxytyrosol influences exercise-induced mitochondrial respiratory complex assembly into supercomplexes in rats

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ABSTRACT

Hydroxytyrosol (HT) has been demonstrated to improve mitochondrial function, both in sedentary and in exercised animals. Herein, we assessed the effects of two different doses of HT on exercise-induced mitochondrial respiratory complex (C) assembly into supercomplexes (SCs) and the relation of the potential results to OPA1 levels and oxidative stress. Wistar rats were allocated into six groups: sedentary (SED), sedentary consuming 20 mg/kg/d of HT (SED-20), sedentary consuming 300 mg/kg/d of HT (SED-300); exercised (EXE), exercised consuming 20 mg/kg/d of HT (EXE-20) and exercised consuming 300 mg/kg/d of HT (EXE-300). Animals were exercised and/or supplemented for 10 weeks, and assembly of SCs, mitochondrial oxidative status and expression of OPA1 were quantified in the gastrocnemius muscle. Both EXE and EXE-20 animals exhibited increased assembly of CI into SCs, but this effect was absent in EXE-300 animals. Levels of $CIII_2$ assembled into SCs were only increased in EXE-20 animals. Notably EXE-300 animals showed a decreased relative expression of s-OPA1 isoforms. Therefore, HT exerted dose-dependent effects on SC assembly in exercised animals. Although the mechanisms leading to SCs assembly in response to exercise and HT are unclear, it seems that a high HT dose can prevent SCs assembly during exercise by decreasing the expression of the s-OPA1 isoforms.

Original article

Hydroxytyrosol influences exercise-induced mitochondrial respiratory complex assembly into supercomplexes in rats

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1 Equal contribution.

A R T I C L E I N F O

Keywords: Exercise Mitochondria, Oxidative phosphorylation, Polyphenols, Skeletal muscle.

A B S T R A C T

Hydroxytyrosol (HT) has been demonstrated to improve mitochondrial function, both in sedentary and in exercised animals. Herein, we assessed the effects of two different doses of HT on exercise-induced mitochondrial respiratory complex (C) assembly into supercomplexes (SCs) and the relation of the potential results to OPA1 levels and oxidative stress. Wistar rats were allocated into six groups: sedentary (SED), sedentary consuming 20 mg/kg/d of HT (SED-20), sedentary consuming 300 mg/kg/d of HT (SED-300); exercised (EXE), exercised consuming 20 mg/kg/d of HT (EXE-20) and exercised consuming 300 mg/kg/d of HT (EXE-300). Animals were exercised and/or supplemented for 10 weeks, and assembly of SCs, mitochondrial oxidative status and ex- pression of OPA1 were quantified in the gastrocnemius muscle. Both EXE and EXE-20 animals exhibited in- creased assembly of CI into SCs, but this effect was absent in EXE-300 animals. Levels of CIII2 assembled into SCs were only increased in EXE-20 animals. Notably EXE-300 animals showed a decreased relative expression of s- OPA1 isoforms. Therefore, HT exerted dose-dependent effects on SC assembly in exercised animals. Although the mechanisms leading to SCs assembly in response to exercise and HT are unclear, it seems that a high HT dose can prevent SCs assembly during exercise by decreasing the expression of the s-OPA1 isoforms.

1. Introduction

Oxidative phosphorylation (OXPHOS) is the primary source of ATP production for metabolic and mechanical work in mammals. This process occurs in the inner mitochondrial membrane, where the OXPHOS system couples the transport of electrons to protons pump. Thus, ATPase uses these protons for ATP production. The efficiency and potential physiological adaptations of the OXPHOS system partially de- pend on the formation of mitochondrial supercomplexes (SCs), which are supramolecular entities formed by complex I (CI), complex III (CIII) and/or complex IV (IV) [1,2]. Indeed, increasing evidence suggests that SCs enhance ETC substrate channeling [2,3] and minimize ROS pro- duction by CI [4], thereby improving cellular and systemic metabolic health [5–10].

Physiological and molecular mechanisms leading to SC assembly are currently under investigation. Recent observations suggest that exercise induces an increase in mitochondrial SCs, which is associated with improved mitochondrial function [10,11]. Additionally, the GTPase OPA1 controls mitochondrial metabolism and cristae integrity, contributing to SC maintenance [12,13]. It has also been reported that excessive reactive oxygen species (ROS) prevent SC assembly [14], and, consequently, molecules with antioxidant or pro-oxidant capacities may modulate SC assembly.

Hydroxytyrosol (HT) is the main polyphenol found in extra virgin olive oil. Recent observations in fibroblasts reported that HT improves mitochondrial function by inducing de novo assembly of mitochondrial complexes [15]. Moreover, studies in rodents have shown that supplementation with low doses of HT (∼20 mg/kg/d) for 10 weeks enhances mitochondrial function [16,17]. In addition to these studies, in vitro observations reported that HT can reverse mitochondrial dysfunction through increasing OPA1 content [18]. Therefore, both the antioxidant properties of HT [16] and its capacity to modulate mitochondrial function could be related to OPA1 overexpression. Thus, it is reasonable to test whether HT influences SC assembly in vivo after endurance exercise and whether there is correlation of the potential effects with the levels of OPA1.

It should be highlighted that the effects triggered by HT might be dose-dependent since previous studies have reported that 20 mg/kg/d of HT supplementation for 10 weeks improves mitochondrial and antioxidant adaptations induced by exercise in rodents [19], but 300 mg/ kg/d of HT supplementation for 10 weeks in exercised rodents induces a systemic pro-oxidant effect [20]. This phenomenon may reflect lower SC assembly induced by high doses [11]. Therefore, the main purpose of the present study was to describe the effects of both a low and a high HT dose on the

assembly of SCs in both sedentary and exercised animals, as well as to identify any potential correlation of the results with the levels of OPA1 and oxidative stress.

2. Methods

2.1. Animals

Male Wistar rats where purchased from Charles River (USA) at six weeks of age. Rats where acclimated to the experimental conditions for two weeks and maintained in a room under standard conditions of temperature (21 ± 2 °C) and relative humidity (40%–60%) under a reverse 12-h light/12-h dark cycle. Before animals were allocated into experimental groups, all of them were subjected to a maximal velocity running test (Rat 5-lanes Touchscreen Treadmill for rats, LE8710RTS, PanLab, Spain) where rats started at a velocity of 22 cm/s, and the velocity was increased by 5 cm/s every minute until fatigue was achieved. Rats were then allocated into six experimental groups by their maximal velocity and weight: sedentary (SED; n = 7), sedentary consuming 20 mg/kg/d HT (SED-20; n = 5), sedentary consuming 300 mg/kg/d (SED-300; n = 5), exercised (EXE; n = 7), EXE consuming 20 mg/kg/d HT (EXE-20; n = 7) and EXE consuming 300 mg/kg/d HT (EXE-300; n = 8). Sedentary animals were handled daily and run for 10 min every two weeks to maintain their acclimation to the treadmill. Throughout the experimental period, all rats consumed water and standard chow AD libitum (2.9 kcal/g). Daily food and water intake were monitored. All interventions lasted for 10 weeks. Seventy-two hours after the last maximal velocity test was performed, rats were fasted overnight, anesthetized with pentobarbital and sacrificed by exsanguination.

All experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Granada (procedures Granada, Spain N°: 28/06/2016/116).

2.2. Hydroxytyrosol treatment

Supplementation with low (20 mg/kg/d) or high dosage (300 mg/ kg/d) HT began once the rats where allocated into experimental groups and stopped 12 h before euthanasia. HT was diluted in water in an opaque water bottle to avoid oxidation, as previously described [20]. Water intake was controlled daily and HT dilutions were made ac- cording to the average liquid consumed in the previous week and the weight of the animals.

2.3. Exercise study

The exercise programme was divided into two similar 5-wk meso- cycles where rats ran five days per week and rested over the weekends [20]. During the entire protocol,

the animals ran at 75% of their maximal velocity, as calculated from the maximal velocity test performed at the beginning of each mesocycle. Rats started running for 20 min/d, and this period was increased by 5 min every two days up to 65 min/d and was then maintained at 65 min/d until the end of the mesocycle. Daily physical work was monitored using the formula: work (J) = force × vertical distance, where force = body weight (kg) × 9.8 m/s2, and vertical distance = speed (m/min) × time (min) [21].

2.4. Mitochondrial supercomplex quantification

Gastrocnemius muscles were harvested from rats, and mitochondria was isolated as previously described [22]. Next, Blue Native Gel Electrophoresis (BNGE) was performed on crude mitochondrial fractions. One aliquot of the crude mitochondrial fraction was used for protein determination. The remaining samples were then centrifuged at 13,000×g for 3 min at 4 °C. Mitochondrial pellets were suspended in an appropriate volume of medium C (1 M aminocaproic acid, 50 mM Bis- Tris-HCl [pH 7.0]) to create a protein concentration of 10 mg/ml, and membrane proteins were solubilized with digitonin (4 g/g) and incubated for 10 min on ice. After 30 min of centrifugation at 13,000×g (4 °C), supernatants were collected, and 3 µL of 5% Brilliant Blue G dye prepared in 1 M aminocaproic acid was added. Mitochondrial proteins (100 µg) were then loaded and run on a 3–13% gradient native gel [23]. After electrophoresis, complexes were electroblotted onto PVDF mem- branes and sequentially tested with specific antibodies against CI (anti- NDUFA9, Abcam, ab14713), CIII (anti-ubiquinol-cytochrome c reductase core protein I, Abcam, ab110252) and Vdac1 (Abcam, ab14734).

2.5. Mitochondrial protein carbonyls and hydroperoxides

The concentration of hydroperoxides (HPx) was determined with a Sigma PD1 kit (St Louis, MO, USA). Absorbance changes at 560 nm were monitored by spectrophotometry. 100 µg of protein from the mitochondrial fraction was used to determine the mitochondrial con- centration of HPx.

Protein carbonyls were measured in duplicate using an ELISA-based assay according to the manufacturer's instructions (OxiSelect Protein Carbonyl ELISA Kit; Cell Biolabs Inc., San Diego, USA).

2.6. Western blotting

Mitochondrial samples were harvested in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, and a protease inhibitor cocktail (Thermo Scientific) and were then placed on ice for 20 min. After centrifugation (30 min, 13,000 g, 4 °C), the protein content in the supernatant was measured using a Protein Assay Kit II (Bio-Rad Laboratories, California, USA). Samples containing 30 µg of protein were mixed with 3X SDS-PAGE sample buffer (100 mM Tris- HCl, pH 6.8, 25% SDS, 0.4%

bromophenol blue, 10% β-mercaptoethanol, and 2% glycerol), separated via SDS-PAGE using TGX Any kD gel (Bio-Rad Laboratories, California, USA), and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, California, USA). After incubation in blocking buffer (5% non-fat milk and 1% Tween 20 in Trisbuffered saline, TBS), membranes were probed with the following antibodies: mouse anti-OPA1 antibody (dilution 1:1000 in 5% non-fat milk) acquired from BD Biosciences (New Jersey, USA, 612,606) and anti-Hsp-70 (internal control; 1:500 in 5% non-fat milk) acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA, sc-7298). Immunoreactive signals were detected via enhanced chemiluminescence (SuperSignal West Dura Chemiluminescent Substrate, 34,075, Thermo Scientific, Europe), and membranes were digitally imaged and quantified by densitometry using ImageJ software. Results are re- presented as fold-change (Fc) in expression relative to the control.

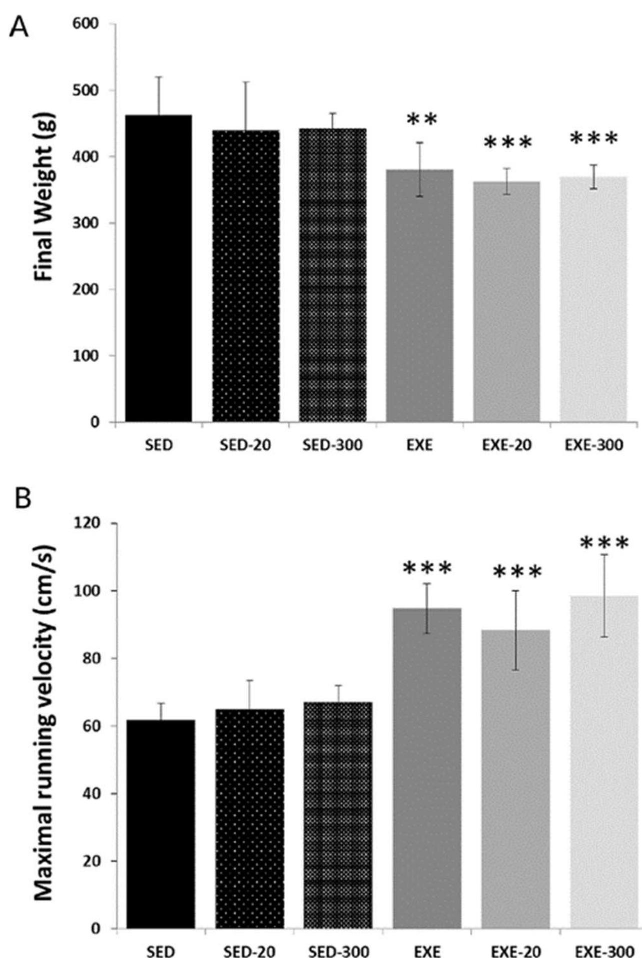

Fig. 1. Effects of exercise and HT on final weight and maximal running velocity.a) All the exercised animals showed lower final weight than the sedentary animals, but no effect of HT were observed. b) Similarly, exercised animals increased their maximal running velocity, however, EXE-20 animals showed lower maximal running velocity than EXE-300. Data are shown by means ± SD. **p < 0.01 and ***p < 0.001 vs. SED. SED, sedentary (n = 7); SED-20, sedentary and supplemented with 20 mg/kg/d hydroxytyrosol (n = 5); SED-300, sedentary and supplemented with 300 mg/kg/d hydroxytyrosol (n = 5); EXE, exercised (n = 7); EXE-20; exercised and supplemented with 20 mg/kg/d hydroxytyrosol (n = 7); EXE-300; exercised and supplemented with 300 mg/kg/d hydroxytyrosol (n = 7).

2.7. Statistics

Results are presented as the mean ± standard deviation. Homoscedasticity and normality were tested by the Levenne and Kolmogorov–Smirnov tests, respectively. One-way ANOVA was used to analyse data. Post hoc analysis was performed using Bonferroni, unless otherwise indicated. Results were considered statistically significant

at $p < 0.05$. Statistical analyses were performed using SPSS (version 22 for Windows; IBM Corp., Armonk, NY, USA).

3. Results

Neither final weight (Fig. 1a) nor maximal running velocity (Fig. 1b) were affected by HT intake in sedentary animals. Final weights, however, were lower in all exercised groups compared to sedentary animals ($p < 0.01$ for EXE and EXE-300; $p < 0.001$ for EXE- 20). No differences in final weight were identified among exercised groups (Fig. 1a) and the amount of food ingested was similar in all experimental groups (data not shown). As expected, the exercised groups, i.e., EXE ($p < 0.001$), EXE-20 ($p < 0.01$) and EXE-300 ($p < 0.001$), showed higher maximal running velocities than the se- dentary animals (Fig. 1b).

Fig. 2 illustrates the effects of HT and exercise on mitochondrial SCs assembly. The low HT dose in sedentary animals increased 30% the SC/ Free CI ratio if compared with SED animals but this effect did not reach a statistical significance ($p = 0.089$). However, the increase of CI assembled into SCs reached by the high HT dose was lower. In addition, endurance exercise was able to induce SC/free CI ratio ($p < 0.05$), and low dose HT (EXE-20) did not modify this effect ($p < 0.01$). However, high dose HT partially suppressed the effect exercise on CI assembly. The SC/Free CIII2 ratio was similar between sedentary animals. Notably, EXE-20 animals exhibited increased CIII2 assembly into SCs ($p < 0.05$) compared with SED, EXE and EXE-300 animals. In order to rule out the possibility that the higher level of supercomplexes found in EXE-20 was due to increased work being performed during the exercise period, we monitored the daily amount of work performed by each rat. As shown in Fig. S1, there was no correlation between the total amount of work performed during the experimental period and assembly of CI and CIII2 into SCs and, therefore, these results suggest that HT may exert a modulatory effect on the assembly of SCs in response to exercise. We next explored whether HT and/or endurance exercise modulate OPA1 expression (Fig. 3). We found that none of the interventions in- creased the expression of total OPA1, calculated as the sum of all the bands detected (Fig. 3A). However, we were able to identify two short (s)-OPA1 isoforms and two long (l)-OPA1 isoforms as previously de- scribed [24]. As s-OPA1 isoforms are important for SCs assembly [12], we analyzed the relative expression of these two isoforms (i.e. s-OPA1). In sedentary animals, s-OPA1 expression increased in a dose dependent manner (Fig. 3b). Indeed SED animals reported a relative expression of the two s-OPA1 isoforms of 6.8% and this was increased to 8.8% in SED-20 ($p = 0.09$) and to 19.5% in SED-300 ($p < 0.001$). It is note- worthy that we were unable to detect the highest molecular weight band from the l-OPA1 isoform in SED-300 animals (Fig. 3b). However, we must acknowledge that this might be an artifact due to the low protein loaded. Nevertheless, exercise did not

increase the relative ex- pression of s-OPA1 isoforms (6.5% for EXE animals). Similarly, the low HT dose did not modify the relative expression of the two s-OPA1 isoforms in exercised animals (6.3% for EXE-20). In contrast, EXE-300 animals showed a significant decrease in the relative expression of s- OPA1 (4.9%; $p < 0.05$) if compared with SED. These findings suggest that the inability of EXE-300 animals to induce SCs assembly may be due to the lower relative content of the s-OPA1 isoforms.

Because mitochondrial oxidative status could be directly related to the assembly of individual complexes into SCs [14,25], we next analyzed mitochondrial oxidation status. Fig. 4a shows that mitochondrial hydroperoxides, a marker of lipid peroxidation, did not differ between experimental groups. Mitochondrial protein carbonyls, however, were decreased in EXE groups ($p < 0.05$) compared to sedentary animals (Fig. 4b), and HT did not modify this effect, either at the low or high dose. Moreover, low dose HT supplementation in sedentary animals did not alter mitochondrial oxidative status (Fig. S2).

4. Discussion

The primary finding of the present study is that 20 mg/kg/d HT supplementation for 10 weeks enhances exercise-induced mitochondrial complex assembly into SCs. While a high HT dose of 300 mg/kg/d supplemented for 10 weeks hampers exercise-induced CI assembly into SCs. Notably, we found that the high HT dose decreases the relative expression of the s-OPA1 isoforms in trained animals, which may suggest that high HT doses may prevent SCs assembly during exercise by altering the relative expression of s-OPA1.

Exercise training promotes a unique antioxidant environment by enhancing both enzymatic and non-enzymatic adaptations [26,27]. Additionally, we recently reported that exercise-induced CI assembly into SCs reduces mitochondrial oxidative stress [11].

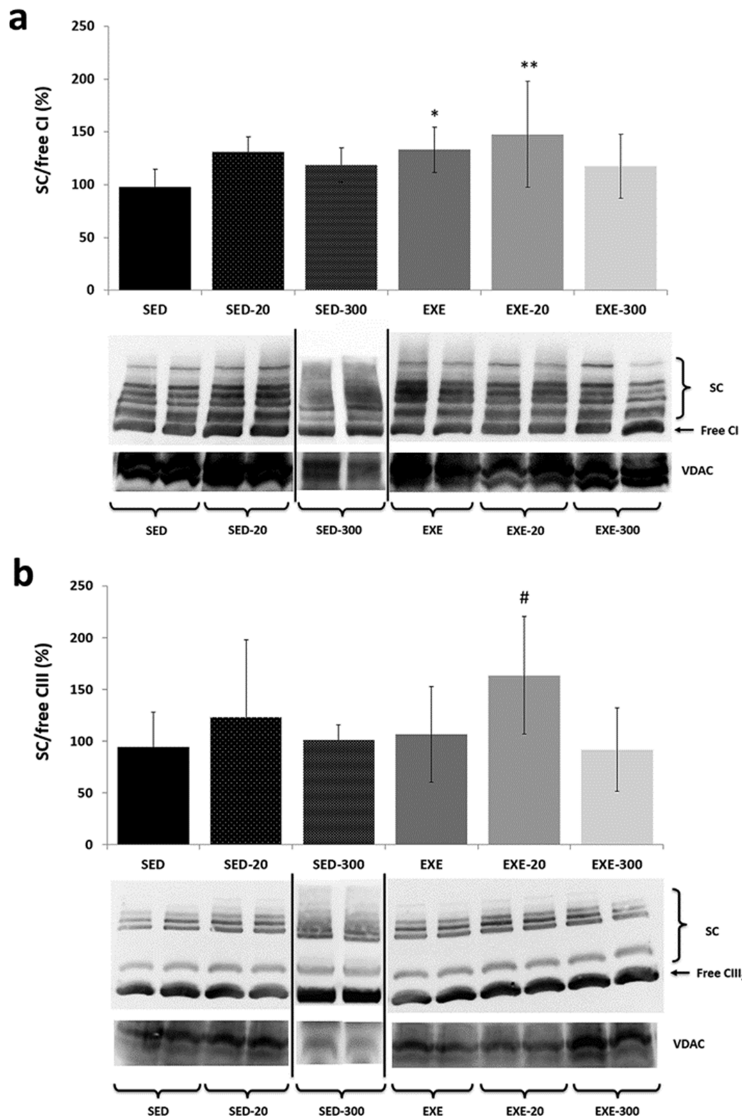

Fig. 2. Effects of exercise and HT on supercomplexes (SCs) assembly. a) Assembly of mitochondrial com- plex I (CI) into supercomplexes (SC) is induced in EXE and EXE-20 animals, but this effect was not observed for EXE-300 animals. b) Assembly of dimeric mitochondrial complex III (CIII2) into SCs was only induced in EXE-20 animals. Data are shown as means ± SD. *p < 0.05 and **p < 0.01 vs. SED rats. . #p < 0.05 compared with SED, EXE and EXE300. SED, sedentary (n = 7); SED-20, sedentary and supplemented with 20 mg/kg/d hydroxytyrosol (n = 4); SED-300, sedentary and supplemented with 300 mg/kg/d hydroxytyrosol (n = 3); EXE, exercised (n = 7); EXE-20; exercised and supplemented with 20 mg/kg/d hydroxytyrosol (n = 7); EXE-300; exercised and supplemented with 300 mg/kg/d hydroxytyrosol (n = 6).

This is in accordance with findings showing that the assembly of CI into SCs minimizes ROS production from this particular complex [4]. Here we found that EXE-20 animals showed an enhanced capacity to assemble CI and CIII2 into SCs compared to EXE-300. It should be noted that the HT dose used for EXE-20 animals increases skeletal muscle antioxidant adaptations [16,19], which may help to maintain mitochondrial SCs [14,20]. Although not statistically significant, the lowest level of both PC and HPx were found in the EXE-20 group which may suggest that the experimental procedures may not be sensitive enough in order to discriminate the antioxidant potential of HT in healthy rats. In fact, in mice consuming a high fat diet, it has been reported that HT doses ranging from 10 to 50 mg/kg/d ameliorates diet-induced skeletal muscle PC rise [16]. Nevertheless, the potential antioxidant mechanisms of HT within skeletal muscle are unknown. It has been described that HT modulates glutathione redox status [28] thus leading to Nrf2- related phase II antioxidant response in adipose and liver tissue [28,29]. A similar antioxidant response has been described within contracting skeletal

muscle, where glutathione oxidation leads to transcription factors (including Nrf2) translocation to the nucleus [30]. Nevertheless, the relationship between HT, exercise and Nrf2-related antioxidant gene expression needs to be determined. Under our experimental conditions, however, the enhanced SC assembly capacity induced by EXE-20 cannot be attributed to improvements in the mitochondrial antioxidant environment. In addition, it is important to remark that high dose HT blunts exercise-induced SC assembly but this effect is not associated with mitochondrial oxidative stress alterations. Another mechanism that may explain the higher SCs assembly showed in EXE-20 comes from the stoichiometry of mitochondrial complexes. Indeed, while most CI is found in SCs, one third of dimeric CIII (CIII2) may not be bound to monomeric CI [31,32].

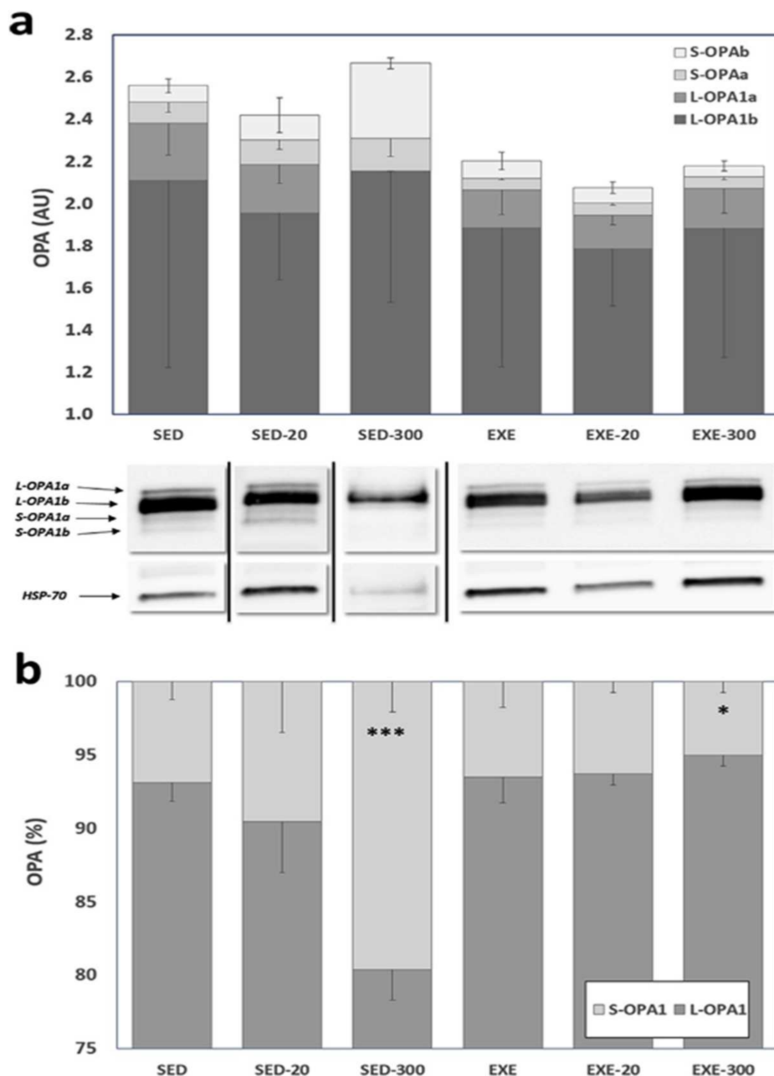

Fig. 3. Effects of exercise and HT on OPA1 expression. a) None on the interventions increased the expression of total OPA1. b) Relative expression (%) of the long (l)- and short (s)-OPA1 isoforms. SED-300 animals raised the amount of s-OPA while EXE-300 showed a decreased relative expression of s-OPA. Data are shown as means ± SD and analyzed using a one-way ANOVA, specific differences between mean values were located using Student's t-test for in- dependent samples. *p = 0.01 if compared with SED; ***p < 0.001 if com- pared with SED, EXE, EXE-20 and EXE-300. SED, sedentary (n = 6); SED-20, sedentary and supplemented with 20 mg/kg/d hydroxytyrosol (n = 4); SED- 300, sedentary and supplemented with 300 mg/kg/d hydroxytyrosol (n = 3); EXE, exercised (n = 5); EXE-20; exercised and supplemented with 20 mg/kg/d hydroxytyrosol (n = 5); EXE-300; exercised and supplemented with 300 mg/ kg/d hydroxytyrosol (n = 5).

It has been demonstrated that HT increases the biogenesis of CI. In fact, Feng et al. [19] showed that 25 mg/kg/d HT supplemented to exercised mice in- creases levels of CI within skeletal muscle. In addition, doses between 10 and 50 mg/kg/d HT increase the levels of CI and CII within skeletal muscle [16] and within brain cortex [17].

Importantly, all of these studies failed to show an increment in CIII content in response to HT doses ranging from 10 to 50 mg/kg/d. Therefore, it can be proposed that doses ranging from 10 to 20 mg/kg/d HT stimulate the biogenesis of CI but not CIII. This may increase the amount of free CI available to bind to CIII2 to form SCs. This mechanism may explain the higher levels of CIII2 assembled into SCs in exercised rats supplemented with 20 mg/ kg/d HT.

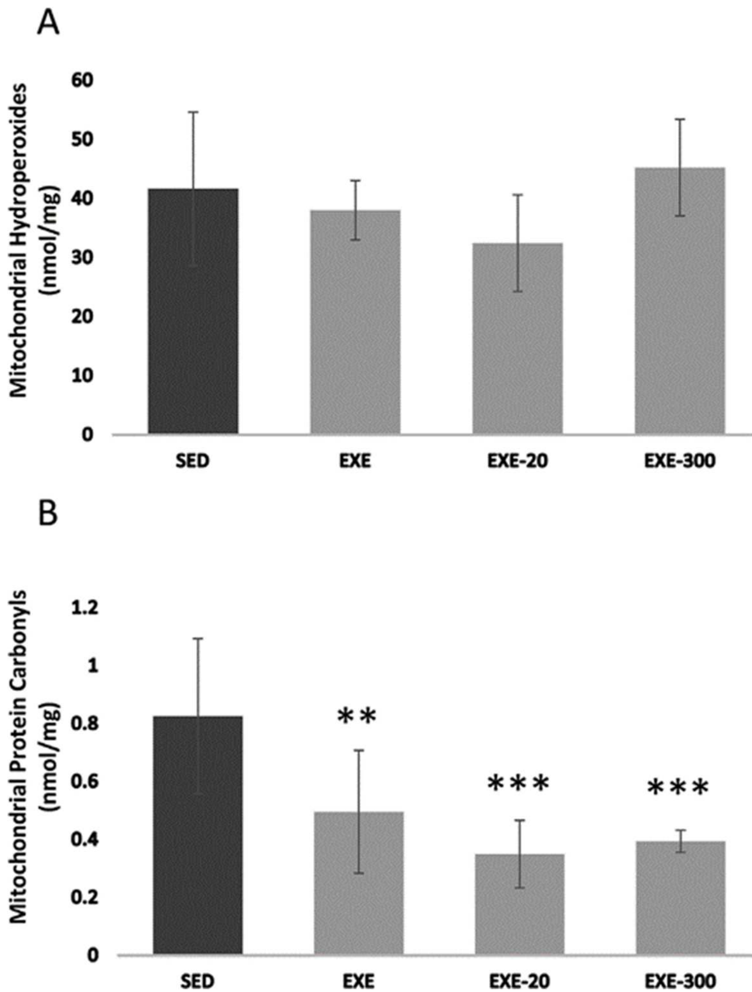

Fig. 4. Mitochondrial oxidative status in exercised animals. a) Mitochondrial hydroperoxides remained unchanged between experimental groups (n = 5 per group). b) Exercise reduces mitochondrial protein carbonyls, and this effect is augmented by both hydroxytyrosol doses (n = 7 per group). Data are shown as means ± SD. **p < 0.01 and ***p < 0.001 vs. sedentary rats. SED, sedentary; EXE, exercised; EXE-20; exercised and supplemented with 20 mg/kg/d hydroxytyrosol; EXE-300; exercised and supplemented with 300 mg/kg/d hydroxytyrosol.

In addition, early studies on polyphenols suggested that their modulatory effects on mitochondrial function were through NAD+- dependent deacetylase sirtuin 1 (SIRT1) [33,34]. However, when supplemented during exercise, the relationship between polyphenols and SIRT1 is not evident [35–37]. Notably, SIRT3 is the main mitochondrial protein that, under stress conditions, acts as an upstream regulator of OPA1 [38]. Therefore, the observation that HT rescues mitochondrial dysfunction in a mechanism related to OPA1 [18], provides a link be- tween HT, sirtuins and mitochondrial function. Nevertheless, we found that neither exercise nor HT increased total OPA1 (i.e. the sum of all the bands detected) expression within skeletal muscle. A recent study showed that s-OPA1 isoforms regulate cristae integrity and SCs assembly while l-OPA1 isoforms

control mitochondrial fusion events, but all the OPA1 isoforms are needed to reach the mitochondrial network [12]. Thus, the observation of a blunted capacity of EXE-300 animals to assemble SCs may be due to the fact that s-OPA1 isoforms only re- present a 4.9% of the total OPA1. It is important to note that exercise do not alter the relative expression of s-OPA1 isoforms while in sedentary animals, HT seems to increase the relative expression of the two s-OPA1 isoforms in a dose dependent manner. Notably, we were unable to detect one of the bands belonging to the l-OPA1 in SED-300 animals. This may suggest that high dose HT supplementation may impede the development of the mitochondrial network. However, future studies must confirm such finding because we cannot exclude the possibility that this might be an artifact due to the low protein loaded. Taken together, it seems that the high HT dose can prevent exercise-induced SCs assembly because it decreases the relative expression of the s-OPA1 isoforms.

An important observation in the present study is that 300 mg/kg/d HT hampers exercise-induced CI assembly into SCs. In addition to the lower s-OPA1 isoforms content found in EXE-300 animals, it has been recently demonstrated that coenzyme Q (COQ) metabolism plays a key role in the stability of complex I. In fact, the inability to oxidize COQH2 leads to a forward pump of electrons to CI via reverse electron transport (RET), thereby producing unique ROS signalling that induces de- gradation of CI and prevents assembly of CI with CIII2 [39]. Therefore, high antioxidant doses might impede COQH2 oxidation, leading to its accumulation during periods of high energy demands. It should be noted that although ROS accumulation via RET induces CI degradation, and this effect ultimately improves mitochondrial function and in- creases lifespan [40]. This is in accordance with the observation that EXE-300 animals showed a remarkable increase in maximal running velocity. Importantly, this occurred in spite of the fact that SC assembly is known to enhance cellular metabolism and mitochondrial respiration [10]. It should, however, be noted that the running test applied is a short test where velocity progressively increases until exhaustion. In a previous study, Wistar rats submitted to the same running test show blood lactate levels above 8 mmol/L [41]. Thus, the main limiting performance factor can be attributable to anaerobic glycolysis and not to oxidative phosphorylation potential. Therefore, a high HT dose hampers exercise-induced CI aggregation into SCs within skeletal muscle without affecting high-intensity skeletal muscle performance.

The present study has several limitations and strengths that need to be mentioned. The main strength is that we performed a 10 weeks highly demanding exercise training regime where workloads were adjusted at week 5. Thus, allowing to maintain the exercise stimuli throughout all the intervention. Moreover, we were able to separate four OPA-1 isoforms, thereby providing important information on the relative contributions of the long and short isoforms On the other hand, the main limitation of the present study is the lower number of se- dentary animals consuming HT. In

addition, we have been unable to explore further potential mechanisms induced by HT which may regulate SC assembly. For instance, our results highlight s-OPA1 as a potential mechanism to maintain SC assembly. Therefore, it would be important to address whether the interaction of OPA-1 with mitochondrial solute carriers such as SLC25A [42,43] is modulated either by exercise and/or by HT.

In conclusion, 20 mg/kg/d HT supplemented over 10 weeks of exercise provides a more powerful stimuli than exercise alone to induce SC formation. However, the consumption of 300 mg/kg/d of HT during exercise hampers exercise-induced CI assembly into SCs. This harmful effect observed when a high HT dose is supplemented during exercise can be explained by a down-regulation of the relative expression of s-OPA1 isoforms. Future studies should be performed to unravel the molecular mechanisms by which HT and exercise modulates SC aggregation.

Competing interest

None declared.

Authors contributions

RAC was involved in the conception, design, acquisition and statistical analysis of the data and drafting the manuscript; SAF was involved in the conception, design, acquisition and analysis of the data and drafting the manuscript; AHG was involved in the conception, design, acquisition and analysis of the data; LCL was involved in the conception and design; JPD and ARD were involved in acquisition of the data; JRH was involved in the conception and analysis of the data; All authors approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2019.01.027.

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Artículo III

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RESEARCH ARTICLE Open Access

Effects of hydroxytyrosol dose on the redox status of exercised rats: the role of hydroxytyrosol in exercise performance

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Abstract

Background: Hydroxytyrosol (HT) is a polyphenol found in olive oil that is known for its antioxidant effects. Here, we aimed to describe the effects of a low and high HT dose on the physical running capacity and redox state in both sedentary and exercised rats.

Methods: Male Wistar rats were allocated into 6 groups: sedentary (SED; $n = 10$); SED consuming 20 mg/kg/d HT (SED20; $n = 7$); SED consuming 300 mg/kg/d HT (SED300; n = 7); exercised (EXE; n = 10); EXE consuming 20 mg/kg/d HT (EXE20; n = 10) and EXE consuming 300 mg/kg/d HT (EXE300; n = 10). All the interventions lasted 10 weeks; the maximal running velocity was assessed throughout the study, whereas daily physical work was monitored during each training session. At the end of the study, the rats were sacrificed by bleeding. Hemoglobin (HGB) and hematocrit (HCT) were measured in the terminal blood sample. Moreover, plasma hydroperoxide (HPx) concentrations were quantified as markers of lipid peroxidation.

Results: In sedentary rats, HT induced an antioxidant effect in a dose-dependent manner without implications on running performance. However, if combined with exercise, the 300 mg/kg/d HT dosage exhibited a pro-oxidant effect in the EXE300 group compared with the EXE and EXE20 groups. The EXE20 rats showed a reduction in daily physical work and a lower maximal velocity than the EXE and EXE300 rats. The higher physical capacity exhibited by the EXE300 group was achieved despite the EXE300 rats expressing lower HGB levels and a lower HCT than the EXE20 rats.

Conclusions: Our results suggest that a high HT dose induces a systemic pro-oxidant effect and may prevent the loss of performance that was observed with the low HT dose.

Keywords: Polyphenols, ROS, Exercise, Hemoglobin, Oxidative stress, Antioxidants

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Background

Hydroxytyrosol (HT), the main polyphenol found in extra virgin olive oil, is in part responsible for the health-related effects of the Mediterranean Diet [1]. Polyphenols function as antioxidants due to their chemical structure com- posed of several hydroxyl groups on aromatic rings [2]. Accordingly, among other biological properties, HT has powerful in vivo antioxidant effects [3]. Moreover, recent data suggests that HT can improve mitochondrial function [4]. This feature has been previously described for different polyphenols [5–7]. However, despite mitochondria playing a key role in energy production during exercise [8], no evidence has been found to support any ergogenic potential of polyphenols [9, 10]. On the other hand, polyphenol supplementation has been reported to hinder exercise- induced skeletal muscle adaptations in both rodents and humans [11–13].

A plausible explanation comes from the antioxidant effects of polyphenols. Indeed, exercise adaptation is preceded by transient bursts of reactive oxygen species (ROS) production within contracting muscles [14]. These acute alterations in redox

homeostasis lead to a chronic, systemic enhancement of antioxidant machinery by improving the function and content of both enzymatic and non-enzymatic antioxidants [14–16]. In addition, ROS are important molecular messengers that regulate redox-sensitive proteins involved in vascularization, mitochondrial biogenesis, immune response and growth factor signaling [17, 18]. Accordingly, subjects who show high oxidative stress in response to acute exercise are known to have greater training adaptations than those subjects who show low oxidative stress in response to the same exercise [19]. Therefore, blunting the acute ROS production within each training session with antioxidants may prevent some long-term exercise-induced adaptations. Accordingly, rats that were supplemented with 25 mg/kg/d HT and exercised for 10 weeks do not increase their performance when compared with sedentary rats [20]. This effect can be attributed to the antioxidant effects of HT, as the anti- oxidant effect of HT rises with the dosage from 10 mg/kg/ d to 50 mg/kg/d HT in rodents [21].

However, under special conditions, polyphenols might become pro-oxidants [22]. In vitro studies show that as a result of their antioxidant activity, some oxidized metabolites are produced [22–24]. These metabolites in- duce oxidative damage to key proteins such as glutathione [23]. This paradoxical effect suggests that high polyphenol doses may induce a pro-oxidant environment. Similarly, high HT doses in vitro have been shown to increase ROS generation within tumor cells, leading to their apoptosis [25–27].

Since HT dosages up to 300 mg/kg/d are known to be safe for rodents [28], and a dosage of 20 mg/kg/d is known to exert an antioxidant effect [20, 21], we hypothesized that the consumption of a low (20 mg/kg/d) dosage of HT during endurance exercise would induce an antioxidant effect, while a high (300 mg/kg/d) dosage would reverse this effect by inducing systemic oxidative stress. Our purpose was to describe the effects of a low and high HT dose for 10 weeks on the physical capacity and redox state of both sedentary and exercised rats.

Methods

Animals

Male Wistar rats were purchased from Charles River (USA) at six weeks-old. The rats initially weighed 212 ± 13.5 g and were maintained in a well-ventilated room. This room was maintained under standard conditions of temperature (21 ± 2 °C) and relative humidity (40% to 60%) and under a reverse 12-h light/12-h dark cycle. Throughout the experimental period, all rats consumed water and standard chow ad libitum (2.9 kcal/g). Daily food and water intakes were monitored. All interventions lasted for 10 weeks. Rats were weighed weekly. Seventy-two hours after the last exercise was performed, rats were fasted overnight, anesthetized with pentobarbital

and sacrificed by bleeding. The experiments were approved by the ethics committee of the University of Granada (Granada, Spain; N°: 28/06/2016/116).

Hydroxytyrosol treatment

Rats receiving HT (Biomaslinic, S.L., Granada, Spain) were supplemented with a low dosage (20 mg/kg/d) or a high dosage (300 mg/kg/d) of HT. Supplementation began once the rats were allocated into the experimental groups and stopped 12 h before rats were euthanized. HT was diluted in water in an opaque drinking bottle. Water and HT were replaced every day in order to pre- vent HT oxidation. The dilution was adjusted weekly ac- cording to the weight of each rat and its average water intake. Based on our preliminary studies, this procedure is reliable for HT supplementation.

Sedentary rats

Twenty-four rats were allocated into 3 groups: sedentary (SED; n = 10), SED consuming 20 mg/kg/d HT (SED20; n = 7), and SED consuming 300 mg/kg/d HT (SED300; n = 7). All the rats performed a maximal velocity performance test prior to the beginning of the study (Test 1) and after 10 weeks of treatment (Test 2). The maximal velocity test was a progressive intensity running test (PanLab treadmill for 5 rats, model LE 8710R) starting at a velocity of 22 cm/s and increasing by 5 cm/s every minute, similar to the previous study [29].

Exercised rats

Thirty rats were allocated into 3 groups: exercised (EXE;n = 10), EXE consuming 20 mg/kg/d HT (EXE20; n = 10), and EXE consuming 300 mg/kg/d HT (EXE300; n = 10). Exercise training was divided into 2 mesocycles of 5 weeks each. Rats ran at 75% of their maximal velocity. Therefore, all rats allocated into exercised groups per- formed three maximal velocity tests (MVTs), as de- scribed above: Test 1 to establish their velocity during the first mesocycle; Test 2 to adjust the velocity for the second mesocycle; and Test 3 was performed 72 h after the last training session to evaluate maximal velocity capacity. After Test 1, rats were grouped by similar maximal velocity capacity as follows: EXE, 76.5 ± 10.12 cm/s; EXE20, 77.0 ± 8.50 cm/s; and EXE300, 77.4 ± 6.50 cm/s. Throughout the entire protocol, fatigue was defined as the point at which rats remained at the back of the treadmill on an electric shock pad for 5 s.

Each running mesocycle was identically designed. Rats started running for 20 min/d; this period was increased by 5 min every other day up to 65 min/d and was then maintained at 65 min/d until the end of the mesocycle. Moreover, physical work performed during each session was monitored as a reliable and continuous marker of exercise capacity [30]. We calculated the physical work performed by applying the formula: work (J) = force × vertical distance, where force = body weight (kg) × 9.8 m/ s2, and vertical distance = speed (m/min) × time (min). Therefore, rats becoming

fatigued before finishing the target time of a certain training session were removed from the treadmill and the time was recorded. This exercise protocol is known to induce both cellular and systemic redox adaptations [29].

Feed efficiency

Feed efficiency measures the ability of an animal to transform the calories ingested into body weight. We followed the formula: feed efficiency = weight gain (g) × caloric intake (kcal)− 1 [31]. The caloric intake was calculated by the daily average food consumed in each cage containing five rats.

Blood measurements

A portion of the blood obtained during the bleeding procedure was collected into heparin tubes for the measurement of hemoglobin (HGB) and hematocrit (HCT) using a KX-21 Automated Hematology Analyzer (Sysmex Corporation, Kobe, Japan). The remaining blood was centrifuged for 10 min at 3000 rpm in order to isolate plasma.

Mitochondrial isolation

Mitochondrial isolation was performed as previously de- scribed [32, 33]. One aliquot of the crude mitochondrial fraction was used for protein determination. The remaining samples were then centrifuged at 13000 x g for 3 min at 4 °C. The mitochondrial pellets were sus- pended in an appropriate volume of medium C (1 M aminocaproic acid, 50 mM Bis-Tris-HCl [pH 7.0]) to obtain a protein concentration of 10 mg/ml.

Plasma lipid peroxidation and mitochondrial organic HPx

The concentration of hydroperoxides (HPx), a specific and direct biomarker of lipid peroxidation, was deter- mined using a Sigma PD1 kit (St Louis, MO, USA). Absorbance changes at 560 nm were monitored by spectrophotometry.

Blood collected from the bleeding procedure was centrifuged for 10 min at 3000 rpm to isolate the plasma. Then, 40 µl of plasma was used for the quantification of HPx concentration in plasma. A total of 100 µg of protein from the mitochondrial fraction was used to deter- mine the mitochondrial concentration of organic HPx.

Statistical analysis

Results are shown as the mean ± SD. Homoscedasticity and normality were tested by Levene's test and the Kolmogorov-Smirnov test, respectively. Two-way re- peated measures ANOVAs were used to analyze performance during the maximal velocity tests, as well as to analyze weekly and mesocycle work. The different groups represented the between-subjects variable, and time was the intersubjects variable. A post hoc analysis was performed, and confidence intervals were adjusted using the Bonferroni correction when the effect was significant. One-way ANOVAs were used to

analyze the remaining data. The level of significance was set at $p < 0.05$. All analyses were performed using the Statistical Package for Social Sciences (SPSS, version 22 for Windows; IBM Corp., Armonk, NY).

Results

The one-way ANOVAs showed that weight gain (Fig. 1A) and feed efficiency (Fig. 1B) were unaffected by HT either in sedentary or in exercised rats throughout the study. However, exercise induced less weight gain and reduced feed efficiency in all the exercised groups ($p < 0.05$ for all groups and for both variables). The results (Fig. 1C) show that 300 mg/kg/d HT functions as anti- oxidant in the SED300 rats since this dose decreases the concentration of plasma HPx compared with the SED group ($p < 0.05$). However, we found that 300 mg/kg/d HT functions as a pro-oxidant agent in exercised animals. Indeed, the one-way ANOVA showed that the EXE300 group exhibited a higher plasma HPx concentration than the EXE and SED300 groups ($p < 0.05$ for both). Moreover, there was a trend ($p = 0.09$) toward a higher plasma HPx concentration in the EXE300 group compared with the EXE20 group. We isolated mitochondria from the gastrocnemius muscle of exercised rats, in order to determine the mitochondrial oxidative status. Despite organic mitochondrial HPx being 9%, 11% and 15% higher for EXE300 than for SED, EXE and EXE20 respectively, these results were not significant (Fig. 2).

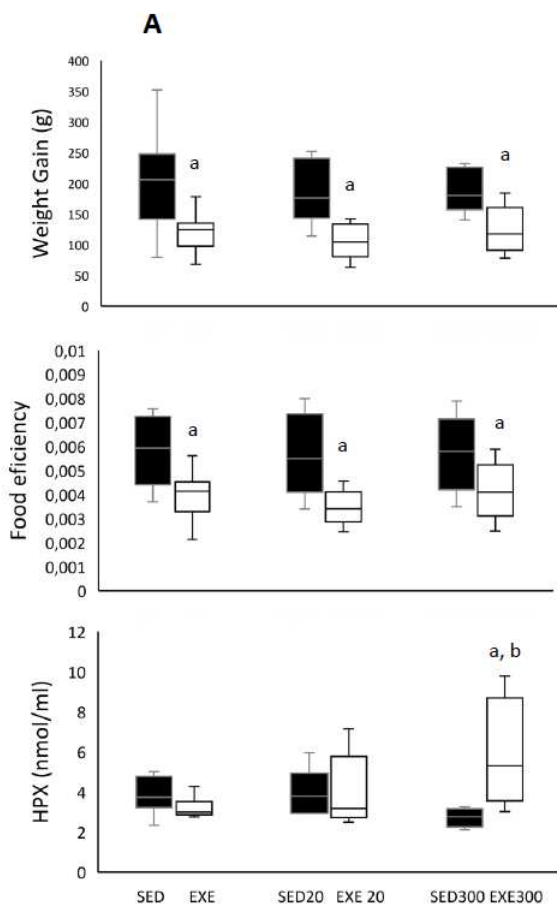

Fig. 1 High hydroxytyrosol intake becomes pro-oxidant in exercised rats. Weight gain (A) and feed efficiency (B) reported throughout the study. Hydroperoxides (C) were quantified in plasma at the end of the study. ap < 0.05 compared with the sedentary group. bp < 0.05 compared with the EXE group. cp < 0.05 compared with the SED group. SED, sedentary; EXE, exercised; 20, group supplemented with 20 mg/kg/d of hydroxytyrosol; 300, group supplemented with 300 mg/kg/d of hydroxytyrosol

One-way ANOVA was also used to analyze hematological data (Table 1). We found that the SED20 ($p < 0.001$) and SED300 ($p < 0.05$) groups had lower HGB levels than the SED group. Similarly, the SED20 group ($p < 0.01$) had a lower HCT than the SED group. The fact that the EXE300 group showed a lower HCT and lower HGB values ($p < 0.05$ for both) than the EXE20 group is noteworthy.

Two-way repeated measures ANOVA showed a significant group × time interaction ($p < 0.001$, eta2 = 0.645, 1- β = 0.999) when analyzing maximal running velocity (Fig. 3). A more thorough analysis showed that all sedentary groups lost their performance when comparing Test 1 with the final test (Fig. 3A; $p < 0.05$ for all groups). The analysis of the exercised animals is shown in Fig. 3B. The EXE and EXE300 groups increased their maximal velocity from Test 1 to Test 2 ($p < 0.05$) and a further increase was observed from Test 2 to Test 3 ($p < 0.05$). Both EXE ($p < 0.05$) and EXE300 ($p < 0.05$) groups showed a higher maximal velocity than the EXE20 group at the end of the study. Indeed, the maximal velocity of the EXE20 group remained unchanged during the three tests performed.

	HGB (g/dl)	HCT (%)
Sedentary		
SED	14.7±0.68	42.5± 2.09
SED20	10.8±1.54***	36.7±2.21**
SED300	12.7±1.75*	40.3±2.08
Exercised		
EXE	14.9±0.88	42.0±2.57
EXE20	15.2±0.51	43.9±2.36
EXE300	13.9±0.37#	40.3±2.02#

Table 1. Hematological parameters at the end of the study. HGB, hemoglobin; HCT, hematocrit; SED, sedentary; EXE; exercised; 20, 20 mg/kg/d of hydroxytyrosol; 300, 300 mg/kg/d of hydroxytyrosol. * p< 0.05; ** p < 0.01; *** p < 0.001 compared to the SED group. # p < 0.05 compared to the EXE20 group.

Daily physical work was monitored during each training session (Fig. 4). One-way ANOVA showed that rats in the EXE20 group ran less than those in the EXE group (p

< 0.001) and the EXE300 group ($p < 0.001$) when analyzing daily physical work throughout the entire protocol (Fig. 4A). Moreover, a significant group × time interaction ($p = 0.038$, eta2 = 0.155, 1- β = 0.622) was found when analyzing work performed during each mesocycle (Fig. 4B). Both the EXE and EXE300 rats increased ($p < 0.05$) their physical work from mesocycle 1 to mesocycle 2, while the EXE20 rats decreased their physical work ($p < 0.05$). Indeed, the EXE20 group performed less work ($p < 0.05$) than the EXE and EXE300 groups at mesocycle 2.

Discussion

We aimed to describe the effects of a low and a high HT dose on the physical capacity and redox state of both sedentary and exercised rats. Our results suggest that 20 mg/kg/d of HT intake over 10 weeks may hinder training-induced physical capacity enhancement, whereas a dosage of 300 mg/kg/d HT does not. This effect may be related to changes in the systemic redox environment, as the 300 mg/kg/d dosage increases plasma HPx levels. All the supplemented groups (i.e., exercised and sedentary) showed similar weight gain, suggesting no evidence for potential toxicity for the doses used [28]. However, the lower levels of HGB together with the higher HPx levels reported in the EXE300 group should be further studied as they may reflect a harmful effect of the higher HT dose when combined with exercise.

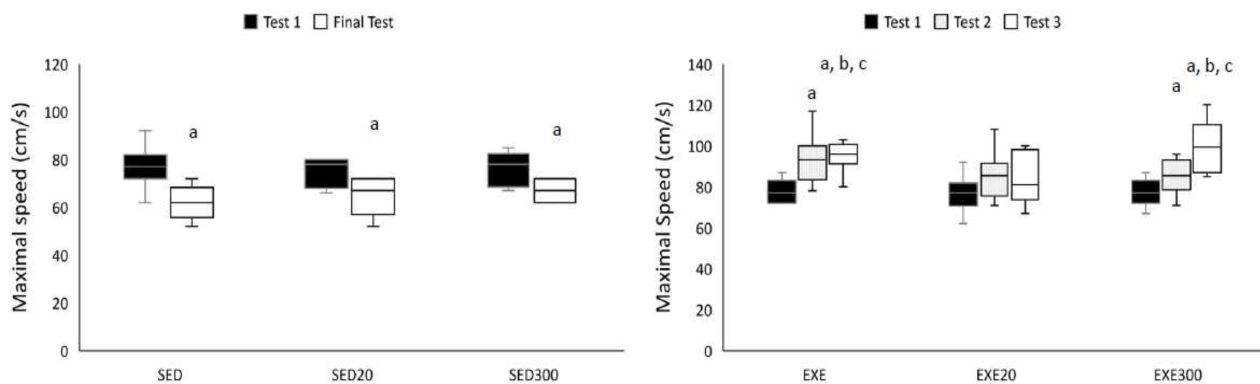

Figure 3. Maximal velocity performance is influenced by hydroxytyrosol. Maximal velocity tests in sedentary rats (A) were performed prior to and at the end of the study. The maximal velocity test in exercised animals (B) was performed before the study, after 5 weeks and at the end of the study. a $p < 0.05$ compared to Test 1. b $p < 0.05$ compared to Test 2. c $p < 0.05$ compared to the SED20 group in Test 3. SED, sedentary; EXE, exercised; 20, group supplemented with 20 mg/kg/d of hydroxytyrosol; 300, group supplemented with 300 mg/kg/d of hydroxytyrosol.

The antioxidant capacity of the endurance exercise is well recognized. This systemic effect is achieved because within contracting muscles, there are multiple sites of ROS production, including the mitochondrial respiratory chain [34] and the plasma membrane [35]. These ROS act as signaling molecules that activate molecular pathways, which chronically lead to improvements in the function and content of endogenous antioxidants [15, 36]. It should be highlighted that the exercise protocol applied induces a strong antioxidant effect within skeletal muscles, which is reflected in blood plasma as a decrease in HPx concentration [29]. In this scenario, the loss of performance reported in the EXE20 group may be a consequence of the antioxidant effect of this dose. However, we have not found statistical evidence supporting that the 20 mg/ kg/d dosage of HT has antioxidant effects. This occurred even though this dose was previously described as an anti- oxidant in rodents [21]. A plausible explanation is that the antioxidant effect induced by endurance exercise may mask any additional antioxidant effects induced by the low HT dose. Moreover, Feng et al. [20] showed that 25 mg/kg/d HT blunts the autophagic response of exercised rats. Importantly, the autophagic response to exercise is known to be ROS-dependent [37, 38]. Taken together, our results and those previously published suggest that dosages close to 20 mg/kg/d may hinder some exercise adaptations, probably due to their antioxidant effect.

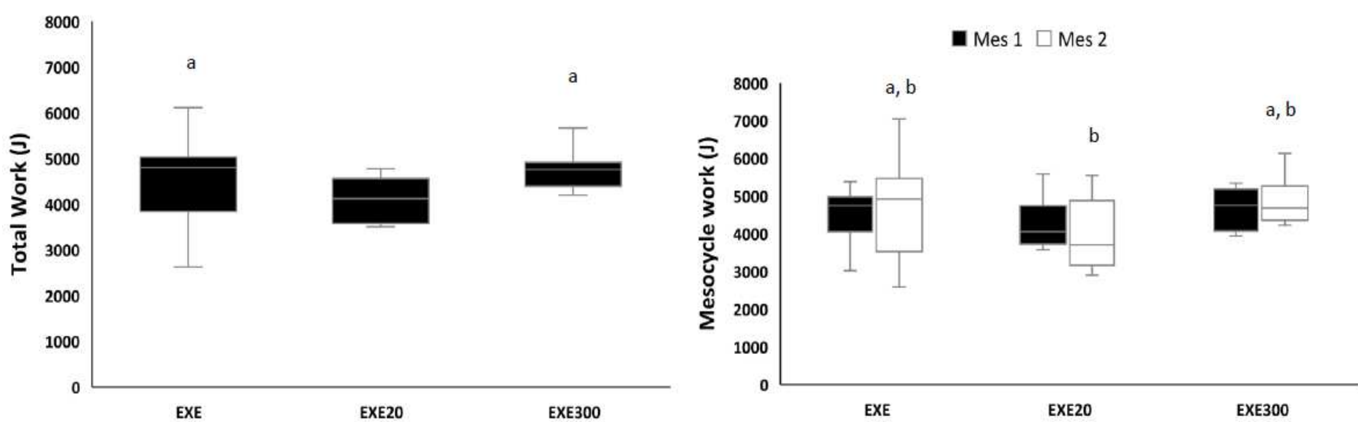

Figure 4. Hydroxytyrosol affects physical work performed during the training protocol. Total work (A), mesocycle work (B) and weekly work were measured as the average of the daily work (C). a $p < 0.05$ compared to the EXE20 group. b $p < 0.05$ compared to MES1. c $p < 0.05$ compared to the EXE300 group. d $p < 0.05$ compared to the EXE group. EXE, exercised; EXE20, EXE supplemented with20 mg/kg/d of hydroxytyrosol; EXE300, EXE supplemented with300 mg/kg/d of hydroxytyrosol; MES, mesocycle.

It should be highlighted that we have not found evidence for mitochondrial redox alterations within exercised animals. Several potential explanations for the differences found between the plasma and mitochondrial compartments, especially for the EXE300 group, are suggested. First, mitochondria can alter their inner membrane structure in response to exercise in order to prevent their oxidative damage. Recent findings from our group showed that physical exercise stimulates the assembly of mitochondrial complexes in Supercomplexes (SCs), and prevents excessive ROS production [29]. Exercise can increase mitochondria resilience to excessive oxidative stress. In addition, previous studies on contracting muscles have reported that the main source of ROS production in response to contractile activity is the enzyme NADPH-oxidase, wich is mainly located in the sarcoplasmic reticulum [35]. Finally, data on other polyphenols suggest that plasma proteins and lipids can be rapidly oxidized in response to high polyphenol concentrations [39, 40]. Altogether, these data suggest that exercise training may adapt mitochondria to be protected from excessive oxidative stress. However, the high polyphenol dose in conjunction with the ROS produced by the extra mitochondrial enzymes may result in in- creases in circulating lipid peroxide.

Table 1 shows that sedentary animals consuming HT may undergo hematological dysfunction. However, Wistar rats are known to show high variability in response along hematological parameters. Previous studies on sedentary rats subjected to an intake of 25 mg/kg/d of the polyphenol quercetin showed lower hematological values than exercised rats, wich is consistent with the data of the present study. Indeed, HCT values below 35% and HGB values below 11 g/dL have been reported [41]. These results are consistent with the concept that polyphenols may interfere with circulating proteins [39, 40]. However, it seems that exercise can prevent such an effect but only when a low dose of HT is administered. In fact, we found a lower HGB concentration in the EXE300 group com- pared to the EXE20 group. Therefore, even though the EXE300 rats may have maintained their physical capacity, if HPx and HGB are considered in conjunction, these data may reflect the beginning of a harmful process. The optimal HT dose and the supplementation period required to maximize benefits (i.e., endurance performance) without inducing harmful effects must be elucidated in future studies. Furthermore, the high variability found in HGB and HCT data reported here and previously in response to polyphenol intake [41] suggests that Wistar rats may not respond homogenously to HT intake.

Conclusions

In summary, HT dosages ranging from 20 mg/kg/d to 300 mg/kg/d for 10 weeks induced an antioxidant response in a dose-dependent manner in sedentary animals. However, 20 mg/kg/d HT decreased the running capacity when this dose was supplemented during exercise, whereas 300 mg/kg/d HT was able to maintain and even increase the running capacity. This effect might be due to a systemic pro-oxidant effect induced when a high HT dose is supplemented during exercise training. However, mechanistic studies should address the optimal HT doses and supplement duration necessary to increase physical capacity through inducing oxidative stress with- out resulting in any harmful effects.

Abbreviations

EXE: exercised; HCT: hematocrit; HGB: hemoglobin; HPx: hydroperoxide; HT: hydroxytyrosol; ROS: reactive oxygen species; SED: sedentary

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Availability of data and materials

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

Authors' contributions

RAC was involved in the conception, design, and acquisition and analysis of the data as well as drafting the manuscript; AFS was involved in the conception, design, and acquisition and analysis of the data as well as drafting the manuscript; FJRH was

involved in the conception, design, and acquisition and analysis of the data as well as drafting the manuscript; AVJ was involved in the design and analysis of the data; CC was involved in the conception and design. All authors have given final approval of the version to be published.

Ethics approval and consent to participate

The experiments were approved by the ethics committee of the University of Granada (Granada, Spain; N°: 28/06/2016/116).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Estudios enviados a revisión (no publicados)

Estudio I (enviado a revisión)

UNIVERSIDAD DE GRANADA

Hydroxytyrosol modifies skeletal muscle GLUT4/AKT/RAC1 axis in trained rats.

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Abstract

Exercise induces a number of healthy effect including a rise in skeletal muscle (SKM) glucose uptake. These adaptations are at least in part due to the reactive oxygen species produced within SKM. Which is in agreement with the notion that antioxidant supplementation blunts these adaptations. Here we tested whether hydroxytyrosol (HT), the main polyphenol of olive oil, would modify the molecular regulators of glucose uptake when HT is supplemented during exercise. Rats were included into sedentary and exercised (EXE) groups. EXE group was further divided into a group consuming the minimum HT dose inducing an antioxidant effect (0.31 mg/kg/d; EXElow), a moderate HT dose (4.61 mg/kg/d; EXEmid) and a control group (EXE). EXE raised GLUT4 protein content in SKM, and RAC1 and AKT activity as well. Furthermore, EXElow blunted GLUT4 protein content and AKT activity while EXEmid showed a down-regulation of the GLUT4/AKT/RAC1 axis. Therefore, a low to moderate dose of HT, when supplemented as an isolated compound, might alter the beneficial effects of exercise regarding insulin signaling and glucose uptake in rat SKM.

Key words: insulin; exercise; glucose; skeletal muscle

Abbreviations

SKM: skeletal muscle

ROS: reactive oxygen species

AKT: protein kinase B

GLUT4: glucose transporter 4

RAC1: Ras-related C3 botulinum toxin substrate 1

HT: hydroxytyrosol

EXE: exercised rats

EXElow: exercised rats supplemented with 0.31 mg/kg/d of HT

EXEmid: exercised rats supplemented with 4.61 mg/kg/d of HT

1. Introduction

Exercise training improves insulin resistance in subjects at risk of develop type 2 diabetes[1]. ROS producing within contracting muscles may be important transductor for this adaptations since antioxidants intake prevents the induction of molecular regulators of insulin sensitivity by exercise[2]. In fact, ROS triggers insulin-signaling through protein kinase b (AKT) activation[3]. AKT increases glucose uptake by translocating GLUT4 to the cellular membrane[4]. Moreover, a novel mechanism suggest that RAC1 regulates exercise-induced glucose uptake in a mechanism mediated by ROS[5].

High dose olive oil intake improves exercise performance[6] and enhances cellular membrane integrity during training[7]. The main polyphenol found in olive oil is HT which at doses of 20mg/kg/d is highly antioxidant[8] and blunts endurance capacity in trained rats[9], suggesting that a lower HT dose may preserve SKM adaptations to exercise. Here, we aimed to study the effects of the lower HT dose inducing an antioxidant effect and a moderate HT dose on GLUT4/AKT/RAC1 axis in SKM of trained rats.

2. Methods

2.1. Animals

Male Wistar rats were purchased from Charles River (USA) at six weeks-old. The rats initially weighed 200 ± 15.8 g and were maintained in a well-ventilated room under standard conditions. All interventions lasted for 10 weeks. Rats were randomly allocated into a sedentary (n = 6) or exercised groups (n=18) for ten weeks. Exercised groups were divided into 3 groups (EXE, n=6; EXElow, n=6; EXEmid, n=6). Exercise training and testing has been previously described[9]. Briefly, rats run 5 d/week for 10 weeks at 70% of their maximal velocity which was adjusted after 5 weeks of training. Seventy-two hours after the last exercise was performed, rats were fasted overnight, anesthetized with pentobarbital and sacrificed by bleeding. The soleus muscle was harvested for analysis. The experiments were approved by the ethics committee of the University of Granada (Granada, Spain; N°: 28/06/2016/116).

2.2. Hydroxytyrosol treatment

ExeLow animals were supplemented with 0.31mg/kg/d of HT which is the minimum antioxidant dose described[10]. ExeMid group received a moderate dose of HT (4.61 mg/kg/d). HT was diluted in water in an opaque drinking bottle to prevent oxidation. The dilution was adjusted weekly according to the weight of each rat and its average water intake. This procedure is reliable for HT supplementation[9]. Supplementation stopped 12 h before rats were euthanized.

2.3. *Quantitative real time (qRT)-PCR*

We used the Real-time Ready Custom Panel 96 (Roche, Barcelona, Spain), which is a two-step qRT-PCR platform. Which has been previously described in detail[11]. The RealTime Ready Custom Panel 96 (Roche, Barcelona, Spain) included the following specific primer pairs: Slc2a4 (Assay ID 500810, Roche, Barcelona, Spain). The expression level of each gene was analyzed with RT2 Profiler PCR Array Data Analysis software (version 3.4, SABiosciences). Changes in gene expression were expressed as fold changes (Fc).

2.4. *Western blot*

Samples were proceded as we previously described[11] and the membranes were probed with the following antibodies: anti-GLUT4 (sc-53566) (1:100 in 5 % non-fat milk), anti-AKT (C67E7) and anti-phospho-AKT (Ser473, D9E) both 1:1000 in 5% BSA were acquired from Cell Signalling Technologies (Beverly, MA, USA), anti-Hsp-70 (sc-7298) (1:500 in 5% non-fat milk).

2.5. *Rac1 activation assay*

Ra1 activity was measured in the supernatant of muscle lysates using a commercially available Rac1 activation assay kit (ab211161). In short, lysates were incubated with agarose beads to selectively isolate and pull-down the active form of Rac (GTP-Rac) from samples. GTP-Rac was detected by western blot analysis using an anti-Rac1 specific monoclonal antibody, which is provided by the kit.

2.6. *Statistical analysis*

Results are shown as the mean ± SD. Homoscedasticity and normality were tested by Levene's test and the Kolmogorov-Smirnov test, respectively. One-way ANOVAs were used to analyse the data. A post hoc analysis was performed, and confidence intervals were adjusted using the Bonferroni correction when the effect was significant. The level of significance was set at $p < 0.05$. All analyses were performed using the Statistical Package for Social Sciences (SPSS, version 22 for Windows; IBM Corp., Armonk, NY).

3. Results

We observed that trained animals showed higher maximal running velocity and lower weight gain than sedentary rats. However, HT did not alter these parameters when supplemented during exercise (Table 1).

GLUT4 mRNA levels tended to raise in the soleus of EXE animals but this effect was significant in HTMid while no effect was detected in HTLow rats (Figure 1A). However, at the protein level, GLUT4 raised only in EXE animals (Figure 1B). Similarly, we found that insulin signaling was impaired in the animals consuming HT read by the phosphor-

AKT(Ser473)/AKT within SKM (Figure 1C). We next assessed RAC1 activity as a possible compensatory mechanism for GLUT4 translocation to the plasma membrane independently from the canonical insulin signaling[12]. We observed that exercise increased RAC1 activity in skeletal muscle and that this effect was maintained in EXELow but hampered in EXEMild (Figure 1D).

4. Discussion

SKM adaptations to exercise are highly dependent upon ROS production within contracting muscles. Despite that olive oil consumption improves glucose uptake in type 2 diabetes subjects[13] and that it improves exercise adaptations in rats[8], in the present study we show that low doses of HT supplemented during exercise are able to disturb the molecular regulators of insulin signaling in SKM. In fact, we observed that exercise raises basal AKT and RAC1 activity as well as GLUT4 protein content within SKM whereas HT supplementation blunts those adaptations.

Deleterious effects on exercise performance have been reported in rodents consuming 20mg/kg/d9, a dose with high antioxidant potential within SKM[8]. Notably, here we used significant lower HT doses, but they are still able to rise plasma antioxidant capacity[10]. Previous findings support the notion that antioxidant supplementation can blunt exercise-induced insulin signalling by impeding AKT activation[3]. In addition, it is known that RAC1 controls glucose uptake during exercise in a ROS-dependent manner[4]. Here we report for the first time that antioxidant supplementation prevents the training-induced adaptation on AKT and RAC1 activity. It should be noted that RAC1 is a non-canonical insulin-stimulated protein that controls skeletal muscle glucose uptake within exercised muscle by promoting GLUT4 translocation to the plasma membrane[15]. In fact, glucose uptake via RAC1 occurs independently from insulin signalling through AKT12. Importantly, we do not detect changes in maximal exercise capacity which may indicate that 1) low to moderate HT doses hampers exercise-induced basal glucose metabolism but not glucose uptake during exercise and/or that 2) the blunted molecular signalling regulating glucose uptake precedes the loss of exercise capacity. Moreover, the moderate HT dose blunts the entire AKT/RAC1 axis whereas the low dose only hampers AKT activity. Thus, suggesting that RAC1 activity may be essential for basal glucose uptake as it appear less sensitive to mild SKM homeostatic changes.

It should be noted that we observed that GLUT4 mRNA significantly raised in response to a moderate dose of HT together with a numerical but not significant increase in GLUT4 protein content. This is a conspicuous finding as ROS induces GLUT4 transcription through p38 mitogen-activated protein kinase[16]. In this regards, we must acknowledge that the main limitation of the present study is that we have not quantified SKM oxidative status. However, the redox reactions produced in living cells following polyphenol supplementation are poorly understood. For instance, following

its antioxidant activity, some of the metabolites produced became pro-oxidant[17]. This pro-oxidant effect has been reported in exercised rats supplemented with 12mg/kg/d of quercetin[18]. On the other hand, in vitro experiments show that low HT doses ranging from 0.1 to 10 ɥM can modulate mitochondrial metabolism by activating PGC-1α promoter activity[19]. Thus, it is therefore possible moderate HT dose might modulate GLUT4 gene expression through epigenetic mechanisms and/or by redox mechanisms.

In conclusion, HT supplementation reduces the basal AKT and RAC1 activity induced by endurance exercise within SKM in rats, and this effect seems to be in a dose-dependent manner based on the low to moderate dose results, which blunted the AKT/RAC1 axis.

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7. Author contributions

Conception and design of the study: RAC and JRH. Acquisition of data: SAF, JAV, FJRO, JPD, ARR. Analysis and interpretation: RAC, JRH, JAV, FJRO. Drafting the article: RAC and SAF. All the authors approved the final version.

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Figure Caption

Figure 1. Molecular regulators of glucose uptake within soleus muscle

A) Moderate hydroxytyrosol dose increases GLUT4 mRNA levels in exercised animals. B) GLUT4 protein content raises following 10 weeks of training. C) Both a low and a moderate hydroxytyrosol dose blunts the exercise-induced increment of the ratio between AKT phosphorytaltion at Ser^{473} and total AKT. D) Exercise increases RAC1 activity. This effect is maintained when a low HT intake is supplemented but hampered when a moderate HT dose is consumed. SED, sedentary animals; EXE, exercised animals; EXElow; exercised and supplemented with a low hydroxytyrosol dose (i.e. 0.31mg/kg/d); EXEmid; exercised and supplemented with a low hydroxytyrosol dose (i.e. 4.61mg/kg/d). * $p < 0.05$ if compared with SED.

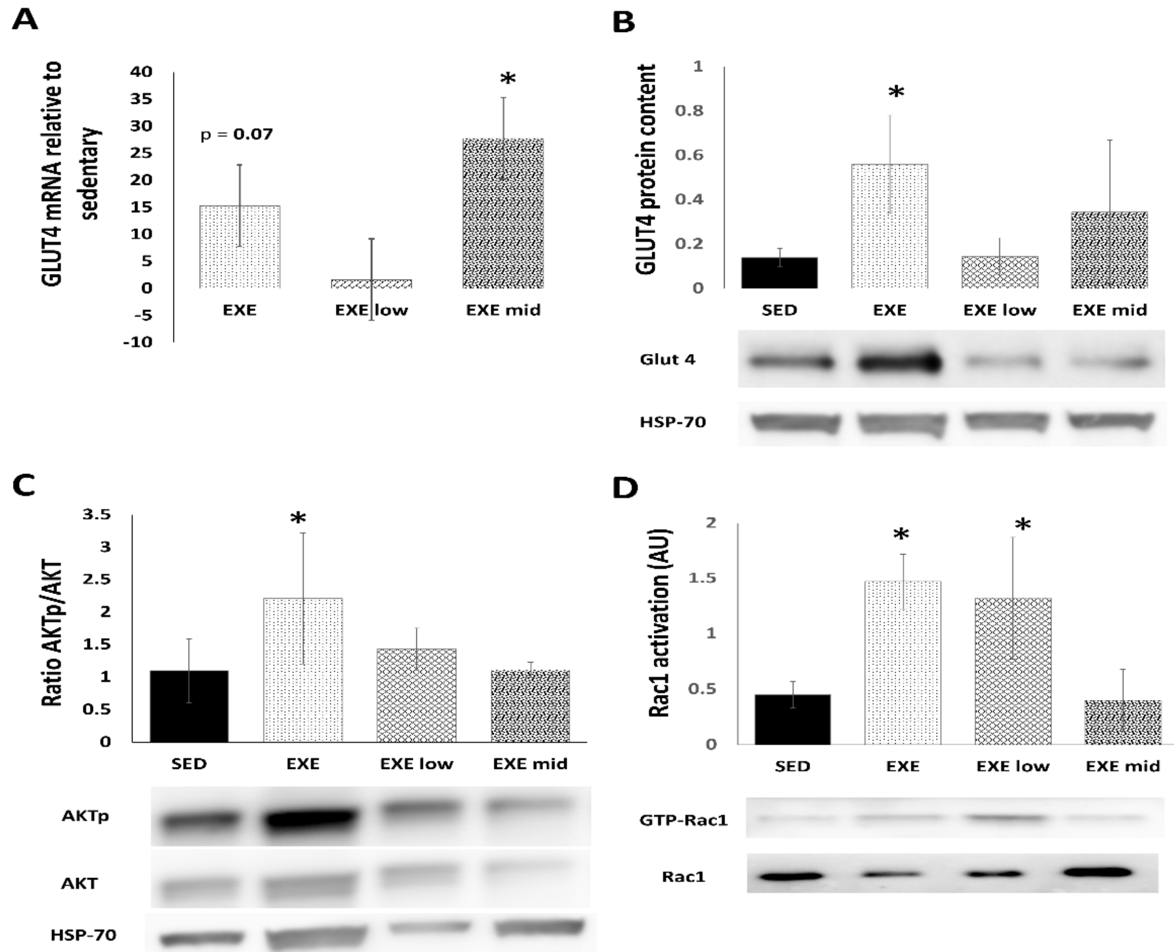

Table 1. Physical performance and weight gain.

	Control	EXE	HTLow	HTMild
Preliminary Maximal Velocity (cm/s)	76.4 ±5.9	77.8 ± 5.8	76.2 ± 8.0	75.2 ± 5.1
Final Maximal Velocity (cm/s)	65.3 ± 7.2*	92.3 ± 7.6 *†	86.6 ± 13.8*†	93.8 ± 7.9*†
Weight Gain (g)	216 ± 42.3	126 ± 26.5†	118 ± 19.5 †	134 ± 23.7†

Data is shown as mean ± SD (n = 6). EXE; exercised; HTLow; exercised and supplemented with 0.31 mg/kg/d of hydroxytyrosol; HTMild; exercised and supplemented with 4.61 mg/kg/d of hydroxytyrosol. * Statistically different vs preliminary measurement; † statistically different vs controls.

Estudio II (enviado a revisión)

Physiological Doses of Hydroxytyrosol Modulate Gene Expression in Skeletal Muscle of Exercised Rats.

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Abstract: In the present study we tested whether physiological doses of hydroxytyrosol (HT) may enhance the mRNA transcription of key metabolic genes in exercised skeletal muscle. Two groups of exercise-trained Wistar rats, HTlow and HTmid, were supplemented with 0.31 and 4.61 mg/kg/d of HT, respectively, for ten weeks. These dosages can be achieved by consuming a nutraceutical extra virgin olive oil. Another two groups of rats were not supplemented with HT, one remained sedentary and the other one exercised. After the experimental period, the animals were overnight fasted and the soleus muscle was removed for qRT-PCR analysis. Our results show that the consumption of 4.61 mg/kg/d of HT during exercise increases the mRNA expression of important metabolic proteins. Indeed, 4.61 mg/kg/d of HT may upregulate long-chain fatty acid oxidation, lactate and glucose oxidation as well as mitochondrial Krebs cycle in trained skeletal muscle.

Keywords: polyphenols; mitochondria; glycolysis; gene expression

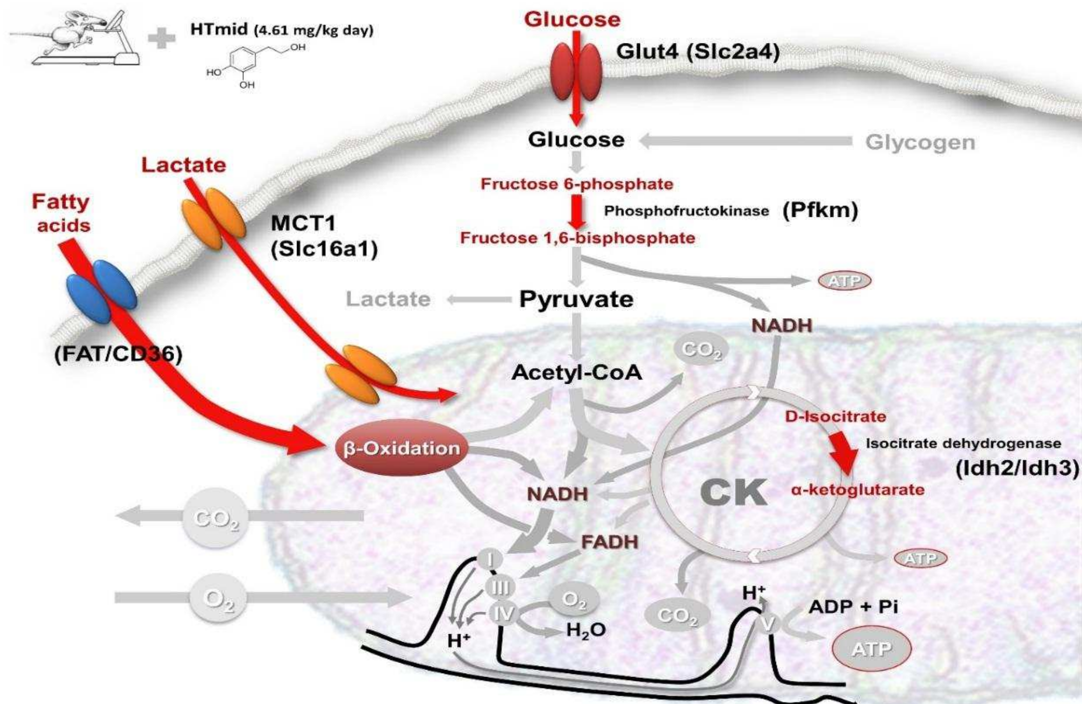
1. Introduction

Extra virgin olive oil (EVOO) is a monounsaturated fatty acid widely consumed as part of the Mediterranean diet. Consumption of EVOO has been inversely associated

with the development and management of a number of metabolic diseases, including cardiovascular diseases and type 2 diabetes [1,2]. Moreover, when combined with exercise, EVOO can improve the fatty acid composition of the biological membranes [3], thereby EVOO improves mitochondrial resistance against exercise-induced oxidative stress [4]. Accordingly, 30 days of EVOO supplementation to rats reduced the deleterious effect that highly intense exercise has on mitochondrial ultrastructure [5]. During moderate exercise intensity, EVOO has been reported to improve exercise performance [6]. It is known that during a moderate exercise intensity, mitochondria plays a primary role in skeletal muscle performance [7]. Consequently, EVOO supplementation may boost mitochondrial metabolism during exercise.

However, the potential molecular mechanisms by which EVOO may improve skeletal muscle and mitochondrial function is unclear. For instance, polyphenols present in olive oil are known to have antioxidant and anti-inflammatory systemic properties [8,9]. In fact, high concentrations of HT can modulate skeletal muscle metabolism [10-13]. Nevertheless, whether physiological doses of HT (i.e. doses that can be achieved with EVOO supplementation) can regulate skeletal muscle metabolism is unknown.

Recent reports suggest that old people consuming a Mediterranean diet supplemented with EVOO influences the methylation of genes related to metabolism in peripheral white blood cells [14]. Moreover, *in vitro* studies suggest that physiological doses of HT ranging from 0.1–10 μM activate the PPARG coactivator 1 alpha (PGC-1α) promoter activity, thereby increasing mitochondrial content in adipocytes [15]. These results suggest that physiological doses of HT , which can be achieved by nutraceutical EVOO consumption, could improve skeletal muscle metabolism by modulating epigenetic changes in key DNA promoters. A synergistic effect of EVOO on mitochondrial membranes when consumed during an exercise period has been described [3]. Therefore, we tested whether physiological doses of HT can induce the mRNA expression of key metabolic genes regulating mitochondrial fuel oxidation



(Figure 1). Figure 1. Schematic representation of the metabolic pathways studied (red arrows). Exercised rats supplemented with 4.61 mg/kg/d of HT increased the mRNA levels of all the studied proteins. HT, hydroxytyrosol; GLUT4, glucose transporter 4; MCT1, monocarboxylate transporter 1; FAT/CD36, fatty acid translocase/CD36; CK; Krebs cycle. 2. Materials and Methods

Animals

Six-weeks-old male Wistar rats were purchased from Charles River (USA). The rats initially weighed 200 ± 15.8 g and were maintained in a well-ventilated room. This room was maintained under standard conditions of temperature (21 ± 2 °C) and relative humidity (40–60%) and under a reverse 12 h light/12 h dark cycle. All rats were allowed ad libitum access to water and a standard chow (2.9 kcal/g) throughout the experimental period. Daily food and water intakes were monitored. All interventions lasted for 10 weeks. Rats were randomly allocated into a sedentary group (SED, n = 6) or one of the exercised groups for ten weeks. The exercised group were three: EXE (n = 6), HTlow (n = 6) and HTmid (n = 6) (see descriptions below). 72 h after the last exercise was performed, rats were fasted overnight, anesthetized with pentobarbital and killed by bleeding. The soleus muscle was collected for analysis. The experiments were approved by the ethics committee of the University of Granada (Granada, Spain; N°: 28/06/2016/116).

Hydroxytyrosol treatment

Hydroxytyrosol was kindly provided by EXTRACTOS Y DERIVADOS, S.L. (Granada, Spain). We calculated the low HT dose as 2.5-fold the daily amount consumed by the Spanish population (i.e. 0.31 mg/kg/d: HTlow group). We also tested a HT dose that can be achieved by nutraceutical enrichment of EVOO, this mid-level dose was calculated as 35-fold the daily amount consumed by the Spanish population (i.e. 4.61 mg/kg/d: HTmid group). Note that these dosages are quite lower than the usually tested *in vivo,* which range from 10–50 mg/kg/d (11,16). Hydroxytyrosol was diluted in water in an opaque drinking bottle to prevent oxidation. The dilution was adjusted weekly according to the weight of each rat and its average water intake. This procedure is reliable for HT supplementation (13). Supplementation stopped 12 h before rats were euthanized. The exercised control group (EXE) were not supplemented with HT.

Quantitative real time (qRT)-PCR

We used the RealTime ready Custom Panel 96 (Roche, Barcelona, Spain), which is a two-step qRT-PCR platform. Briefly, total ribonucleic acid (RNA) was extracted from the soleus muscle using the PeqGOLD HP Total RNA kit (Peqlab, Germany), according to the manufacturer's recommendations. Isolated RNA was then treated with Turbo DNase (Ambion, Life Technologies, Carlsbad, CA, USA). Final RNA concentration and quality were determined using a NanoDrop2000 (NanoDrop Technologies, Winooski, Vermont, USA). Complementary DNA (cDNA) was synthesized from total RNA using the iScript advanced cDNA Synthesis Kit (Bio-Rad Laboratories, California, USA). The RealTime ready Custom Panel 96 (Roche, Barcelona, Spain) included the following specific primer pairs: Slc2a4 (Assay ID 500810, Roche, Barcelona, Spain), Slc16a1, (Assay ID 506515, Roche, Barcelona, Spain), Cd36, (Assay ID 506518, Roche, Barcelona, Spain), Pfkm, (Assay ID 506511, Roche, Barcelona, Spain), Idh3a (Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial Precursor (EC 1.1.1.41)(Isocitric dehydrogenase subunit alpha)(NAD(+)-specific ICDH subunit alpha) [Source:UniProtKB/Swiss-Prot;Acc:Q99NA5]), Idh2 (Assay ID 506513, Roche, Barcelona, Spain). The Rat 18S rRNA sequence (Assay ID 502300, Roche, Barcelona, Spain) and hydroxymethylbilane synthase (Hmbs) (Assay ID 502305, Roche, Barcelona, Spain) were used as reference genes. The cDNA was then subjected to qRT-PCR analysis with the LightCycler® 480 Probes Master Kit (Roche, Barcelona, Spain) on a LightCycler® 480 Instrument II detector (Roche, Barcelona, Spain). The PCR conditions were 1 cycle at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s, and 1 cycle at 40° C for 30 s. The expression level of each gene was analyzed with RT^2 Profiler PCR Array Data Analysis software (version 3.4, SABiosciences). Changes in gene expression were expressed as fold changes (Fc).

Statistical analysis

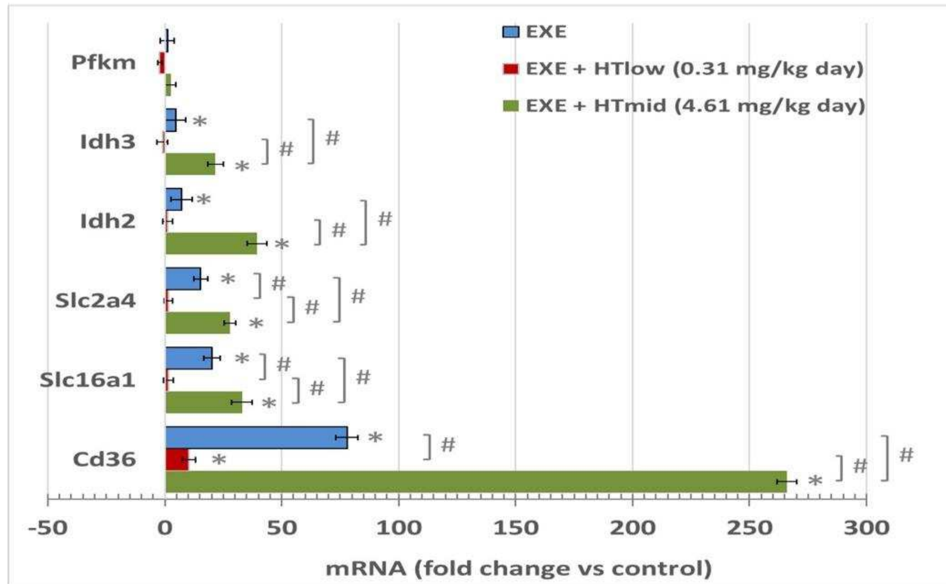
Results are shown as the mean ± SD. Homoscedasticity and normality were tested by Levene's test and the Kolmogorov-Smirnov test, respectively. One-way ANOVA was used for data analysis. A *post hoc* analysis was performed, and confidence intervals were adjusted using the Bonferroni correction when the effect was significant. The level of significance was set at $p < 0.05$. All analyses were performed using the Statistical Package for Social Sciences (SPSS, version 22 for Windows; IBM Corp., Armonk, NY).

3. Results

First, we analyzed the mRNA level of the selected glycolytic genes found in the soleus skeletal muscle. EXE animals showed an increased expression of the glucose transporter 4 (GLUT 4) and the monocarboxylate transporter 1 (MCT1) when compared with sedentary animals (Figure 2). Notably, this effect was magnified in HTmid animals (Figure 1 and 2), but absent in the HTlow group (Figure 2). None of the experimental groups showed an increase in the main regulatory enzyme of glycolysis, that is, phosphofructokinase (PFK).

We next assessed the expression of isocitrate dehydrogenase (IDH), which is an enzyme belonging to the Krebs cycle. This enzyme catalyzes the conversion of isocitrate to α-ketoglutarate (Figures 1 and 2). Notably, it has been reported that IDH2 isoform is mainly present in oxidative fibers, while IDH3 isoform is found in glycolytic fibers [17]. Both IDH2 and IDH3 mRNA levels were increased in the skeletal muscle of EXE animals (Figure 2). However, HTmid animals showed an 84-fold expression of IDH2 ($p < 0.001$) and a 2.5-fold expression of IDH3, which were higher than their expression in EXE and HTlow groups.

Finally, we investigated the mRNA levels of the fatty acid transporter fatty acid translocase (CD36) (Figures 1 and 2). Gene expression of CD36 was induced in all the experimental groups (Figure 1). However, HTmid group showed a higher expression of



CD36 than the EXE and HTlow groups (Figure 1 and 2).

Figure 2. Relative mRNA levels of key metabolic pathways. Data are shown as fold change compared to control (sedentary) animals and as mean ± SD. EXE, exercised animals; HT, hydroxytyrosol; PFKm, phosphofructokinase; IDH, isocitrate dehydrogenase; Slc2a4; glucose transporter 4; Slc16a1; monocarboxylate transporter 1; CD36; fatty acid translocase/CD36. * p <0.05 compared to sedentary animals; # p <0.05.

4. Discussion

In the present study, we demonstrate that HT supplementation at physiological doses during exercise can upregulate the expression of key metabolic proteins. Notably, this effect seems to be dose-dependent because we found that 4.61 mg/kg/d HT induce higher mRNA expression of metabolic genes than 0.31 mg/kg/d HT. To date, the *in vivo* effects of HT have been mainly tested in the context of the antioxidant effects induced by dosages ranging from 10–50 mg/kg/d [11, 16]. In addition, HT inducing effects on skeletal muscle metabolism has also been tested in response to ~20mg/kg/d of HT [10,11,13]. Our data demonstrate that the medium physiological dose of HT (4.6mg/kg/d) can increase the expression of key metabolic genes in skeletal muscle of trained rats. This is important because this HT dose can be achieved by nutraceutical EVOO consumption.

Mitochondria are the hub of energy production, however, mitochondria can produce superoxide as a result of the metabolic activity occurring under heavy exercise, thereby mitochondrial oxidative damage is generated [18]. A higher number

of antioxidant enzymes was found in trained skeletal muscle to face oxidative bursts [19]. Moreover, mitochondria in trained skeletal muscle show a high plasticity, being able to dynamically change their shape to maximize energy production and minimize oxidative damage [20, 21]. In this context, antioxidant supplementation, such as the widely tested HT dosages ranging from 10–50mg/kg/d [11], may prevent the skeletal muscle adaptations induced by exercise [22].

Our results show that HT increases the gene expression of various proteins relevant for exercise metabolism. For instance, glucose oxidation is important due to its contribution to both anaerobic and aerobic glycolysis [7]. In this regard, GLUT4 is a transporter facilitating glucose uptake from the blood and its delivery into the cytosol of the skeletal muscle cell [23]. In addition, the monocarboxilate trasporter MCT1 is responsible of the lactate uptake from the circulation for oxidation [24-26]. Another key metabolic transporter is CD36, which can deliver circulating long-chain fatty acids into the mitochondria for B-oxidation [27, 28]. Finally, we also determined IDH gene expression as a marker of Krebs cycle function. It is noteworthy that IDH2 is mainly expressed in oxidative type I fibers, whereas IDH3 is mainly expressed in glycolytic fibers [17,29]. We found a higher expression of IDH2 and a moderate, but significant, expression of IDH3 induced by exercise. This result is in line with the phenotype of the studied muscle, which is considered a red or oxidative muscle due to its high composition of type I oxidative fibers. Moreover, similar expression pattern but magnified was also found in animals consuming 4.6 mg/kg/d of HT compared with EXE animals. In conjunction, our results suggest that physiological doses of HT potentiate the exercise-induced mRNA expression of key metabolic proteins. However, several differences were found between the two doses tested. While 0.31mg/kg/d only modulates fatty acid oxidation as evidenced by an increase in mRNA levels of CD36,a dosage of 4.6 mg/kg/d may enhance the metabolic pathways related to long-chain fatty acid oxidation, lactate and glucose oxidation, as well as mitochondrial Krebs cycle. However, the mechanisms by which HT induces mRNA expression is mostly unknown.

Recently, the biological effects of HT have been tested beyond its antioxidant an anti-inflammatory effects. Indeed, a relatively new research topic aims to identify the epigenetic effects that HT can induce on the DNA promoters of key regulatory proteins. For instance, it has been observed that HT can rescue oxidative stress-induced DNA hypomethylation in the offspring of female mice suffering from intrauterine growth restriction [30]. Moreover, 50 μM of HT increases the expression of the anticancer gene type 1 cannabinoid receptor (CB1) by decreasing the methylation of its DNA promoter site [31]. Notably, significantly lower doses of HT, ranging from 0.1–10 μM, can regulate mitochondrial metabolism by activating PGC-1α promoter activity [15]. Although the later study did not measure epigenetic changes in the PGC-1α promoter, these results are consistent with recent finding reporting that EVOO supplementation can modulate metabolism by changing the methylation levels

of genes related to metabolism in peripheral white blood cells [14]. These studies are in the context of clinical diseases; however, these data collectively suggest that HT can regulate gene expression through modulating the methylation levels of the DNA promoter. These results are noteworthy because they contribute to the study of phytochemicals from another point of view, which may help to gain insight into their biological and medicinal properties. Moreover, our results pave the way to study whether a supplementation with physiological doses of HT or EVOO may boosts exercise adaptation by regulating mRNA expression through an epigenetic modification of DNA promoters.

Surprisingly, our results show that neither exercise nor HT are able to regulate PFK. Indeed, PFK is considered the main enzyme of glycolysis because it allosterically converts glucose-6-phosphate to fructose-6-phosphate [7 and Figure 1]. The lack of induction of PFK expression can also be explained by the epigenetic modulation exerted by both the diet and the level of physical activity on its DNA promoter. An interesting study compared the methylation levels of two groups of wild baboons; one group consisted of wild-feeding animals, while the other group consisted of wild baboons living close to the civilization and having access to human food thus resulting in high feeding efficiency and low daily travel distances [32] The results show that wild-feeding animals show a higher PFK promoter methylation resulting in lower PFK gene expression [32]. These results may explain why all the studied transcripts are highly expressed in response to exercise and to HT intake while PFK remains unchanged. In fact, it seems that, under basal conditions, PFK DNA promoter is slightly methylated, thereby providing a continuous expression of mRNA, while mid-level cell stress may downregulate its expression by epigenetic mechanisms.

5. Conclusions

The consumption of 4.61 mg/kg/d of HT can boost the mRNA levels of key metabolic proteins induced by exercise. The molecular mechanisms triggered by HT inducing such effects are yet to be studied. Nevertheless, future studies must test the epigenetic modifications induced by HT at physiological doses and/or by EVOO supplementation. In fact, we suggest that physiological doses of HT may regulate the methylation levels of key DNA promoters.

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Conclusiones

1. **1.** El ejercicio induce el ensamblaje crónico de CI en SC, proporcionando protección mitocondrial contra el estrés oxidativo.
2. **2.** El rango de dosis de HT de 20 a 300 mg/kg/d durante 10 semanas induce una respuesta antioxidante dependiente de la dosis en ratas sedentarias.
3. **3.** La dosis de 20 mg/kg/d de HT durante 10 semanas disminuye la capacidad de carrera de las ratas ejercitadas.
4. **4.** La dosis alta de HT (300 mg/kg/d) induce un efecto prooxidante y aumenta la capacidad de carrera de las ratas ejercitadas.
5. **5.** La dosis 20 mg/kg/d de suplementación con HT durante 10 semanas de ejercicio proporciona un estímulo más poderoso que únicamente el ejercicio, para inducir la formación de SC.
6. **6.** El consumo de 300 mg/kg/d de HT durante el ejercicio dificulta el ensamblaje de CI en SC, inducido por el ejercicio.
7. **7.** Una dosis baja a moderada de HT (0,31 y 4,61 mg/kg/d), cuando se complementa como un compuesto aislado, podría alterar los efectos beneficiosos del ejercicio con respecto a la señalización de insulina y la absorción de glucosa en SKM de rata (resultados no publicados).
8. **8.** El consumo de 4,61 mg/kg/d de HT puede aumentar los niveles de ARNm de proteínas metabólicas claves en el músculo de ratas ejercitadas (resultados no publicados).

Conclusions

1. Exercise induces the chronic assembly of CI into SCs, which provides mitochondrial protection against oxidative stress.
2. HT dosage ranging from 20 to 300 mg/kg/d for 10 weeks induce an antioxidant response in a dose-dependent manner in sedentary rats.
3. 20 mg/kg/d of HT during 10 weeks decrease the running capacity of exercised rats.
4. High HT dose (300 mg/kg/d) induce a pro-oxidant effect and increase the running capacity of exercised rats.
5. 20 mg/kg/d of HT supplementation over 10 weeks of exercise provides a more powerful stimulus than exercise alone to induce SC formation.
6. The consumption of 300 mg/kg/d of HT during exercise hampers exercise-induced CI assembly into SCs.
7. A low to moderate dose of HT (0.31 and 4.61 mg/kg/d), when supplemented as an isolated compound, might alter the beneficial effects of exercise regarding insulin signaling and glucose uptake in rat SKM (unpublished results).
8. The consumption of 4.61 mg/kg/d of HT can boost the mRNA levels of key metabolic proteins induced by exercise (unpublished results).

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