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TESIS DOCTORAL

QUERCETINA Y ENTRENAMIENTO. ANÁLISIS DEL ESTRÉS OXIDATIVO Y LA BIOGÉNESIS MITOCONDRIAL EN TEJIDOS CON DIFERENTE ACTIVIDAD METABÓLICA

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A mi familia

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QUERCETIN AND EXERCISE. AN ANALYSIS OF OXIDATIVE STRESS AND MITOCHONDRIAL BIOGENESIS IN TISSUES WITH DIFFERENT METABOLIC RATES

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AUTORIZACIÓN DE LOS DIRECTORES DE LA TESIS PARA SU PRESENTACIÓN

El Dr. Antonio Martínez Amat, el Dr. Fidel Hita Contreras y el Dr. Emilio J Martínez López como Directores de la Tesis Doctoral titulada "*Quercetina y entrenamiento. Análisis del estrés oxidativo y la biogénesis mitocondrial en tejidos con diferente actividad metabólica",* realizada por Don. **Rafael A. Casuso Pérez** en el Departamento de Ciencias de la Salud **autorizan su presentación a trámite** dado que reúne las condiciones necesarias para su defensa.

Lo firmo, para dar cumplimiento a los Reales Decretos 56/2005 y 778/98, en Jaén a 3 de Febrero de 2014.

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II. **Casuso RA**, Martínez-López EJ, Hita-Contreras F, Camiletti-Moirón D, Martínez-Amat A. Quercetin effects on weight gain and caloric intake in exercised rats. Biol Sports 2014;31:63-7

III. **Casuso RA**, Martínez-Amat A, Martínez-Romero R, Camiletti-Moirón D, Hita-Contreras F, Martínez-López E. Plasmatic nitric oxide correlates with weight and red cell distribution width in exercised rats supplemented with quercetin. Int J Food Sci Nutr 2013; 64(7):830-5

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VI. **Casuso RA**, Martínez-Amat A, Hita-Contreras F, Camiletti-Morión D, Aranda P, Martínez-López EJ. Quercetin and exercise induced adaptations in rats cerebellum are hampered when quercetin is supplemented during exercise. Submitted

RESUMEN

La quercetina es un flavonoide que podría mimetizar el efecto del ejercicio en la biogénesis mitocondrial. La quercetina se está aconsejando a deportistas por mejorar este proceso en músculo y cerebro, pero también a sujetos no entrenados para mejorar su salud integral. El efecto a largo plazo, sin embargo, puede resultar perjudicial para las adaptaciones celulares inducidas por el entrenamiento

El objetivo general de esta Tesis Doctoral es describir los efectos ergogénicos derivados de una suplementación de larga duración de quercetina, así como analizar el efecto de la quercetina en las adaptaciones biológicas inducidas por el ejercicio en tejido muscular y cerebro.

Se utilizaron un total de 34 ratas Wistar macho de 6 semanas de edad que se distribuyeron en 4 grupos: quercetina +entrenamiento (n=9); quercetina+sedentario (n=8); no quercetina +entrenamiento (n=9) y no quercetina+sedentarios (n=9). Los grupos entrenados realizaron 6 semanas de entrenamiento en cinta rodante, durante este periodo, a los grupos suplementados se les introdujo 25mg/kg de quercetina cada 2 días por sonda gástrica. Los grupos no suplementados ingirieron solo el vehículo (metilcelulosa). 24h tras la última sesión de entrenamiento las ratas realizaron una prueba de esfuerzo hasta la fatiga y al día siguiente un test incremental de consumo de oxígeno máximo. 48h después del último test las ratas fueron sacrificadas y cuádriceps y cerebro fueron extraídos. Se analizó los niveles de daño oxidativo tanto en proteínas como en lípidos, así como la actividad enzimática antioxidante. También se cuantificó el contenido mitocondrial y la transcripción de genes clave del metabolismo oxidativo y la biogénesis mitocondrial.

Los principales resultados de la Tesis muestran que: a) La quercetina no proporciona ningún efecto ergogénico ni en ratas sedentarias ni entrenadas. b) La quercetina incrementa el daño oxidativo en las estructuras proteicas que puede estar siendo contrarrestado por una modulación de la actividad enzimática. c) Cuando la quercetina se suplementa durante el entrenamiento, además del daño oxidativo observado, la actividad antioxidante está noqueada. d) En condición sedentaria la quercetina parece mimetizar los efectos del ejercicio, especialmente en cerebro y cerebelo, al incrementar el contenido mitocondrial. Este efecto parece ser mediado por la transcripción de la sirtuina 1 (SIRT1). e) Cuando la quercetina se suplementa durante el ejercicio los efectos del ejercicio sobre la biogénesis mitocondrial se ven comprometidos probablemente por la disminución de la transcripción de SIRT1. f) La quercetina parece actuar sobre SIRT1 más que sobre PGC-1α para ejercer sus efectos en el metabolismo oxidativo.

Estos descubrimientos ponen de manifiesto que la quercetina suplementada durante el ejercicio impide el desarrollo de las adaptaciones celulares inducidas por el entrenamiento en órganos con diferente actividad metabólica.

QUERCETIN AND EXERCISE. AN ANALYSIS OF OXIDATIVE STRESS AND MITOCHONDRIAL BIOGENESIS IN TISSUES WITH DIFFERENT METABOLIC RATES

SUMMARY

Quercetin is a flavonoid which may mimic exercise-induced mitochondrial biogenesis. Quercetin has being recommended to athletes in order to improve the biological process in skeletal muscle, the brain, and also for sedentary subjects as a health enhancer. However long-term supplementation could be a disadvantage for exercise-induced cellular adaptations.

The overall objective of this Thesis is to describe possible ergogenic effects induced by long-term quercetin supplementation, as well as to analyze quercetin´s effect on exercise-induced biological adaptations on brain and muscle.

34 male Wistar rats aged 6 weeks were used and they were allocated into 4 groups: quercetin exercised (n=9); quercetin sedentary (n=8); no-quercetin exercised (n=9); no-quercetin sedentary (n=8). The exercised groups were trained on a treadmill for a period of 6 weeks consisting of 5 days per week. During this period rats in the quercetin groups were supplemented with 25mg/kg of quercetin every other day via gavage. No-quercetin groups were also supplemented using the same procedure but only with the vehicle. 24 hours after the last training session we carried out an incremental running test to exhaustion in order to determine VO2 peak, using a treadmill gas analyzer. Following 24 hours of rest a low-intensity run to exhaustion test was administered. 48h after the last exercise test rats were sacrificed and brain, cerebellum and quadriceps were removed. Oxidative damage as well as antioxidant enzymatic activity was assessed in all the tissues. Moreover, mitochondrial content and transcription of some key genes related to oxidative metabolism and mitochondrial biogenesis were also quantified.

The main findings and conclusions are: a) Quercetin is not an ergogenic agent neither in sedentary or in exercised rats. b) Quercetin increases oxidative damage to proteins which may be counteracted by an increased modulation of the antioxidant activity. c) When quercetin is supplemented during exercise, oxidative damage is also evident for protein structures, however, antioxidant enzymatic activity was knocked out. d) In the sedentary condition quercetin seems to mimic exercise effects on the mitochondrial biogenesis, especially in the brain and cerebellar tissue. This effect may be due to the increase of the transcription of sirtuin 1 (SIRT1). e) When quercetin is supplemented during exercise, exercise-induced effects on mitochondrial biogenesis are

compromised perhaps by a decrease in SIRT1 transcription. f) Quercetin seems to target on SIRT1 rather than PGC-1α in order to exert its biological effects on oxidative metabolism.

These findings highlight that exercise with concomitant quercetin supplementation hampers exercise-induced cellular adaptations in organs with different metabolic rates.

ABREVIATURAS [ABBREVIATIONS]

ANOVA	Analysis of Variance
AMPK	AMP-activated Protein Kinase
ATP	Adenosine Triphosphate
CAT	Catalase
CS	Citrate Synthase
ERRs	Estrogen Related Receptors
NO	Nitric Oxide
NQ-EX	No-quercetin+exercise group
NQ-SED	No-quercetin+exercise group
NRF	Nuclear Respiratory Factor
mtDNA	Mitochondrial ADN
PCC	Protein Carbonyl Content
PGC-1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-α
PPARs	Peroxisome Proliferator-Activated Receptors
Q-EX	Quercetin+exercise group
Q-SED	Quercetin+sedentary group
RDW	Red Cell Distribution Width
SIRT1	Sirtuin 1
TBARs	Thiobarbituric Acid Reactive Substances
Tfam	Mitochondrial Transcription Factor A
tSOD	Total Superoxide Dismutase
VO2 max	Maximum Oxygen Uptake

INTRODUCCIÓN [INTRODUCTION]

Quercetin bioavility and metabolism

Fruits and vegetables rich diet improve cardiovascular health[1]. Mediterranean diet is characterized by the consumption of a variety of food and high intake of fruits and vegetables. When supplemented with olive oil, mediterranean diet can significantly enhance cardiovascular health[2]. Healthy effects from olive oil[3] as well as from fruits and vegetables[4,5] are due to a high content of polyphenols. These healthy effects of polyphenols include free radical scavenge, modulation of transduction signals, cellular signaling, gene expression and cellular communication[6].

Several thousand molecules that have a polyphenol structure (ie, several hydroxyl groups on aromatic rings) have been identified. These compounds may be classified into different groups as a function of the number of phenol rings that they contain and of the structural elements that bind these rings to one another[4]. More studied polyphenols, flavonoids, share a common structure consisting of 2 aromatic rings that are bound together by 3 carbon atoms that form an oxygenated heterocycle.

The main representative flavonoid is quercetin because it is mainly found in plant food which is consumed by humans[7,8,9]. The richest quercetin sources are onions (up to 1.2 g/kg fresh wt), curly kale, leeks, broccoli, and blueberries, but red wine and tea also contain up to 45 mg flavonols/L[4,10].

Once ingested, quercetin shows a quite extensive metabolism, it can be absorbed by the stomach[11], but most quercetin is absorbed by the small intestine[4,12]. In food, quercetin is present in the glycosilated forms, the sugar moiety is very often glucose but other sugars may also be involved. Flavonoids in general and quercetin in particular undergo an extensive phase I and phase II metabolism[13]. Phase I consist in de-glycosylation[14], and phase II consists of the metabolism of the resulting aglycones to glucuronides, sulphates and O-methylated forms during transfer across the small intestine[15,16].

Biochemical changes through metabolism occur to allow quercetin to cross the gastrointestinal barrier[14] Then, quercetin goes to the liver, through the hepatic portal vein, where quercetin metabolites undergo further phase I and II metabolism[12-16]. Nevertheless, it is interesting to note that quercetin´s conjugated metabolites formed during the absorption can be de-conjugated

in the liver by the deconjugating enzyme beta-glucuronidase, resulting in the aglycone form[17]. Thus, target tissues can receive quercetin as aglycone or in the glucuronide, sulphated and O-methylated forms.

Biological effects of quercetin

Quercetin and their methylated derivatives are distributed in most tissues after long-term ingestion[18]. Once in tissues quercetin modulates the activity of a great variety of enzymes and cellular receptors[19]. *In vitro* it has been shown that quercetin has powerful antioxidants and anti-inflammatory activities[20-21] which explain current scientific interest of quercetin effects on health. Some *in vivo* studies have shown that quercetin is able to reduce virus influenza after exercise [22] and disease development after vigorous exercise in humans[23], perhaps this is due to the enhanced immunological blood response[24].

Regarding the antioxidant potential of quercetin, it modulates the activity of some antioxidant enzymes[19,25-27], moreover it can also indirectly, act as an antioxidant by modulating ferric reductases[28]. However, physiological concentrations of flavonoids and their metabolites in the plasma and organs such as the brain[29] are lower than those recorded for small molecule antioxidant nutrients such as ascorbic acid and a-tocopherol[30]. Furthermore, during quercetin metabolism, other distinct molecules form the original aglycone are formed[16,31,32], resulting in a significant alteration of their redox potential[12]. In fact, beneficial effects of flavonoid metabolites *in vivo* are unlikely to result by their ability to out-compete antioxidants such as ascorbate, which are present at higher concentrations[33]. Thus, over the past decade other biological effects, distinct from antioxidant, of flavonoids in general and quercetin in particular have been investigated.

More recently, it has been demonstrated that the molecules that have a polyphenol structure[34,35] activate mitochondrial biogenesis. These molecules reprogram the skeletal muscle genome by activating PPARs and their activators. PPARs, in fact, are supposed to be exercise mimetics[36], because exercise is a well-known mitochondrial biogenesis promoter[37,38].

Mitochondrial biogenesis process

Mitochondria is a cellular organelle which engage in an array of biochemical activities and are the major sites of oxidative ATP production in eukaryotic cells[39]. A soluble matrix bounded by a double membrane conform the mitochondria, the inner membrane is impermeable to ions while the outer membrane is permeable to bigger molecules. The electron donors, NADH and FADH2,

derived from the oxidation of acetyl-CoA, are utilized by the electron transport chain of the mitochondrial inner membrane to establish an electrochemical proton gradient across the membrane. The resulting proton movement, comprising both a voltage potential and a pH gradient, is used by the membrane-bound ATP synthase to drive the synthesis of ATP[40].

ATP production is crucial for exercise performance and muscle contraction for multiple reasons. Muscular contraction is induced by actin-myosin crossbridge cycling, and hydrolysis of adenosine triphosphate by myosin ATPase which provides the immediate energy source for crossbridge cycling[38,41]. However, ATP can also be fuel for sodium-potassium dynamics and calcium exchange during contraction. In fact, resting intrmuscular ATP levels are small, activation of metabolic pathways triggering ATP generation maintain cellular levels. Maximal intensity exercise induces a 100-fold increase ATP production which, in turn, limits performance[42]. More recently, it has been shown that mitochondrial density is a key determinant for human performance, in fact, CS activity significantly correlates with moderate intensity performance[43]. What is more, high intensity exercise performance is also improved by a high mitochondrial content[44].

Mitochondrial biogenesis is a well-known response to exercise training[37,45], and is defined by an increase in muscle mitochondrial number and volume, as well as concomitant changes in organelle composition. Mitochondrial biogenesis is a complex process in which a small amount of nuclear transcriptional factors coordinate the expression of a high amount of nuclear and respiratory proteins. Tfam binds the mtDNA at multiples sites and functions in both mtDNA maintenance and transcription initiation. The nuclear respiratory factors, NRF-1 and NRF-2, were the first nuclear transcription factors implicated in the expression of multiple mitochondrial functions in vertebrates. NRF-1 acts on genes encoding respiratory subunits[46] and Tfam[47]. Moreover, both NRF-1 and NRF-2 have a direct role in the expression of cytochrome oxidase subunits[48,49]. The PPARa, a member of the nuclear receptor family is thought to regulate nuclear genes encoding mitochondrial fatty acid oxidation enzymes[50]. Other members of the nuclear receptor family such as EERs are associated with the enconding of mitochondrial proteins involved in fatty acid oxidation enzymes[51] and the tricarboxylic cycle [40].

SIRT1-PGC-1α pathway and quercetin. Implications for health

The role of co-regulators in mitochondrial biogenesis is perhaps best illustrated by PGC-1α[52], the master regulator of mitochondrial biogenesis and energy expenditure. Interactions between PGC-1α and specific transcription factors orchestrates the mayor functions of the

mitochondria. PGC-1α regulate de activity of PPARs[52,53], ERRs[54] and NRF1[55], for the assembly of the fatty acid B-oxidation, the tricarboxylic cycle, mtDNA replication, the electron transport chain, in addition to biogenesis of the organelle[40]. There are several pathways which trigger PGC-1α transcription in response to insuline, cold and glucagon[56]. In response to the exercise some Ca+ dependent kinases are thought to initiate the transcription of the PGC-1α gene[57].

Post-translational activation of PGC-1α has been the focus of a lot of research because it is responsible for subsequent metabolic signaling[50]. Exercise compromises the nutrient abailavility resulting in a cellular energy stress. In this scenario, PGC-1α is phosphorilated (activated) by the AMPK[58], a sensor of the ATP levels within the cell. SIRT1 is a NAD+-dependent deacetylase[59], in general, NAD+ levels increase in mammalian tissues in response to energy/nutrient stresses such as exercise[60-62]. In fact, in response to exercise, SIRT1 is considered one of the main activators of PGC-1α [60,63].

Mitochondrial content and activity are key factors for neuronal and metabolic health protecting against neurodegeneration and type 2 Diabetes[64,65]. Exercise training can trigger mitochondrial biogenesis by increasing the transcription of the SIRT1 and PGC-1α genes in skeletal muscle[60,63,66] and in most brain regions[67]. Thus, exercise training can provide a protective effect against neurodegeneration and metabolic diseases despite different metabolic rates of between the brain and skeletal muscle in response to exercise.

The fact that quercetin increase mitochondrial biogenesis in brain and soleus muscle by increasing the transcription of the SIRT1-PGC-1 pathway[68], led researchers to believe that quercetin may mimic on exercise effect. Thus, it has been recommended in order to improve integral health and to reduce aging diseases[69]. What is more, it has also been assumed that quercetin supplementation increases exercise performance and it has been suggested as an ergogenic agent for athletes[70].

Quercetin and cellular adaptations to exercise

When ergogenic effects of quercetin have been tested, there have been contradictory results. Many research papers have failed to attempt to describe any ergogenic effects of quercetin either in untrained[71-75] or in well-trained subjects[76-78]. In contrast, two studies have reported a 3.9% in VO2 peak and 13.2% in time to fatigue[79], as well as 2.9% in a maximal 12-minute test after 60min of preload[80] in untrained subjects. However, a recent meta-analysis classified these possible ergogenic effects of quercetin as trivial and unlikely to improve exercise performance[81].

Recently, quercetin has been shown to inhibits cellular and systemic adaptations to exercise[83]. What is more, resveratrol, a polyphenol which target to SIRT1 in a similar way as quercetin[34,83,84], blunts the positive effects of exercise on vascular function and cardiovascular health when supplemented concomitant to exercise[85]. The fact that quercetin may provide a disadvantage for exercise-induced adaptations can be due to the change in it antioxidant potential[12]. Quercetin is thought to exert it antioxidant effect at the early stage of the supplementation, but later, quercetin's metabolites that are formed during the antioxidant activity might shade the direct positive effects of quercetin supplementation by acting as prooxidant[86,87].

At the early stage of exercise training, before homeostasis is achieved, exercise acts as an oxidative stressor in a similar way in muscle[88] and brain tissue[89]. Thus, oxidative stress induced by exercise concomitant with the oxidative stress induced by quercetin in its target organs can blunt cellular adaptations to exercise. This may explain ineffective effects of quercetin in order to enhance performance and systemic adaptations in response to exercise.

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OBJETIVOS

General:

El objetivo general de esta Tesis Doctoral es describir los posibles efectos ergogénicos derivados de una suplementación de larga duración de quercetina en ratas, así como analizar el efecto de la quercetina en las adaptaciones biológicas inducidas por el ejercicio en tejido muscular y cerebro.

Específicos:

- Analizar la eficacia de la quercetina como potenciador del rendimiento deportivo en ratas entrenadas y no entrenadas (Artículo I)
- Describir el efecto de la quercetina en la ganancia de peso (Artículo II)
- Analizar el efecto de la quercetina en la producción de óxido nítrico y parámetros hematológicos (Artículo III)
- Determinar el efecto de la quercetina sobre el estado oxidativo y el proceso de la biogénesis mitocondrial en músculo esquelético (Artículo IV).
- Determinar el efecto de la quercetina sobre el estado oxidativo y el proceso de la biogénesis mitocondrial en cerebro (Artículo V).
- Determinar el efecto de la quercetina sobre el estado oxidativo y el proceso de la biogénesis mitocondrial en cerebelo (Artículo VI).
- Comparar la capacidad de la quercetina en mejorar la respuesta antioxidante así como la capacidad de la biogénesis mitocondrial en tejidos con diferente actividad metabólica durante el ejercicio como son el músculo esquelético, cerebro y cerebelo (Artículos IV, V y VI).
- Describir los efectos de la suplementación de la quercetina durante el entrenamiento en la transcripción de genes clave del metabolismo oxidativo relacionados con la salud integral (Artículos IV, V y VI)

AIMS

Overall:

The overall objective of this Thesis was to describe possible ergogenic effects derived from long-term quercetin supplementation in rats, as well as to analyze quercetin effect on exercise-induced biological adaptations on brain and muscle.

Specific:

- To analyze quercetin´s efficiency as an exercise performance enhancer in exercised and sedentary rats (Paper I).
- To describe quercetin´s effect on weight gain (Paper II)
- To analyze quercetin effects on nitric oxide and hematological parameters (Paper III)
- To determine quercetin´s effect on oxidative status and mitochondrial biogenesis process in skeletal muscle (Paper IV).
- To determine quercetin´s effect on oxidative status and mitochondrial biogenesis process in brain (Paper V).
- To determine quercetin´s effect on oxidative status and mitochondrial biogenesis process in cerebellum (Paper VI).
- To compare the capacity of quercetin supplementation to enhance the antioxidant response as well as the mitochondrial biogenesis in tissues with different metabolic rates in response to exercise such as skeletal muscle, brain and cerebellum (Papers IV, V and VI).
- To describe the effect of quercetin supplementation in sedentary and exercised rats on the transcription of some oxidative key genes related with health (Papers IV, V and VI).

MATERIAL Y MÉTODOS

El diseño de la presente Tesis se describe a continuación, los análisis bioquímicos realizados en cada uno de los tejidos se encuentran descritos en los artículos tal cual han sido publicados o sometidos.

Animales

Se utilizaron ratas Wistar macho de 6 semanas de edad y con un peso de 147±4g. Los animales se mantuvieron durante 8 semanas en jaulas individuales en una habitación con una temperatura de 21ºC, una humedad de 40-60% y un ciclo de luz de 12h de luz y 12 horas de oscuridad. Tuvieron acceso libre a pienso estándar (Harlan 2014) y agua. 48h tras el test de resistencia (descrito más abajo) las ratas se anestesiaron con pentobarbital y fueron desangradas. Todos los experimentos descritos fueron aprobados por el Comité de Ética de la Universidad de Jaén.

Entrenamiento y suplementación

Las ratas se asignaron de forma aleatoria a los grupos quercetina (Q, n = 18) y no quercetina (NQ, n = 17). Ambos grupos fueron, a su vez, divididos en quercetina+entrenamiento (n = 9), quercetina+sedentario (n = 9); no quercetina+entrenamiento (n = 9) y no quercetina+sedentarios (n = 8). Todas las ratas se aclimataron a las condiciones experimentales durante dos semanas, durante los 3 últimos días de la segunda semana los grupos entrenados se adaptaron a la cinta de correr. El entrenamiento se realizó en una cinta de correr diseñada para ratas (5 rats Panlab Treadmills for LE 8710R) 5 días a la semana durante 6 semanas. Todos los entrenamientos se realizaron a una velocidad constante de 44 cm/s y con una inclinación del 10%. Las ratas empezaron corriendo 20 min y cada dos días se incrementó en 5 min el tiempo de entrenamiento. El último día de la 5ª semana las ratas alcanzaron los 80 min de entrenamiento que se mantuvieron durante la última semana. Por otra parte, las ratas en los grupos quercetina fueron suplementados por sonda gástrica con quercetina (QU99; Quercegen Pharma, MA) en días alternos. La suplementación se realizó por la mañana unas 2h antes del entrenamiento con una dosis de 25mg/kg en una solución de metilcelulosa al 1%, la dosis fue calculada cada semana. La dosis fue elegida porque se ha reportado que suplementaciones entre 12.5 y 25mg/kg de quercetina provocan su efecto biológico. Las ratas en los grupos no suplementados con quercetina fueron igualmente suplementadas pero solo con el vehículo (metilcelulosa al 1%).

Test incremental de carrera y VO2max

24 horas tras la última sesión de entrenamiento todos los animales realizaron un test de incremental hasta la fatiga en una cinta con analizador de gases (Model LE405, Panlab/Harvard Apparatus) para determinar el consumo máximo de oxígeno. Tras calibrar el aparato con mezclas conocidas de O2 y CO2 se inició el test. Las ratas comenzaron corriendo dos minutos a 22 cm/s y la velocidad se incrementó en 11cm/s cada dos minutos. El test finalizó las ratas estaban exhaustas y se quedaron situadas sobre la rejilla eléctrica durante 5 seg tras lo cual fueron rápidamente sacadas. El máximo consumo de oxígeno se definió como la mayor media registrada durante 20". El lactato sanguíneo se midió antes e inmediatamente después del test utilizando un analizador Lactate-Pro, la sangre se obtuvo de un pequeño corte en la cola de la rata.

Test de carrera de resistencia

Tras 24h de descanso, un test de carrera hasta la fatiga a baja intensidad fue pasado a cada rata. El test consistió en una carrera hasta la fatiga a 44cm/s y 10% de inclinación. El test finalizó cuando las ratas estaban visiblemente exhaustas y no eran capaces de mantener la velocidad requerida, lo que resultaba en un incremento de las veces que las ratas contactaban con las rejillas eléctricas de la cinta de carrera. Cuando esto sucedía durante 5 segundos las ratas eran rápidamente sacadas de la cinta y el tiempo hasta la fatiga fue anotado.

Obtención de tejidos

Todas las ratas fueron anestesiadas con pentobarbital y desangradas por canulación de la arteria aorta 48h después del último ejercicio. Cuádriceps, cerebro y cerebelo fueron rápidamente extraídos, sumergidos en una solución salina, congelados en nitrógeno líquido y mantenidos a -80ºC hasta su análisis.

MATERIAL AND METHODS

Animals

This study was performed on male Wistar rats aged 6 weeks and weighing 147 ± 4 g. The animals were maintained for 8 weeks in individual cages under standard conditions of light (12:12 light– dark cycle), temperature (21 °C), and humidity (40–60%) and allowed ad libitum access to food (Harlan 2014, maintenance chow; Indianapolis, Indiana, USA) and water. 48 hours after run to exhaustion test, the rats were anesthetized with pentobarbital and were killed by bleeding. All experiments followed the ACSM animal care standards and were approved by the institutional committee for ethics (University of Jaén).

Exercise and supplementation

Rats were randomly assigned to quercetin (n=17) and no-quercetin (n=17) groups. Both groups were further divided into quercetin exercised (n = 9), quercetin sedentary (n = 8), no-quercetin exercised (n = 9), and no-quercetin sedentary (n = 8). All rats were acclimated to experimental conditions for 2 weeks, and 3 days before the training period, trained groups were acclimated to the treadmill. Exercise training and supplementation were further described previously. Treadmill exercise training took place 5 days a week for 6 weeks (5 rats Panlab Treadmills for LE8710R). The rats ran at a constant speed of 44 cm/s at an angle of 10%. The rats ran for 20 min during the first 2 days and for 25 min on the third day. Training duration increased by 5 min every 2 days. The rats ran for 80 min on the last day of the fifth week and also throughout the last week of training. The rats in the quercetin groups were supplemented with quercetin, via gavage (QU995; Quercegen Pharma, Newton, Massachusetts, USA) on alternate days throughout the experimental period. Supplementation took place in the morning < 2 h before exercise training; a dose of 25 mg/kg diluted in a 1% solution of methylcellulose was used. The no-quercetin groups were also supplemented with the vehicle (1% methylcellulose solution).

Incremental running test and VO2max

Twenty-four hours after the last training session, all animals performed a graded high-intensity treadmill test to determine VO2max using a treadmill gas analyzer (Model LE405, Panlab/ Harvard Apparatus) previously calibrated with mixtures of O2 and CO2 at different concentrations. After an initial two minutes with no grade at 22 cm/s, treadmill speed was increased by 11 cm/s every two minutes. The test was finished when the rat was exhausted and located at the end of the

treadmill, on the shock bar, for 5 seconds, when rats were quickly removed. VO2max was defined as the highest 20" interval recorded during the test. Blood lactate was measured before and immediately after the test using a Lactate-Pro analyzer, blood was taken from a small cut in the rat's tail.

Running time to exhaustion test

After twenty-four hours of recovery a low-intensity en- durance test was performed. Each rat was required to run to exhaustion at 44 cm/s at a 10% grade. The test finished when the animal was visibly exhausted, not able to maintain the appropriate pace, and this resulted in a rising frequency of landings on the electrical shock grid. When the animal was standing for 5 sec on the electrical shock it was quickly removed and the time was recorded.

Tissue collection

All rats were anesthetized with pentobarbital and were bled by cannulation of the aorta 48 h after the last exercise. Quadriceps muscle, brain and cerebellum were immediately collected, rinsed in saline solution, frozen in liquid nitrogen, and stored at -80 °C until their further analysis.

RESULTADOS Y DISCUSIÓN [RESULTS AND DISCUSSION]

La sección de Resultados y Discusión se presenta de la forma en la que han sido originalmente publicados o sometidos.

Results and Discussion section is presented as it has been previously published or submitted

1. EFECTOS ERGOGÉNICOS DE LA QUERCETINA EN RATAS ENTRENADAS Y SEDENTARIAS.

(ARTÍCULO 1)

I

ERGOGENIC EFFECTS OF QUERCETIN SUPPLEMENTATION IN TRAINED RATS

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RESEARCH ARTICLE Open Access

Ergogenic effects of quercetin supplementation in trained rats

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Abstract

Background: Quercetin is a natural polyphenolic compound currently under study for its ergogenic capacity to improve mitochondrial biogenesis. Sedentary mice have exhibited increased endurance performance, but results are contradictory in human models.

Methods: We examined the effects of six weeks of endurance training and quercetin supplementation on markers of endurance performance and training in a rodent model. Rats were randomly assigned to one of the following groups: placebo+sedentary (PS), quercetin+sedentary (QS), placebo+endurance training (PT) and quercetin +endurance training (QT). Quercetin was administered at a dose of 25 mg/kg on alternate days. During six weeks of treatment volume parameters of training were recorded, and after six weeks all groups performed a maximal graded VO_2 max test and a low-intensity endurance run-to-fatigue test.

Results: No effects were found in VO_2 peak ($p>0.999$), nor in distance run during low-intensity test, although it was 14% greater in QT when compared with PT ($P = 0.097$). Post-exercise blood lactate was increased in QT when compared with PT ($p=0.023$) and also in QS compared with PS ($p=0.024$).

Conclusions: This study showed no effects in VO_2 peak, speed at VO_2 peak or endurance time to exhaustion after six weeks of quercetin supplementation compared with placebo in trained rats. Quercetin was show to increase blood lactate production after high-intensity exercise.

Background

Flavonoids are a large family of phenolic compounds or polyphenols with wide therapeutic applications [1]. Quercetin is one of the most widely spread naturally occurring flavonoids, found in onions, garlic, cabbage, leek, broccoli, apples, blueberries, tea and red wine [2]. It is known that quercetin may exhibit anti-oxidant properties due to its chemical structure, particularly the presence and location of the hydroxyl (-OH) substitutions [3]. Despite the fact that after long-term intake there is a wide distribution of quercetin (including its metabolites) in all tissues [4], toxic effects have not been reported until the dose reached 157 mg per kg/d [5].

Quercetin might improve endurance performance since it is known that some polyphenols like quercetin [6] and resveratrol [7] improve aerobic capacity of skeletal muscle by promoting mitochondrial biogenesis in mice. A psychostimulant effect of quercetin has also been reported in vitro [8] in a manner similar to that of caffeine [9], but this effect was not found in human subjects [10]. Quercetin has also been shown to reduce illness after strenuous exercise [11], as corroborated by Davis et al. [12] in a mice model. However, these anti-inflamatory effects seen in vivo are not as powerful as those previously described in vitro [13]. The differences are even greater when the in vivo data is obtained from athletes [14-16].

Quercetin supplementation improves running time to fatigue by stimulating mitochondrial biogenesis in mice [6]. However, this effect has not been observed in humans [16-18]. Research has shown improvements of 3.9% in VO_2 peak and 13.2% in time to fatigue [19], as well as 2.9% in a maximal 12-minute test after an hour of preload [18] in untrained subjects. These findings are in contrast to those of previous studies [11,17,20]. When athletes are studied, most research has failed to find an ergogenic effect [15,16], in contrast to that of a study of

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elite cyclists, who exhibited an improvement of their aerobic performance [21]. Finally, effects of quercetin on pre-exercise and post-exercise blood lactate have not been reported [22].

Based on the data provided, the question arises: could quercetin be an ergogenic supplement for athletes or untrained subjects? Our primary goal is to study, for the first time and using a rat model, the effects of both endurance training and chronic quercetin supplementation on 1) endurance capacity, VO_2 peak, and lactate production, 2) endurance training progress, and 3) distance covered in a low-intensity treadmill test and in a high-intensity treadmill test.

Methods

Animals and experimental design

Thirty-three young (three week old) male Wistar rats were randomly allocated into four groups: quercetin and endurance training (QT, n=9), placebo and endurance training (PT, n=8), quercetin and sedentary (QS, n=8), and placebo and sedentary (PS, n=8). Animals, with an initial body weight of 150 (SD=10) g, were housed in individual stainless steel metabolism cages. The cages were located in a well-ventilated thermostatically controlled room (21 ± 2°C), with relative humidity ranging from 40 to 60%. A reverse 12 h light-12 h dark cycle (08.00-20.00 hours) was implemented to allow exercise training during the day. Throughout the experimental period, all rats consumed water and food ad libitum. Two weeks before the experimental period, rats were allowed to adapt to the diet and experimental conditions, and a week before the experimental period, rats had three days of acclimation to the treadmill. Body weight was measured twice per week during this time. After six weeks of treatment we performed two different exercise tests. Tests were carried out after the treatment so that we could compare four different conditions without assessing the effect of training. The reason for choosing a rat model is that a previous study showed that sedentary mice exhibited higher endurance performance with quercetin intake than with placebo [6]. All experiments were undertaken according to the Directional Guides Related to Animal Housing and Care (European Community Council, 1986), and all procedures were approved by the Animal Experimentation Ethics Committee of the University of Jaén.

Quercetin treatment

Rats were supplemented, during the training period, with quercetin (QU995; Quercegen Pharma, Newton, MA, USA) on alternate days at a dose of 25 mg/kg. This dose has been reported to improve mitochondrial biogenesis and endurance capacity in sedentary mice [6]. Quercetin was diluted in a 1% solution of methilcellulose, and was administered using a metal gavage. Oral gavage was performed to ensure that 25 mg/kg of quercetin was introduced into the stomach. Quercetin also contained vitamins B3 and C, which have been shown to increase the bioavailability of quercetin (personal communication, Quercegen Pharma). The PT and PS groups were also supplemented with methilcellulose and vitamin B3 and C with the same concentration as in QT and QS.

Training protocol

Trained animals were exercised five days per week during six weeks on a motorized treadmill (Panlab TREADMILLS for five rats LE 8710R). We followed a modification of the protocol of Davies et al [23]. Animals ran at a constant speed of 44 cm/s and at 10% grade. The first day's training session was 20-minutes long, and every two days the work period was increased by five minutes. On the last day of the fifth week they were required to run for a full 80 minutes. This work duration was maintained during the sixth week. The untrained group was exercised at the same speed and grade for only 10 minutes twice per week, in order to ensure that they were able to perform the tests performed at the end of the treatment.

Twenty-four hours after the last training session, all animals performed a graded high-intensity treadmill test to determine VO_2 peak using a treadmill gas analyzer (Model LE405, Panlab/Harvard Apparatus) previously calibrated with mixtures of O2 and CO2 at different concentrations. After an initial two minutes with no grade at 22 cm/s, treadmill speed was increased by 11 cm/s every two minutes. The test was finished when the rat was exhausted and located at the end of the treadmill, on the shock bar, for 5 seconds, when rats were quickly removed [24]. VO_2 peak was defined as the highest 20" interval recorded during the test. Blood lactate was measured before and immediately after the test using a Lactate-Pro analyzer, blood was taken from a small cut in the rat's tail.

After twenty-four hours of recovery a low-intensity endurance test was performed. Each rat was required to run to exhaustion at 44 cm/s at a 10% grade. The test finished when the animal was visibly exhausted, not able to maintain the appropriate pace, and this resulted in a rising frequency of landings on the electrical shock grid [24]. The endpoint was marked by the rat's inability to return to the treadmill belt, and to stand on a flat surface.

Statistical methods

Treatment effect between trained (QT vs PT) and sedentary (QS vs PS) groups was analyzed with a t test for independent samples, using study groups as independent variables and each of the performance parameters

measured as dependent variables (Weight, VO_2 peak, vVO_2 peak, maximum speed achieved, time of endurance test, distance run and distance run until RQ= 1, and VO_2 at exhaustion). Lactate production measured before and after the maximal incremental treadmill test was analyzed using a two-way repeated measures ANOVA, with groups as between-subject variable and exercise time as within-subject variable. When the effect was significant, post hoc analysis was performed and adjustment done through the Bonferroni confidence interval. The level of significance was P≤0.05 for the t-test and P≤0.008 in post hoc Bonferroni's comparisons (P=0.008 needed for significance with an experiment-wise alpha of 0.05 using Bonferroni adjustment in alpha for six comparisons). All analyses were performed using the Statistical Package for Social Sciences (SPSS, version 19.0 for Windows; SPSS, Inc., Chicago, IL, USA).

Results

Training progress

The training protocol and the effect of time on the meters run is presented in Figure 1. The QT and PT groups were subjected to a six-week duration training with an increase of five minutes every two days up to a maximum of 80 minutes, which represented an average increase of the load between intervals of 11.9 and 10.6% in QT and PT respectively. The final training volume increased by 399% to 349% in QT and PT compared with baseline. There were no differences in the distance run by the two groups at any time of training (P> 0.05). The average/day of meters walked were 986 and 1002 in the QT and PT groups respectively. Although the relationship between training time and distance covered showed an almost linear fit in both groups (R2 = 0.992 and 0.986) for QT and PT respectively, there was a sligh improvement in the performance of the QT group.

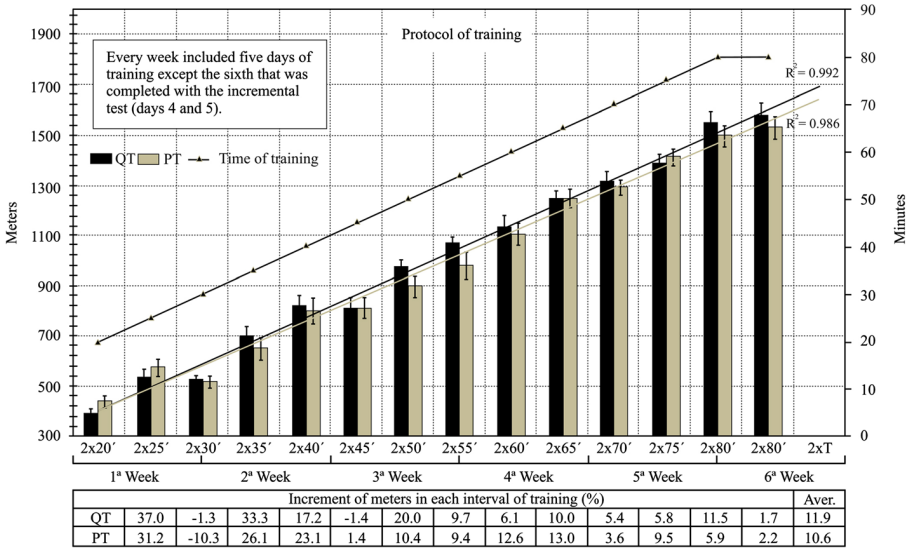
Endurance capacity

There were no significant difference in exercise performance between the quercetin and placebo trials. Although the QT group ran for 5.91% longer (Figure 2) and 14% further (Figure 3B) than the PT group, there were no significant differences in either time [P=0.351, Power=0.147] or distance [P=0.051, Power=0.512)].

Maximal incremental test

During the incremental test VO_2 peak, speed at VO_2 peak and maximum speed achieved did not differ between quercetin and placebo conditions (Table 1). There were no differences between the final weight after treatment, as shown in Table 1. Although the distance achieved by QT was 18.6% greater than PT this result was not significant [P=0.102, Power=0.380] (Figure 3A).

Figure 4B shows that the QT group ran for 56.1% longer before reaching RQ=1 compared with the PT group, but this effect was not significant [P=0.222, Power=0.213]. Similar results are illustrated by Figure 4A, in which VO_2 at exhaustion does not differ after the high-intensity test for the quercetin and placebo exercise groups (P=0.069, Power=0.448). Lactate production was analyzed (pre- and post-high-intensity test) using repeated measures ANOVA, where we observed a group effect P=0.001, Power=0.967 and a group interaction per time unit P=0.001, Power=0.977. Specifically, lactate production



	Increment of meters in each interval of training (%)													Aver.
QT	37.0	-1.3	33.3	17.2	-1.4	20.0	9.7	6.1	10.0	5.4	5.8	11.5	1.7	11.9
PT	31.2	-10.3	26.1	23.1	1.4	10.4	9.4	12.6	13.0	3.6	9.5	5.9	2.2	10.6

Figure 1 Training protocol of six weeks for rats. No significant difference (P>0.05) in distance run between QT and PT at any stage of training. ' = Minutes, Aver = Average, T= Application of tests. The percentage of increase in distance run was computed as ((interval - previous interval) / previous interval) x 100.

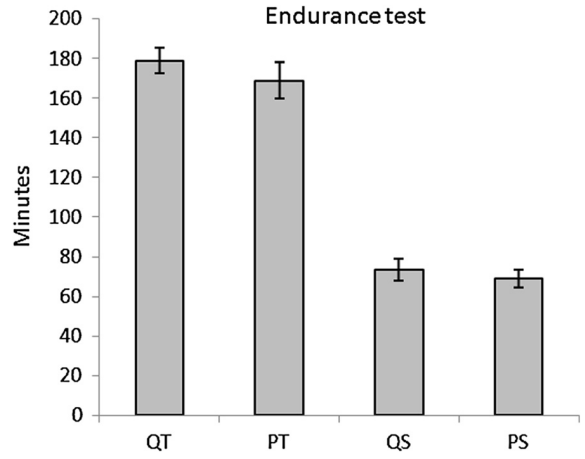


Figure 2 Time run until exhaustion in the low-intensity endurance regime. T- test for independent samples reported no significant differences between QT and PT or QS and PS (P>0.05).

immediately after the high-intensity test was increased in the QT and QS groups compared with the PT and PS groups (P=0.004) [Figure 5]. No differences were found in lactate production between groups prior to the high-intensity test (P>0.05). Lactate production was significantly increased in each group (P<0.001 in QT, QS y PS) and (P=0.004 in either PT) at the end of the high-intensity test (data not shown).

Discussion

A recent study evaluated the effects of short-term quercetin supplementation on exercise performance in mice [6] and demonstrated a significant increase in endurance capacity and mitochondrial biogenesis in comparison with placebo groups. Using a rat model, no significant performance effect (VO2 peak, endurance capacity and training parameters) was measured in trained rats with a quercetin dose of 25 mg/kg on alternative days compared with placebo.

The endurance training protocol used in this study was a modification of a widely used protocol in the literature [23,25,26]. As shown in Figure 1, distance run increased with time. These data suggest that the training workload was well adjusted, since a plateau in the training volume is a sign of overtraining [27]. No difference was found in the average daily distance run between the QT and PT groups. VO_2 peak values in rats vary depending on the methodological test used or on their weight [28]. Our results show that six weeks of quercetin supplementation did not increase VO_2 peak or VO_2 at exhaustion in sedentary or trained rats. It must be noted that our protocol did no alter inclination in order to examine the maximum speed achieved. Protocols that do not use an incline are known to induce a lower VO_2 peak than others with 15°-20° inclination [28,29]. However, our results were similar to those recently reported [17], but were in contrast with the ones that reported an increase of VO_2 peak by quercetin in sedentary humans [19]. Speed at VO_2 peak was also analyzed in this experiment, with no change reported in the quercetin groups. We hypothesized that quercetin would increase VO_2 peak due to its ability to increase mitochondrial biogenesis in mice (6). However, as described above, no differences were observed in any groups on measures related to oxygen uptake by quercetin supplementation. These results are similar to those obtained by Bigelman et al [30]. There are several potential reasons for these results: firstly, VO_2 peak is influenced by muscle mitochondrial oxidative capacity, but relative to endurance capacity, it is limited to a greater extent by oxygen delivery via the cardiovascular system [31]. Secondly, larger doses over extended periods using added flavonoids such as eppigallocatechin gallate (EGCG) may augment quercetin's effects on mitochondrial biogenesis. This could be a more appropriate supplement to increase oxygen consumption [16]. However, previous work did not find any ergogenic effect of quercetin and EGCG supplementation in a moderately trained sample [30].

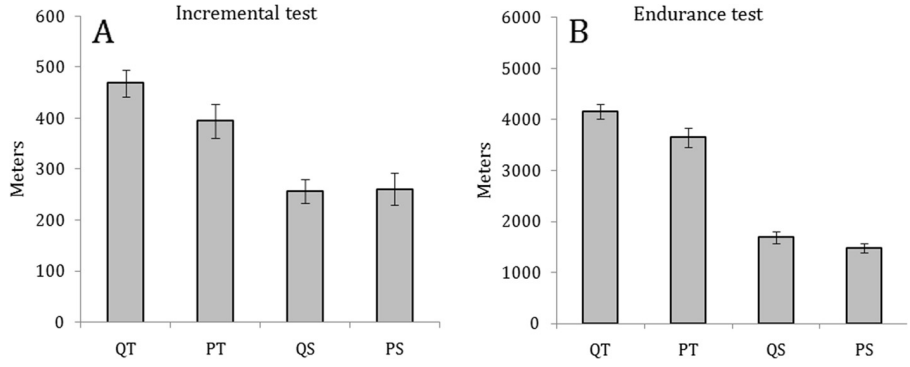


Figure 3 Distance run until exhaustion in A) high-intensity incremental test and B) low-intensity endurance test. T-test for independent samples reported no significant differences between QT and PT or QS and PS (P>0.05).

Table 1 Mean value (standard deviation) after incremental maximal test

	Trained						Sedentary					
	QT	PT	t	df	P	Power	QS	PS	t	df	P	Power
WEIGHT (g)	352.89±31.25	367.25±24.41	1.045	15	0.312	0.161	379.25±52.91	366.63±8.97	0.595	7.298	0.570	0.086
VO_2MAX (ml/kg/min)	63.55±8.58	58.62±7.38	1.272	14.990	0.223	0.219	65.12±8.21	61.87±5.51	0.929	14	0.369	0.139
/vVO_2MAX (cm/s)	47.89±8.17	48.50±16.18	0.100	15	0.922	0.051	46.88±13.21	46.63±10.98	0.041	14	0.968	0.052
MAX. VEL (cm/s)	95.11±7.40	87.50±9.65	0.837	15	0.086	0.405	71.63±8.68	71.63±11.01	0.002	14	0.998	0.050

Compared values for trained (QT vs PT) and sedentary groups (QS vs PS). T-test for independent samples reported no significant differences between QT and PT or QS and PS. VO_2 MAX: Maximum oxygen uptake; vVO_2 MAX: Velocity at VO_2 max; MAX.VEL: Maximal velocity achieved. df: degrees of freedom. Power: statistical power.

To examine additional ergogenic effects of quercetin in rats, oxygen consumption and carbon dioxide production were measured during the incremental exercise test. This enabled the calculation of RQ. In all groups of rats, the average RQ remained fairly constant and did not differ between groups (data not shown). When VCO_2 is greater than VO_2 (RQ>1.0), this point of inflection is correlated with blood lactate accumulation [32]. QT group showed a trend to run longer before reaching an RQ of 1.0 (Figure 4B) indicating that these rats were able to use oxidative metabolism for a longer period.

Fatigue in the endurance test is thought to arise primarily from limitations in the periphery, like the cardiovascular system and muscles [6]. Although it has been reported that antioxidant supplementation may decrease endurance performance [25], the trained groups showed an increase in time to fatigue of 244.96% and 244.93% for QT and PT respectively when compared to PS. However, in contrast with others [6], we did not observe an improvement in QS. When compared to trained groups, there was a non-significant increase of 5.91% in the QT group in time to fatigue. Despite being non-significant, this result was related to recently published results by Kesser et al. [33].

We employed two different types of exercise (a low intensity endurance capacity test and a maximal graded intensity test). Although both are commonly used exercise models, the stimuli are totally different. During the treadmill running endurance test mice run at a given intensity until they can no longer maintain the pace and end up on the electrical shock grid [24,25]. The performance in this type of exercise is known to be related to the oxidative capacity of muscles. However, during the maximal progressive intensity test, rats achieved higher velocities, a performance reflecting their capacity to use glycogen as a source of fuel. Distance run to exhaustion was recorded during these two different regimes (Figure 3). Under the high-intensity regime (test used to analyze oxygen consumption) the QT group ran (18,6%) longer than PT. Under the low-intensity regime (endurance test) QT ran 14% (p=0.097) further than PT. These results were not significant, however they demonstrated a trend that may become significant after a longer treatment.

Although no effects have been previously reported [22], the present study demonstrated that quercetin had an effect on blood lactate immediately after exhaustion. When the QT and QS groups reached exhaustion, their blood lactate levels were elevated when compared with

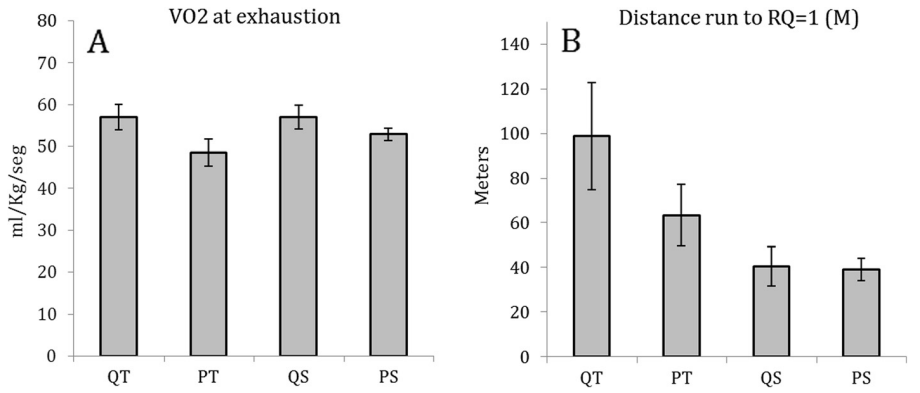


Figure 4 A) VO_2 at the end of the high-intensity incremental test B) Distance run until RQ=1. T-test for independent samples reported no significant differences between QT and PT or QS and PS (P>0.05).

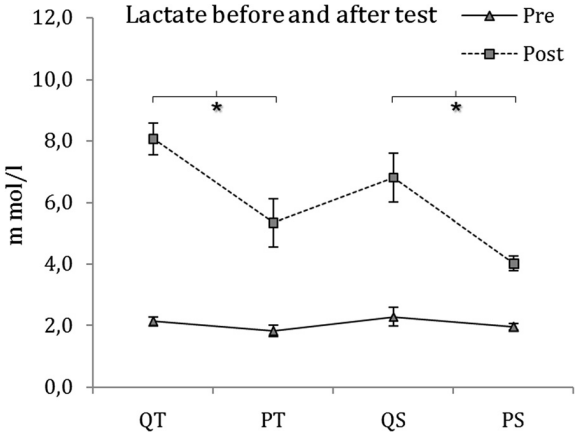


Figure 5 Blood lactate pre- and post-exercise using a two-way repeated measures ANOVA. (P=0.008 needed for significance with an experiment-wise alpha of 0.05 using Bonferroni adjustment in alpha for six comparisons) * Post lactate differences (P=0.004) in QT vs PT and QS vs PS.

PT and with PS respectively (Figure 5). These elevated blood lactate levels were an indication of enhanced glycolysis and lactate production in the skeletal muscle [30] in the quercetin supplemented groups that had run to exhaustion. However, there are other possible reasons that may explain the quercetin effects in addition to improvements in glycolytic flux. The psychostimulant effects of quercetin [8] could increase effort at high intensities and this could result in an increased lactate production. However, further experiments may corroborate this quercetin effect by measuring glycogen depletion in muscle and liver during high-intensity exercise.

In summary, no effects were measured in VO_2 peak, speed at VO_2 peak or endurance time to exhaustion after six weeks of quercetin supplementation compared with placebo in trained rats. No effects were found either in sedentary rats supplemented with quercetin compared with placebo. However, a trend was visible regarding increased performance by quercetin supplementation in some parameters like distance run until exhaustion or distance run until RQ=1. Perhaps after longer treatment, like eight or ten weeks, this effect could be significant. For the first time we have detected an increase in blood lactate production by quercetin, although more research is needed on this topic. No effects on exercise performance were found but this will need to be verified by further studies examining muscle physiology.

Limitations and strengths

The present study has several limitations that must be mentioned. First, the present physiological results obtained in rats must be confirmed in human subjects after long-term quercetin ingestion, since our results cannot be extrapolated to the potential effects over months in trained human subjects. Also, there is a lack of evidence regarding how much quercetin must be supplemented for it to exert its ergogenic effects, although 25 mg/kg is thought to be a good start. In addition, the six-week protocol applied may be insufficient to observe any ergogenic effect, and in fact there are some parameters that started exhibiting a trend and might be significant after 8-13 weeks of treatment. Finally, the lower statistical power observed in most of our results suggests to be cautious in interpreting them, future research with larger samples are needed to draw definitive conclusions. On the other hand, this is the first research that has analyzed the effect of quercetin on both sedentary and trained rats, hopefully paving the road for studies intended to find out if quercetin supplementation can enhance performance in trained athletes.

Competing interests

The authors declare no competing interest.

Authors' contributions

RAC was involved in the conception, design, acquisition and analysis of the data and drafting the manuscript, AM-A was involved in the conception, design, acquisition and analysis of the data and drafting the manuscript, EJM was involved in the conception, design, acquisition and analysis of the data and drafting the manuscript, DC-M was involved in the conception, design, acquisition and analysis of the data and drafting the manuscript, JMP was involved in the analysis of the data and drafting the manuscript and PA was involved in the conception, design, acquisition and analysis of the data and drafting the manuscript. All authors have given final approval of the version to be published.

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2. Suplementación de quercetina, eficiencia alimentaria y peso

(Artículo 2)

II

QUERCETIN EFFECTS ON WEIGHT GAIN AND CALORIC INTAKE IN EXERCISED RATS

Casuso RA, Martínez-Amat A, Martínez-Romero R, Camiletti-Moirón D, Hita-Contreras F, Martínez-López E

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QUERCETIN EFFECTS ON WEIGHT GAIN AND CALORIC INTAKE IN EXERCISED RATS

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ABSTRACT: Quercetin is a flavonoid which activates oxidative metabolism. Quercetin may reduce weight gain by decreasing feed efficiency. The present study aims to evaluate weight gain, caloric intake and feed efficiency in exercised and sedentary rats supplemented with quercetin. Wistar rats were divided into four groups: quercetin-exercise training (QT), quercetin-sedentary (QS), placebo-exercise training (PT) and placebo-sedentary (PS). Rats were exercised and/or orally supplemented with quercetin (25 mg·kg^{-1} on alternate days) during six weeks. Weight gain of the QT group decreased when compared with the PT and PS groups. Exercised groups increased cumulative caloric intake during the experimental period. The QT group rats also reduced their feed efficiency when compared with the QS and PS groups. These results suggest that quercetin is not able to decrease weight gain because no differences were found between placebo and quercetin condition either in the sedentary or in the training condition.

KEY WORDS: flavonoids; body weight; training; feed efficiency.

INTRODUCTION

Flavonoids are a large family of phenolic compounds or polyphenols. Flavonols are the most common of these phenolic compounds with a daily intake ranging from 20 mg to 35 mg [19]. One of the most common flavonols is quercetin. It is mostly found in onions, which contain up to 1.2 g·kg^{-1}, and in most plant foods in quantities of 15-30 mg·kg^{-1} of fresh produce [18]. Quercetin is currently under study because it may have anti-inflammatory and antioxidant effects [22]. Quercetin has been proposed as an ergogenic supplement due to its capacity to increase aerobic exercise performance [9,10,17], because it may increase oxidative metabolism [9]. Moreover, quercetin may also decrease adiposity [1]. However, quercetin's effects on weight gain (WG) differ between studies. While one study performed by Rivera et al. [28] reported lower WG in obese rats supplemented with quercetin, no effect of quercetin on WG was found in lean rats [3,27]. Thus, it seems that quercetin may exert its anti obesity effects on obese rather than lean rats.

It is important to study new strategies to prevent the growth of obesity and related diseases which is expected in the coming years [30]. Exercise seems to be a powerful tool to face obesity problems. However, when studies performed on a rat model are reviewed, the data are contradictory. Resistance training decreased body weight [2]; moreover, high intensity and moderate exercise training may reduce WG [4,8,20,21]. However, other researchers have not found any effect of exercise on WG [6,11,12]. But given that exercise or dietary treatment alone is less effective than the combination of exercise plus dietary treatment to reduce WG [7], it can be suggested that quercetin supplementation during exercise will be more effective to reduce WG. In addition, some polyphenols are thought to decrease body weight when supplemented during exercise [29]. We hypothesize that quercetin combined with exercise will decrease WG in rats.

When WG is evaluated, caloric intake (CI) and feed efficiency must also be assessed [27]. Feed efficiency is the ability to transform the calories into body weight [24]. Thus, the aim of the present study was to assess the ability of quercetin to diminish WG in both sedentary and trained rats. The secondary aim was to find out any effect of quercetin on CI and feed efficiency in order to achieve deeper conclusions. Finally, given that quercetin intake can increase the

weight of some organs such as the liver [3], the third goal was to assess long-term quercetin intake on muscle, heart and liver weight.

MATERIALS AND METHODS

Design. During the experimental period, rats had free access to water and maintenance chow (Table 1). Rats were weighed twice a week at the same hour, and food intake was recorded daily. Feed efficiency was calculated as previously described in the literature [26]: WG (g) ·CI (Kcal)$^{-1}$.

TABLE I. CALORIE COMPOSITION OF THE MAINTENANCE CHOW

Macronutrients	
Energy Density (Kcal·g^{-1})	2,9
Calories from Protein (%)	20
Calories from Fat (%)	13
Calories from Carbohydrates (%)	67

At the end of the treatment, and 48 hours after any exercise, the rats were anaesthetized with pentobarbital and were bled by cannulation of the aorta. All experiments were conducted according to ethical standards in sport and exercise science research [14].

Animals

The experiment was carried out on 33 young male inbred Wistar rats (Janvier, Fr), distributed into four groups: quercetin+exercise training (QT, n=9), placebo+exercise training (PT, n=8), quercetin+sedentary (QS, n=8), and placebo+sedentary (PS, n=8). Initial groups comprised nine rats, but three rats, from PT, QS and PS respectively, died during the first week. Animals were placed for eight weeks in individual cages in a thermoregulated (21 ± 2°C), well-ventilated room, with relative humidity ranging from 40% to 60%.

Exercise and quercetin supplementation

After the two weeks allowed for acclimation to experimental conditions, treadmill training took place five days a week for six weeks (on Panlab Treadmills for five rats, LE 8710R). The rats ran at a constant speed of 44 cm·s^{-1} at an angle of 10 degrees. The rats ran for 20 minutes the first two days, and for 25 minutes the third day. Training duration was increased by five minutes every two days. The rats ran for 80 minutes on the last day of the fifth week and also throughout the last week of training [5].

The rats were supplemented with quercetin (QU995; Quercegen Pharma, Newton, MA), via gavage, on alternate days throughout the experimental period. A dose of 25 mg·kg^{-1} diluted in a 1% solution of methylcellulose was used. Quercetin dose and supplementation length used in the present study were chosen because in a preliminary study we found <3-fold increase in plasma quercetin (unpublished data). Moreover, this dosage was found to activate oxidative metabolism [9].

Statistical analyses

Results are presented as mean and standard deviation. A repeated-measures ANOVA was used to analyse between-group differences within six weeks of study. Weight, CI and feed efficiency were included as dependent variables, and exercised groups as an independent variable. To analyse the results at the end of the study (haematological parameters, WG, cumulative CI and final feed efficiency), a one-way ANOVA was used, using the groups of the study as an independent variable. When the effect was significant ($P < 0.05$) a post-hoc analysis was performed (Bonferroni). Analyses were performed using the Statistical Package for Social Sciences (SPSS, v. 19.0 for Windows; SPSS, Chicago).

RESULTS

The results of the twelve measurements of weight during the six weeks of study, and WG of each group are presented in Figure 1. The ANOVA for repeated measures revealed that the QT group weighed less than the PS ($P = 0.018$) and QS groups ($P = 0.038$) from the third and the fourth weeks respectively (Fig. 1A). After the experimental period, the weight of the QT group rats was significantly lower than the QS ($P = 0.030$) and PS ($P = 0.006$) groups. There were no statistically significant differences between

TABLE 2. FINAL HAEMATOLOGICAL PARAMETERS AND ORGAN RELATIVE WEIGHT

	QT	PT	QS	PS	QT vs. PT	QT vs. QS	QT vs. PS	PT vs. QS	PT vs. PS	QS vs-PS
					p - value					
HGB (g/L)	13.66 ± 2.47	13.47 ± 1.80	12.68 ± 2.06	13.38 ± 1.98	ns	ns	ns	ns	ns	ns
HCT (%)	45.42 ± 6.03	42.81 ± 5.99	38.18 ± 6.54	40.80 ± 6.16	ns	ns	ns	ns	ns	ns
Quad R (g)	0.0069 ± 0.0007	0.0070 ± 0.0005	0.0062 ± 0.0010	0.0070 ± 0.0005	ns	ns	ns	ns	ns	ns
Quad L (g)	0.0066 ± 0.0006	0.0066 ± 0.0012	0.0072 ± 0.0008	0.0064 ± 0.0009	ns	ns	ns	ns	ns	ns
Liver (g)	0.0249 ± 0.0023	0.0217 ± 0.0022	0.0211 ± 0.0027	0.0209 ± 0.0017	p<0.05	p<0.02	p<0.01	ns	ns	ns
Brain (g)	0.0055 ± 0.0004	0.0053 ± 0.0002	0.0053 ± 0.0004	0.0053 ± 0.0002	ns	ns	ns	ns	ns	ns
Heart (g)	0.0031 ± 0.0001	0.0029 ± 0.0002	0.0028 ± 0.0002	0.0028 ± 0.0001	ns	p<0.02	p<0.01	ns	ns	ns

Note: Values are means (± SD). QT - quercetin+exercise training, PT - placebo+exercise training, QS - quercetin+sedentary, PS - placebo+sedentary, HGB - haemoglobin, HCT - hematocrit, quad L and R - quadriceps left and right. ns - not statistically significant

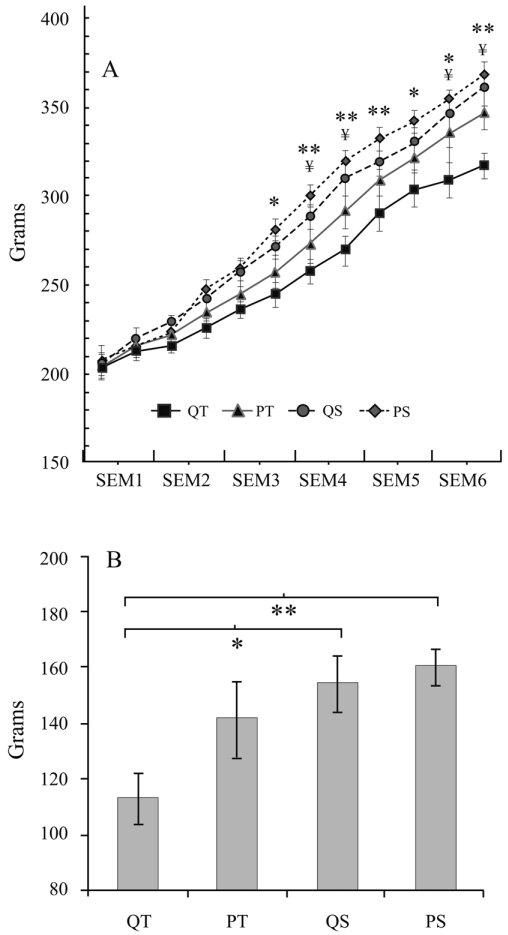

FIG. 1. WEIGHT EVOLUTION DURING THE 6 WEEKS OF STUDY (A) AND WEIGHT GAIN (B) IN EACH GROUP.
Note: Data are presented as the mean ± SD. * P<0.05. ** P<0.01 in QT vs PS (A) and in weight gain (B). ¥ P<0.05 in QT vs QS

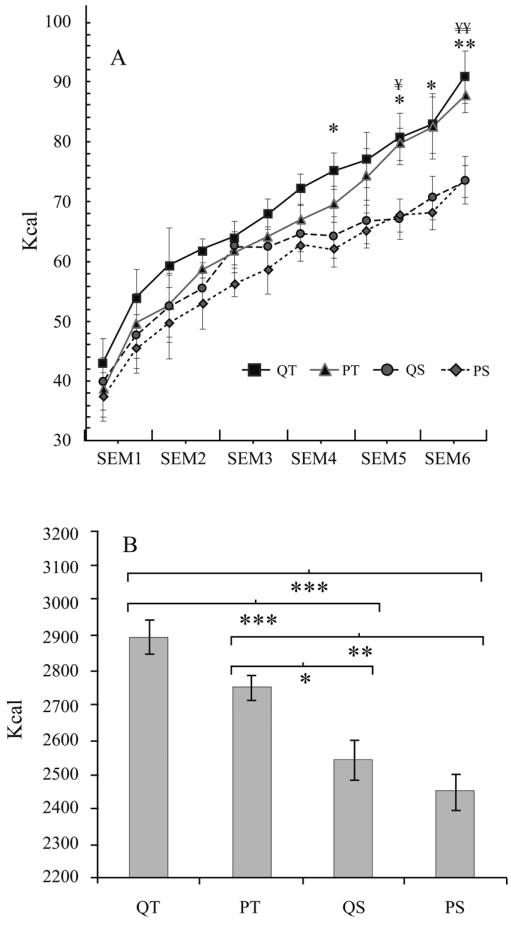

FIG. 2. CALORIC INTAKE (CI) EVOLUTION (KCAL). SAMPLE OBTAINED ON THE SAME DAYS THAT THE RATS WERE WEIGHED (A) AND CUMULATIVE CI (B).
Note: Data are presented as the mean ± SEM *P<0.05 and **P<0.01 in QT vs PS, and ¥ P<0.05, ¥¥ P<0.01 in QT vs QS for fig. 3A. * P<0.05, ** P<0.01 and ***P<0.001 for fig. 3B.

the weights of QT and PT rats in any of the measured intervals (P> 0.05). Moreover, the one-way ANOVA analysis performed on the WG data revealed a lower weight in the QT than in the QS (P = 0.012) and PS (P = 0.009) groups. There were no significant differences in WG between the QT and PT groups (Fig. 1B).

The relative weight of organs (organ weight/total weight) and final haematocrit (HCT) and haemoglobin (HGB) results are presented in Table 2. Data were analysed using a one-way ANOVA. The QT group achieved a higher relative liver weight than the other groups (P = 0.042 compared with PT, P = 0.011 compared with QS, and P = 0.007 compared with PS). The relative weight of the heart was also higher in the QT group when compared with QS (p = 0.015) and PS (p = 0.008). There were no significant differences between groups in hypertrophy rates of right or left quadriceps muscle and of brain (P> 0.05). No differences were found either in HGB or HCT between the study groups, nor in running distance between trained groups (data not shown).

A sample of twelve measurements of food intake, obtained on the same days that the rats were weighed, and the cumulative CI of six weeks are presented in Figure 2. The ANOVA for repeated measures revealed that the QT group had a higher intake than the PS group (P = 0.029) from week four, and than the QS group (P = 0.05) from week five (fig. 2A). At the end of the study daily food intake of the QT group showed higher levels (P <0.01) when compared with the QS and PS groups. No significant differences were found between the QT and PT groups on any day (P> 0.05). The one-way ANOVA revealed that cumulative CI was significantly higher in the QT group when compared with the QS and PS groups (Ps <0.001), and in the PT group when compared with the QS (P = 0.041) and PS groups (P = 0.002) (Fig. 2B).

Results of feed efficiency and total efficiency are presented in Figure 3. The analysis of repeated measures ANOVA performed on the results of feed efficiency did not find statistically significant differences between groups in any of the measurements (P>0.05) (Fig. 3A). However, total feed efficiency revealed a low-

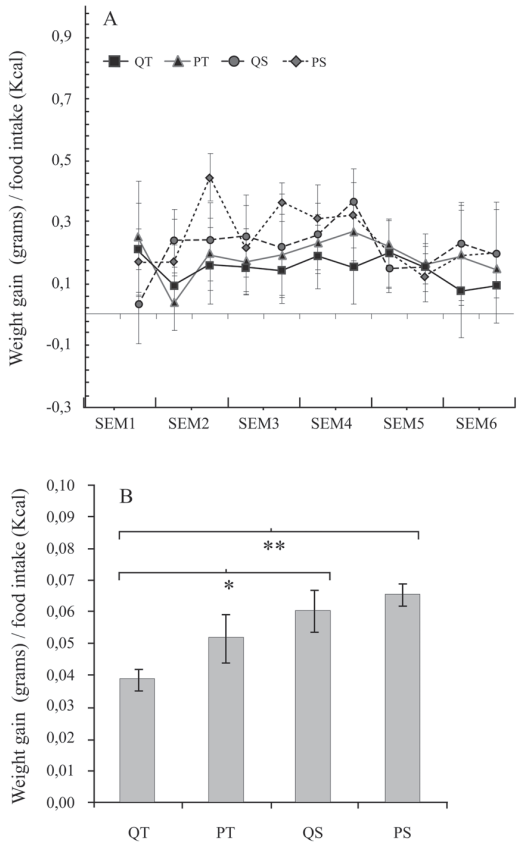

FIG. 3. FEED EFFICIENCY EVOLUTION DURING EXPERIMENTAL PERIOD (A) AND TOTAL FEED EFFICIENCY (B).
Note: Data presented as mean ± SEM * P<0.05, ** P<0.01

er rate in QT (0389 ± 0.010 Kcal) when compared with QS (0.051 ± 0.021 kcal, P = 0.045) and PS (0.060 ± 0.018, P = 0.009). There were no significant differences among the other comparisons (P> 0.05) (fig. 3B).

DISCUSSION

Taken as a whole, the results indicate that six weeks of exercise did not reduce body WG in lean rats fed with maintenance caloric chow. Although there was a lower WG in the QT group when compared with QS and PS groups, no effect was found between quercetin and placebo groups. Moreover, feed efficiency was lower in the QT group than in the QS and PS groups, but no quercetin effect was also found. It must be highlighted that exercise increased CI in both supplemented and non-supplemented groups. Moreover, when quercetin was supplemented during exercise training, the heart and liver became heavier.

In the coming years there will be a rising prevalence of obesity and related diseases [30], and it is therefore necessary to explore new strategies to avoid this problem. Polyphenols are under study as dietary compounds to decrease body weight [21]. Although it appears that polyphenols may hamper WG, quercetin seems to have a greater effect in obese rats than in lean ones [28]. Previous data have shown that polyphenol intake during exercise decreases body weight [25,29]. Some researchers, moreover, support the theory that both diet and exercise have to be controlled to reduce WG [7]. However, our results are not as clear as in previous experiments performed with mixed polyphenols. Although it seems that quercetin supplementation during six weeks of exercise training could decrease WG when compared with both sedentary groups, we believe that this effect must be attributed to exercise rather than to quercetin, because neither in the sedentary nor in the training condition is quercetin able to decrease WG when compared to placebo.

Feed efficiency measures the animal's ability to transform the calories into body weight [24]. The results of the present study show that the QT group had a lower feed efficiency than the QS and PS groups. But no differences in feed efficiency were found between the QT and PT groups, nor between the QS and PS groups. Moreover, the same results were obtained for CI and feed efficiency; in fact, the QS group had higher CI and lower feed efficiency than the QS and PS groups. Contrary to the results reported by others [2,12], our results show that exercise training increases CI in a rat model. Quercetin was supposed to be an activator of oxidative metabolism [9] and it also may inhibit adipogenesis [1]. But taken together, our data show that quercetin is not able to decrease either weight gain or feed efficiency as hypothesized, because no differences were found between placebo and the quercetin group. However, it seems that exercise impairs WG when caloric intake is increased, probably by inducing a lower feed efficiency.

In addition, our results show that the relative weight of the liver is higher in the QT group when compared to the other groups. Azuma et al. [3] described the same effect in the liver with a toxic dose of over 315 $mg \cdot kg^{-1} \cdot d^{-1}$. Thus, it is possible that long-term quercetin supplementation during exercise training may become toxic, but at the present stage we cannot reach further conclusions and more studies are needed. In addition, relative heart weight was higher in QT when compared to QS and PS, but no differences were found between placebo and quercetin condition. These data suggest that, as previously described [16], the increase in relative heart weight observed in the QT group may be an adaptation to exercise. Moreover, muscle weight results confirmed the difficulty of finding muscle hypertrophy with endurance training [13]. However, it can be stated that quercetin supplementation during exercise has a greater effect on central organs, such as heart or liver, than in peripheral organs such as muscle.

CONCLUSIONS

Our results show that quercetin supplementation is not able to decrease WG either in exercised or in sedentary rats. Moreover, no effect of quercetin was found when CI and feed efficiency were assessed. On the other hand, WG and feed efficiency are lower in the QT group. But given that these statistical differences were not found when compared to the PT group and that there is a higher CI induced by exercise training, we believe that exercise and not quercetin is responsible

for the lower WG and feed efficiency found when QT is compared to PS and QS. In addition, when liver weight was assessed, it was greater in the QT group when compared with the other groups. But more studies are needed to reach further conclusions on this topic.

Conflict of interest: The authors declare no conflicts of interest.

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3. **Efecto de la quercetina en marcadores hematológicos y óxido nítrico**

(Artículo 3)

III

PLASMATIC NITRIC OXIDE CORRELATES WITH WEIGHT AND RED CELL DISTRIBUTION WIDTH IN EXERCISED RATS SUPPLEMENTED WITH QUERCETIN

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RESEARCH ARTICLE

Plasmatic nitric oxide correlates with weight and red cell distribution width in exercised rats supplemented with quercetin

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Abstract

Quercetin is suggested as a nitric oxide regulator which may in turn influence blood parameters and weight gain. Wistar rats were classified as: quercetin-exercise training, QT; placebo-exercise training, PT; quercetin-sedentary, QS; and placebo sedentary, PS. After 6 weeks of treatment with quercetin and/or exercise, an incremental test was run to measure oxygen consumption. QT had lower levels of NO compared with PS ($p = 0.029$) and QS ($p = 0.002$). Red cell distribution width increased in both exercised groups, especially in the QT group ($p < 0.001$). Pearson correlation analysis showed that nitric oxide levels were associated with weight ($r = 0.675$) and red distribution width ($r = -0814$) in the QT group. Quercetin effect on NO production seems to be more powerful when it is supplemented during exercise training. Moreover, RDW relationship with NO production need to be further investigated in regards to health.

Keywords

Blood, flavonoids, training, VO_2max

History

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Introduction

Quercetin is a flavonoid present in many plant foods. It is found in significant amounts in onions, garlic, leeks, cabbages, apples, blueberries, tea and red wine (Manach et al., 2004). It is relevant for health studies for its anti-inflammatory and antioxidant capacity (Middleton et al., 2000). Quercetin is currently under study as an ergogenic supplementation because it is thought to improve aerobic performance by increasing mitochondrial biogenesis (Davis et al., 2009). Thus, flavonoids such as quercetin (Davis et al., 2009), resveratrol (Lagouge et al., 2006) and isoflavones (Rasbach & Schnellmann, 2008) are supposed to mimic exercise effects on mitochondrial biogenesis (Davies et al., 1981; Yan et al., 2012).

Quercetin is also suggested as a nitric oxide (NO) regulator (Qureshi et al., 2011), in fact, quercetin is associated with the decrease of NO in obese rats (Rivera et al., 2008). Given that chronic blockade of NO with L-NAME (N (G)-nitro-l-arginine methyl ester) is known to prevent weight gain (Tsuchiya et al., 2007), probably by increasing oxidative metabolism (Joost & Tschöp, 2007). Quercetin-regulatory effects on NO production may alter oxidative metabolism. Quercetin, in fact, decreases adipogenesis (Ahn et al., 2008), and it is thought to decrease weight gain in obese rats (Rivera et al., 2008). In addition, supplementation with green tea flavonoids increases oxidative metabolism in rats during exercise (Shimotoyodome et al., 2005). Thus, we believe that quercetin will increase fat utilization during exercise which, in turn, may impair weight gain in trained rats supplemented with quercetin.

Moreover, oral quercetin can be accumulated in the erythrocyte membrane to improve its reducing activity, providing greater integrity to the membrane against oxidative attack (Fiorani & Accorsi, 2005). Thus, quercetin may have a direct influence on erythrocytes and blood parameters. Given that NO is linked to some diseases such as anemia (Ballas & Marcolina, 2006) and haemolysis-related diseases (Ballas & Marcolina, 2006; Mack & Kato, 2006), it is of importance to clarify if quercetin effects on NO are related to blood parameters.

Given the information described above, quercetin may regulate NO production, which in turn may influence weight gain, fat utilization during exercise and blood parameters. Thus, the primary aim of the present study was to evaluate the hypothesis that quercetin supplementation decreases NO production in both sedentary and trained rats. Moreover, this article sets up to evaluate relationships, in trained and sedentary rats supplemented with quercetin, between NO levels and (1) weight; (2) consumption of fats and carbohydrates during exercise and (3) blood parameters.

Material and methods

Design

A sample of 33 male young inbred Wistar rats (Janvier, Fr) were classified as four groups: quercetin + exercise training (QT, $n = 9$), placebo + exercise training (PT, $n = 8$), quercetin + sedentary (QS, $n = 8$) + sedentary placebo (SP, $n = 8$). Initial groups comprised nine rats, but three rats, from PT, QS and PS, respectively, died during the first week. The rats were kept for 6 weeks in individual cages in a thermoregulated, well-ventilated

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room ($21 \pm 2\,^{\circ}C$) with a relative humidity ranging from 40 to 60%. The rats were weighed twice a week at the same time of the day, and their food intake was recorded daily. Throughout the experimental period, the rats had free access to water and maintenance chow. Blood was taken twice, immediately after the incremental running test blood was taken in order to be able to relate fat consumption during exercise and NO production during exercise. The second blood sample was taken after the completion of the study, 48 h after the last exercise, the rats were anesthetized with pentobarbital and were killed by bleeding, this blood was used to measure blood parameters and NO, in order to evaluate relationships between NO production, weight gain during the study and blood parameters. All experiments were undertaken according to the Directional Guides Related to Animal Housing and Care (Estoppey-Stojanovski, 1986), and were approved by the Ethics Committee for Animal Research of the University of Jaén.

Supplementation and exercise control

After acclimation to experimental conditions and to the treadmill, treadmill training took place 5 days a week for 6 weeks (5 rats Panlab Treadmills for LE 8710R). The rats ran at a constant speed of 44 cm/s at an angle of 10°. The rats ran for 20 min the first two days, and for 25 minutes the third day. Training duration increased by 5 min every two days. The rats ran for 80 min on the last day of the fifth week and also throughout the last week of training. This training protocol was further described previously by our research group (Casuso et al., 2013).

The rats were orally supplemented with quercetin, via gavage (QU995; Quercegen Pharma, Newton, MA) on alternate days throughout the experimental period. A dose of 25 mg/kg diluted in a 1% solution of methylcellulose was used. Quercetin also contained vitamin B3 and C, because these vitamins improve quercetin's bioavailability (personal communication, Querceguen Pharma). The placebo groups were also supplemented with methylcellulose and vitamin B3 and C (Sigma Aldrich, St. Louis, MO) at the same concentration as the QT and QS groups. Quercetin dose and supplementation length used in the present study was chosen because in a preliminary study we found a 3-fold increase in plasma quercetin (unpublished data).

Incremental running test

After 6 weeks of exercise training, an incremental test to fatigue was performed using the gas analyzer (Model LE405, Panlab/Harvard Apparatus) after due calibration. The test was performed without incline as previously described (Casuso et al., 2013), after an initial set of 2 min at 22 cm/s, the speed was increased by 11 cm/s every 2 min. The test was considered complete when the rat was visibly exhausted and on the shock bar for 5 s. The use of fats and carbohydrates during exercise was measured by indirect calorimetry as described elsewhere (Shimotoyodome et al., 2005). We used the mean VO_2 and RQ during the test, which were recorded every 20 seconds.

Blood collection and analysis

The first blood collection was performed immediately after the incremental running test in order to evaluate relationships between fat consumption during exercise and NO production [NO(1)]. The rats were anesthetized with pentobarbital and blood was removed from the subclavian vein. The specimens were centrifuged for 5 min at 13 500 rpm, and the plasma obtained was immediately frozen and stored at $-80\,^{\circ}C$ until it was used to evaluate NO production.

The second extraction was performed when rats were killed by bleeding, 48 h after the incremental running test. A portion was collected using heparin as an anticoagulant for the measurement of blood parameters, hemoglobin, haematocrit and red cell distribution width (RDW), (KX-21 Automated Hematology Analyzer, Sysmex Corporation, Kobe, Japan). The remainder of the blood collected from the rats bled was centrifuged at 13 500 rpm for 5 min. The plasma obtained was immediately frozen and stored at $-80\,^{\circ}C$ until it was used to measure NO production. This second measurement was intended to relate NO(2) with the blood parameters of this extraction and with the final weight.

Nitric oxide quantification

NO production was indirectly quantified in plasma by determining nitrate/nitrite and S-nitroso compounds (NOx), using an ozone chemiluminescence-based method. To carry out the analysis, the thawed plasma aliquots were mixed in 1/2/2 (w/v/v) deproteinization buffer (0.5 N NaOH and 10% $ZnSO_4$), briefly shaken and allowed stand at room temperature for 15 min. After that, samples were centrifuged for 5 min at 13 500 rpm and supernatants were collected and maintained at $4\,^{\circ}C$ until analysis. The total amount of NOx in the deproteinized samples was determined using the purge system of Sievers Instruments, model NOA 280i. A saturated solution of vanadium chloride (VCl_3) in 1 M HCl was added to the nitrogen-bubbled purge vessel fitted with a cold water condenser and a water jacket to permit heating of the reagent to $90\,^{\circ}C$, using a circulating bath. HCl vapors were removed by a gas bubbler containing 1 M NaOH. The gas flow rate into the detector was controlled by a needle valve adjusted to yield a constant pressure. Once the detector signal was stabilized, samples were injected into the purge vessel to react with the reagent, converting NOx to NO, which was then detected by ozone-induced chemiluminescence. NOx concentrations were calculated by comparison with standard solutions of sodium nitrate. NOx data were normalized with total protein concentration of each sample.

Statistical analysis

Results are presented as mean ± SD. Variables were tested for normal distribution by the Kolmogorov–Smirnov test. *t*-Test for dependent samples was used to compare NO production after the incremental test [NO(1)] and after training completion [NO(2)] during bleeding. One-way ANOVA was using as independent variable studying groups (QT, PT, QS and PS) and as dependent variable NO(1), NO(2), initial weight, final weight, mean food intake, VO_2, RQ, carbohydrate utilization, fat utilization, and haematological parameters. Significance was set at $p < 0.05$. When the effect was significant, a post-hoc analysis was done (Bonferroni). Correlations between NO and other parameters were studied using Pearson. Analyses were performed using the Statistical Package for Social Sciences (SPSS, v. 19.0 for Windows; SPSS, Chicago, IL).

Results

Nitric oxide production

Plasmatic NO end-metabolites were measured as an indicator for NO production (Table 1). Immediately after the incremental test, NO production [NO(1)] was measured in order to compare NO production and fat utilization during exercise. In addition, after the completion of the exercise training period, NO production [NO(2)] was also measured in order to assess the effects of exercise training and quercetin supplementation on NO production. The one way ANOVA analysis showed a significant decrease in the QT group when compared with the QS group ($p = 0.001$) in NO(1). A decrease in the QT group compared with QS and PS ($p = 0.002$ and $p = 0.029$) was also found in NO(2). The *t* test for

Table 1. End-metabolites of plasma nitric oxide (NO) after incremental test (1) and after completion of the training program (2).

					P					
	QT	PT	QS	PS	QT versus PT	QT versus QS	QT versus PS	PT versus QS	PT versus PS	QS versus PS
NO(1)	4.25 ± 0.77	5.03 ± 1.26	6.61 ± 1.72	5.40 ± 0.58	>0.999	0.001	0.298	0.068	>0.999	0.279
NO(2)	2.94 ± 0.74	3.41 ± 0.57	4.14 ± 0.58	3.82 ± 0.39	0.676	0.002	0.029	0.130	>0.999	>0.999
P NO (1–2)	0.001	0.026	0.005	0.001						

Mean value ± SD. Value comparison by groups, measured by quantification of nitrites and nitrates and expressed as µmol/l.
P NO (1–2): Differences between NO(1) and NO(2).

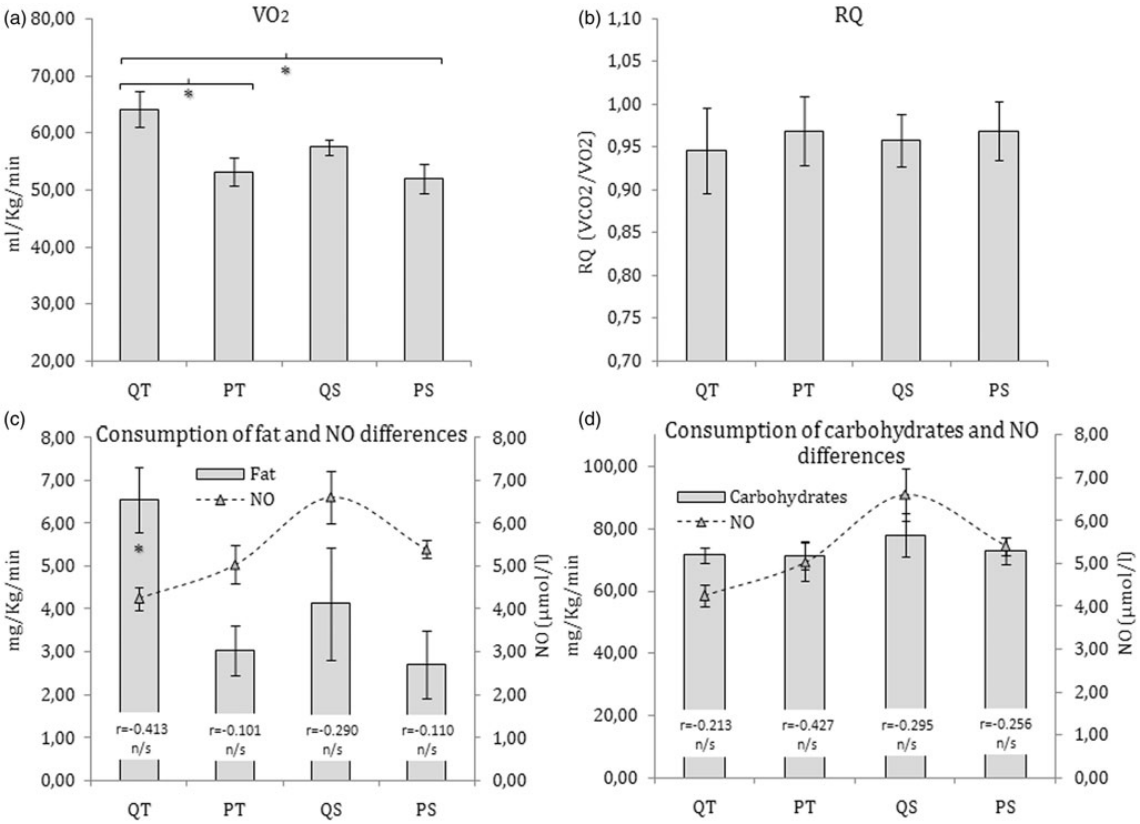

Figure 1. Results of VO_2 (a) and RQ (b) during the maximal test based on the mean of all measurements recorded every 20″. Use of fat (c) and carbohydrates (d) during the test, correlation with NO(1) levels in the blood sample after the test (smooth curve). Comparisons between groups.* $p < 0.05$. * in (c) = $p < 0.05$ compared with PT and PS. n/s: not significant correlation.

dependent samples found that all values of NO(1) were higher than those obtained from NO(2) (QT, $p = 0.001$; in PT, $p = 0.026$; in QS, $p = 0.005$; in PS, $p = 0.001$).

Incremental test and indirect calorimetry

In order to assess fat utilization during exercise, oxygen consumption was measured during an incremental test to fatigue (Figure 1). Moreover, for the proper calculation of fat and carbohydrates consumption (see 'Methods' section) during the incremental test, an average of oxygen consumption during the whole exercise was needed, for that reason the VO_2 data showed here is the mean of all VO_2 data recorded during the test (every 20″). The RQ was also measured as the mean of all measurements taken every 20″. The QT group used a greater amount of oxygen during the test compared with the PT ($p = 0.028$) and the PS group ($p = 0.012$) (Figure 1a). The analysis between groups showed no significant differences in RQ. No differences were found in peak oxygen consumption or RQ at the maximal oxygen consumption (data not shown). An increase in fat consumption was found in the QT group during the incremental test compared with the PT ($p = 0.042$) and the PS group ($p = 0.028$). No differences were found in carbohydrate utilization in any of the other groups ($p > 0.999$). Pearson correlation analysis was not significant as regards the relation between the consumption of fats or carbohydrates and NO(1) after the incremental test (Figure 1c and d).

Completion of the exercise training program

After the completion of the study, 48 h after last exercise, the rats were anesthetized with pentobarbital and killed by bleeding, this blood was used to measure blood parameters and NO, NO(2), in order to evaluate relationships between NO production, final

Table 2. Initial weight, mean food intake and haematological parameters obtained during bleeding.

				P				
	QT	PT	QS	PS	QT versus PT	QT versus QS	QT versus PS	PT versus QS	PT versus PS	QS versus PS
Initial weight (g)	146.11 ± 7.01	143.22 ± 8.24	147.01 ± 6.66	147.15 ± 13.43	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999
Average food intake (g)	22.41 ± 2.05	22.17 ± 1.22	18.47 ± 1.44	17.28 ± 1.46	>0.999	<0.001	<0.001	<0.001	0.001	0.886
HGB (g/dL)	13.66 ± 2.47	13.47 ± 1.80	12.68 ± 2.06	13.38 ± 1.98	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999
HCT (%)	45.42 ± 6.03	42.81 ± 5.99	38.18 ± 6.54	40.80 ± 6.16	>0.999	0.163	0.883	0.964	>0.999	>0.999

Mean value ± SD. Value comparison by groups. HGB: hemoglobin; HCT: haematocrit.

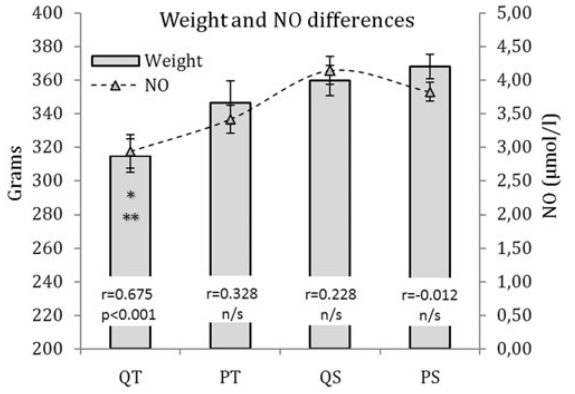

Figure 2. Final weight and correlation with plasma levels of NO(2) obtained from bleeding (smooth curve). Values of final weight compared between groups: $*p<0.05$ QT versus QS; $**p<0.01$ QT versus PS. n/s: non-significant correlation.

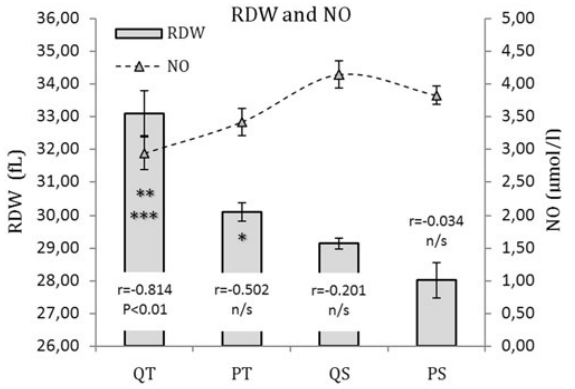

Figure 3. RDW values by groups and correlation with plasma NO(2) levels obtained from bleeding (smooth curve). RDW values compared between groups: $*p<0.05$ PT versus PS, $**p<0.01$ QT versus PT, $***p<0.001$ QT versus QS and PS. n/s: non-significant correlation.

weight and blood parameters. Table 2 presents the results (mean ± SD) of initial weight, food intake, hemoglobin (HMG) and hematocrit (HCT). No differences were found in the initial weight. The average daily food intake throughout the experimental period was significantly greater among the trained groups ($p>0.001$) than among the sedentary groups. There were no significant differences in HGB or HCT between either group. Figure 2 presents the final weight and NO(2) production. The QT group final weight was lower than that in the QS ($p<0.021$) and the PS group ($p<0.004$). But no differences were found between placebo and quercetin groups. However, Pearson correlation analysis showed a positive relationship between NO (2) levels and final weight in the QT group ($r=0.675$, $p<0.001$). Figure 3 shows that the QT group had a higher red cell distribution width (RDW) compared with the PT ($p<0.01$), the QS and the PS group ($p<0.001$). The PT group also showed a higher RDW compared with the PS group ($p<0.05$). The correlation analysis showed a strong negative relationship between NO(2) and (red cell distribution width) RDW in the QT group ($r=-0.814$, $p<0.01$)

Discussion

NO production was lower in QT when compared to QS and PS, but no differences were found between placebo and quercetin

condition. Moreover, when quercetin is supplemented during exercise training, quercetin seems to increase fat consumption during exercise when compared to QS and PS. In addition, data reported here show a lower final weight in QT when compared to QS and PS and this effect is related to NO production. In addition there is a strong negative relationship between NO production and a higher RDW in the QT group.

Quercetin (3,3′,4′,5,7-Pentahydroxyflavone) is a natural polyphenolic flavonoid present in significant amounts in onions, garlic, leeks, cabbages, apples, blueberries, tea and red wine (Manach et al., 2004). Antioxidant activities of flavonoids may decrease oxidative stress, which in turn reduces disease risk (Heim et al., 2002). Given that skeletal muscle fibers continually generate reactive oxygen species at a slow rate that increases during muscle contraction (Reid, 2008). It is possible that the effects that we discuss, regarding to the QT group, could be due to the antioxidant effect of quercetin on the production of reactive oxygen species during exercise.

Although this article was not designed for the study of the effect of acute exercise on end-metabolites of NO, nitric oxide was higher immediately after progressive exercise than after a 48 hour rest period following the completion of the program. These results are similar to those found in humans (Rassaf et al., 2007). This response may be due to the fact that acute exercise increases the activity of eNOS (Caicedo et al., 2011). In mammals, up to 70 or 90% of plasma nitrites are derived from the activity of this enzyme (Kleinbongard et al., 2003). This may be an explanation for the results found, although no firm conclusions can be drawn at the present stage.

RQ is a ratio that compares the volume of carbon dioxide produced by an organism during a given period of time (VCO_2) with VO_2 ($RQ = VCO_2/VO_2$). Our results show an increase in VO_2 average (measured every 20″) during the incremental test in the QT group compared with both placebo groups. Although the RQ average did not change (measured every 20″), fat consumption during exercise was greater in the QT group, because it depends on both factors, VO_2 and RQ (Shimotoyodome et al., 2005). The increase in mitochondrial density caused by quercetin (Davis et al., 2009) might also cause an increase in fat oxidation (Glatz et al., 2010). Quercetin is known to activate transcription factors related to the oxidative metabolism (Davis et al., 2009). Our results provide new information about this topic. Quercetin supplementation causes an increase in fat consumption in exercised rats during progressive exercise. Therefore, quercetin had a similar effect as green tea extract (Shimotoyodome et al., 2005). No changes were found in carbohydrate utilization; and, contrary to our hypothesis, fat consumption during exercise was independent of NO levels. This is because our hypothesis was based on a chronic application of the established NO synthase inhibitor L-NAME (Tsuchiya et al., 2007). However the NO levels obtained in QT could be too high to increase energy dissipation and oxygen consumption as proposed by Joost & Tschöp (2007).

Quercetin had a different effect on exercised and sedentary groups, it significantly increased NO production in the sedentary group compared with the exercised group. However, the PT group showed no difference with the others groups. This confirms the difficulty to see adjustments in the NO-dependent vasodilator system with exercise in healthy (Green et al., 2011). The results of nitric oxide should be considered carefully because healthy animals show a small response to exercise of the final metabolites of NO (Jasperse & Laughlin, 2006). So, the discussion of the results of this signalling molecule can easily become speculative. Furthermore, it should be taken into account that our observations on NO metabolites are limited only to nitrates and nitrates without considering the radical production that may affect the response during training. In fact exercise itself is known to increase NO production (Stamler & Meissner, 2001; Reid, 2008). Thus, given that NO production do not differ between placebo and quercetin neither in the sedentary nor in the trained groups, it is possible that results obtained in the QT group could be an exercise effect, rather than an adaptation induced by quercetin. Similarly, Rivera et al. (2008) found a quercetin effect on NO production in obese rats but not on lean rats. However, our results and those reported by Rivera et al. (2008) are in contrast to those showed by Qureshi et al. (2011). Thus, there is an increase on NO production in QT group, however at this stage no direct effect of quercetin on NO can be stablished, and further studies should be performed to clarify the higher NO production induced by quercetin when it is supplemented during exercise.

Although quercetin reduces adipogenesis (Ahn et al., 2008), we do not believe that the lower weight found in the QT group is induced by quercetin, because no differences were found between quercetin and placebo groups. A previous study show a lower weight in quercetin-supplemented rats compared with the control group (Rivera et al., 2008). Although we have not obtained the same results, regarding to QS group, these differences might be because Rivera's data were obtained after a longer treatment. Our study, otherwise, shows for the first time that lower NO plasmatic level, shown in QT, correlates with lower final weight. Some authors have suggested that NO plays an important role in controlling lipid and glucose metabolism (Joost & Tschöp et al., 2007; Tsuchiya et al., 2007). The latter authors propose that chronic blockade of NO production is followed by weight loss caused by a decrease in adipogenesis. So, a significant decrease in NO that occurs after six weeks of quercetin intake during exercise training is associated with lower weight.

Based on previous analysis (Fiorani & Accorsi, 2005) we thought that quercetin could be able to increase HCT and HGB. Contrary to our hypothesis, quercetin, neither combined with exercise nor in the sedentary condition, does not alter blood HCT or HGB. However, there is an increase in RDW with exercise, especially, in the QT group. There is also a strong negative correlation between RDW and NO(2) level in QT after the treatment. This relationship is known to occur in certain types of anemia (Ballas & Marcolina, 2006) in which there is a decrease in NO and an increase in RDW. Moreover, RDW has been usually studied in patients with coronary diseases (Tonelli et al., 2008; Bazick et al., 2011). Thus, it is possible that quercetin supplementation during exercise compromises erythrocyte integrity. Because when there is a decrease in NO and an increase in RDW, this process results from hemolysis and the decrease in erythrocyte hemoglobin (Ballas & Marcolina, 2006; Mack & Kato, 2006). However, our results show a normal level of hemoglobin in these groups after completion of exercise training (Table 1). Moreover, following training period there is an increase in RDW in women (McClung et al., 2009). Thus, it must be clarified the effect of quercetin supplementation during exercise on anemia disease, because at the current stage we cannot confirm that the relationhip between NO and RDW in the QT is beneficial, or not, for health.

Conclusions

In summary, the results obtained indicate that chronic ingestion of quercetin during training decreases NO production when compared to QS and PS. But given that no effect was found between quercetin and placebo neither in the sedentary nor in the trained conclusion, we cannot confirm yet that this result is due to a quercetin effect. Similarly, when final weight was assessed the QT group showed a lower weight when compared to QS and PS probably because of the increase in fat oxidation reported in the QT group. It is important to note that in the QT group the final

weight correlates with NO production, providing evidence for a physiological pathway induced by quercetin which involve NO and may result in a lower weight. Furthermore, there is a strong RDW increase in the QT, which also correlates with NO production. This might imply an anemia disease, but given that hemoglobin data are not altered, RDW data in response to exercise and quercetin supplementation should be further evaluated.

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Declaration of interest

The authors declare no competing interest.

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2. ADAPTACIONES CELULARES EN RESPUESTA AL EJERCICIO. EFECTOS DE LA SUPLEMENTACIÓN DE QUERCETINA EN DIFERENTES TEJIDOS

(ARTÍCULOS IV, V y VI)

IV

ORAL QUERCETIN SUPPLEMENTATION HAMPERS SKELETAL MUSCLE ADAPTATIONS IN RESPONSE TO EXERCISE

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Oral quercetin supplementation hampers skeletal muscle adaptations in response to exercise training

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We aimed to test exercise-induced adaptations on skeletal muscle when quercetin is supplemented. Four groups of rats were tested: quercetin sedentary, quercetin exercised, placebo sedentary, and placebo exercised. Treadmill exercise training took place 5 days a week for 6 weeks. Quercetin groups were supplemented with quercetin, via gavage, on alternate days throughout the experimental period. Sirtuin 1 (SIRT1), peroxisome proliferator-activated receptor γ coactivator-1α mRNA levels, mitochondrial DNA (mtDNA) content, and citrate synthase (CS) activity were measured on quadriceps muscle. Redox status was also quantified by measuring muscle antioxidant enzymatic activity and oxidative damage product, such as protein carbonyl content (PCC). Quercetin supplementation increased oxidative damage in both exercised and sedentary rats by inducing higher amounts of PCC ($P < 0.001$). Quercetin supplementation caused higher catalase ($P < 0.001$) and superoxide dismutase ($P < 0.05$) activity in the non-exercised animals, but not when quercetin is supplemented during exercise. Quercetin supplementation increased SIRT1 expression, but when quercetin is supplemented during exercise, this effect is abolished ($P < 0.001$). The combination of exercise and quercetin supplementation caused lower ($P < 0.05$) mtDNA content and CS activity when compared with exercise alone. Quercetin supplementation during exercise provides a disadvantage to exercise-induced muscle adaptations.

Exercise induces muscle mitochondrial biogenesis both when performed as endurance low-intensity exercise (Yan et al., 2012) and high-intensity training (Burgomaster et al., 2008). Mitochondrial biogenesis is a complex process in which a small amount of nuclear transcriptional factors coordinate the expression of a high amount of nuclear and respiratory proteins (for review, see Scarpulla et al., 2012). One of these transcriptional factors, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), is described in the current literature as the master regulator of mitochondrial biogenesis by virtue of its ability to coactivate and augment the expression and activity of several transcription factors, which, in turn, bind to the promoters of distinct sets of nuclear-encoded mitochondrial genes (Handschin & Spiegelman, 2008; Fernandez-Marcos & Auwerx, 2011). In situations of energy/nutrient stress, post-trascriptional activation of PGC-1α is mainly induced by sirtuin 1 (SIRT1), a metabolic sensor regulated by NAD+, which, in turn, induces PGC-1α activation by deacetylation (Gerhart-Hines et al., 2007; Rodgers et al., 2008; Cantó et al., 2009).

Dietary flavonoids induce mitochondrial biogenesis by activating the SIRT1–PGC-1α pathway at the messenger RNA level (Lagouge et al., 2006; Rasbach & Schnellmann, 2008; Davis et al., 2009). Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a natural polyphenolic flavonoid present in significant amounts in onions, garlic, leeks, cabbages, apples, blueberries, tea, and red wine (Manach et al., 2004). Quercetin has been proposed as an ergogenic supplement for athletes because it may mimic training-induced mitochondrial biogenesis in skeletal muscle (Davis et al., 2009). Recent meta-analyses of the currently available literature have concluded that chronic quercetin supplementation appears to improve aerobic capacity and endurance performance, with significant differences seen in untrained populations, but not trained groups (Kressler et al., 2011; Pelletier et al., 2013). However, even when the effects are statistically significant, the magnitude of change is trivial to small and unlikely to have real-world significance (Pelletier et al., 2013).

Quercetin metabolites, in fact, induce oxidative damage (Boots et al., 2005), although paradoxically, this

toxicity seems to be a result of its protection. Quercetin may exert an antioxidant effect at the early stage of the supplementation, but later, quercetin's metabolites that are formed during the antioxidant activity might shade the direct positive effects of quercetin supplementation (Boots et al., 2007). Therefore, quercetin supplementation may provide a disadvantage for adaptive responses to stress. In fact, quercetin supplementation inhibits cellular and systemic adaptations to exercise-heat stress (Kuennen et al., 2011). Given that exercise acts as a muscle stressor by increasing the rate of reactive oxygen species in skeletal muscle (Reid, 2008), exercise-induced oxidative stress may explain why the SIRT1–PGC-1α pathway and mitochondrial biogenesis is not altered in trained subjects supplemented with quercetin (Nieman et al., 2009). Moreover, in a recent study developed by our research team, we found that 6 weeks of quercetin supplementation is not ergogenic neither in exercised nor in sedentary rats (Casuso et al., 2013). Thus, it is necessary to clarify the effect of long-term quercetin supplementation on the muscle SIRT1–PGC-1α pathway and mitochondrial biogenesis when supplemented during exercise training. We hypothesized that quercetin would not provide any advantage to adaptive response of quadriceps muscle to endurance exercise training.

The purpose of this study was to evaluate the role of long-term (6-week) supplementation of quercetin during exercise training on the SIRT1–PGC-1α pathway and mitochondrial DNA (mtDNA) content in rat's quadriceps muscle. The second goal of the present study was to evaluate the effects of quercetin on skeletal muscle redox status following an exercise period.

Methods

Animals

This study was performed on male Wistar rats aged 6 weeks and weighing 147 ± 4 g. The animals were maintained for 8 weeks in individual cages under standard conditions of light (12:12 light–dark cycle), temperature (21 °C), and humidity (40–60%) and allowed *ad libitum* access to food (Harlan 2014, maintenance chow; Indianapolis, Indiana, USA) and water. Four hours after run to exhaustion test, the rats were anesthetized with pentobarbital and were killed by bleeding. All experiments followed the ACSM animal care standards and were approved by the institutional committee for ethics.

Exercise and supplementation

Rats were randomly assigned to quercetin (Q, $n = 17$) and no-quercetin (NQ, $n = 17$) groups. Both groups were further divided into quercetin exercised (QEX; $n = 9$), quercetin sedentary (QSED; $n = 8$), no-quercetin exercised (NQEX; $n = 9$), and no-quercetin sedentary (NQSED; $n = 8$). All rats were acclimated to experimental conditions for 2 weeks, and 3 days before the training period, trained groups were acclimated to the treadmill. Exercise training and supplementation were further described previously (Casuso et al., 2013). Treadmill exercise training took place 5 days a week for 6 weeks (5 rats Panlab Treadmills for LE 8710R). The rats ran at a constant speed of 44 cm/s at an angle of 10%. The rats ran for 20 min during the first 2 days and for 25 min on the third day. Training duration increased by 5 min every 2 days. The rats ran for 80 min on the last day of the fifth week and also throughout the last week of training. The rats in the quercetin groups were supplemented with quercetin, via gavag (QU995; Quercegen Pharma, Newton, Massachusetts, USA) on alternate days throughout the experimental period. Supplementation took place in the morning < 2 h before exercise training; a dose of 25 mg/kg diluted in a 1% solution of methylcellulose was used. Length and dosage was chosen because in a preliminary study we found a < 2.5 time-fold increase in plasma quercetin. The no-quercetin groups were also supplemented with the vehicle.

Run to exhaustion

Twenty-four hours after last bout of exercise, a low-intensity endurance test was administered to each rat; endurance capacity was assessed during a run to exhaustion at 44 cm/s and 10% grade. The test finished when the animal was visibly exhausted, not able to maintain the appropriate pace, and this resulted in a rising frequency of landings on the electrical shock grid, when rats were quickly removed. The endpoint was marked by the rat's inability to return to the treadmill belt and to right itself on a flat surface.

Tissue collection

All rats were anesthetized with pentobarbital and were bled by cannulation of the aorta 48 h after the last exercise. Quadriceps muscle was immediately collected, rinsed in saline solution, frozen in liquid nitrogen, and stored at 80 °C until their further analysis. We chose quadriceps muscle as a whole because it is composed of a mixed-fiber population similar to the m. vastus lateralis, which was previously used to assess quercetin's effect on skeletal muscle (Nieman et al., 2009, 2010). Thus, sport research may have a more applicable information about quercetin's effect on skeletal muscle adaptations.

Quantitative real-time polymerase chain reaction (RT-PCR)

Gene expression of different genes (peroxisome proliferator-activated receptor γ coactivator 1, PGC-1α; NAD(+)-dependent histone deacetylases, SIRT1) was quantitatively assessed by Rt-PCR using β-actin as the normalizing gene. Total RNA was isolated from cell extracts using Trizol reagent (Invitrogen, Carlsbad, California, USA), according to the manufacturer's instructions. After treatment with DNase, cDNA was synthesized from 1.5 µg total RNA using reverse transcriptase (Superscript™ III RT, Invitrogen) with oligo-(dT) 15 primers (Promega, Fitchburg, Wisconsin, USA). RT-PCR was performed on the Stratagene MxPro 3005P qPCR system using the Brilliant II SyBR Green QPCR Master Mix (STRATAGENE, La Jolla, California, USA). The following primer pairs were used: PGC-1, 5′-GCGGACAGAA CTGAGAGACC-3′ and 5′-CGACCTGCGTAAAGTATATCCA-3′; SIRT-1, 5′-CCTGACTTCAGATCAAGAGATGGTA-3′ and 5′-CTGATTAAAAATATCTCCACGAACAG-3′; β-actin, 5′ CTT AGAAGCATTTGCGGTGCCGATG-3′ and 5′-TCATGAAGTGT GACGTTGCATCCGT-3′. Experiments were performed with triplicate, and the relative quantities of target genes corrected with the normalizing gene, β-actin, were calculated using the STRATAGENE MxProTM QPCR Software. Quantification of mRNA expression of PGC-1α and SIRT1 was calculated using the ΔΔCT method as previously described (Livak & Schmittgen, 2001).

Mitochondrial DNA quantification

DNA (mitochondrial and nuclear) was extracted from quadriceps muscle samples using a QIAamp DNA minikit (QIAGEN,

Chatsworth, California, USA), and the concentration of each sample was determined spectrophotometrically at 260 nm. RT-PCR was performed on the Stratagene MxPro 3005P qPCR system using the Brilliant II SyBR Green QPCR Master Mix (STRATAGENE). Mitochondrial content was estimated as the ratio between copy numbers of mtDNA (cytochrome b; forward, 5′-AAAGCCACCTTGACCCGATT-3′; reverse, 5′-GATTCGTAG GGCCGCGATA-3′; probe, 5′-CGCTTTCCACTTCATCTTACC ATT-3′) vs nuclear DNA (β-actin).

Thiobarbituric acid reactive substances (TBARS)

TBARS, major indicators of oxidative stress, were determined in rats muscle following the instructions of the Oxikek TBARS Assay Kit (ZeptoMetrix Corp., Buffalo, New York, USA). Final values were referred to the total protein concentration in the initial extracts.

Protein carbonyls

Determination of the protein carbonyl contents (PCCs), an indicator of oxidative stress, was analyzed by 2,4-dinitrophenylhydrazine method as described by Levine et al. (1990). Results were expressed as mmol/mg protein.

Enzyme assays

For catalase (CAT) activity, muscles were homogenized on ice in 5–10 mL of cold buffer [50 mM potassium phosphate, pH 7.0, containing 1 mM ethylenediaminetetraacetic acid (EDTA)] per gram of tissue. After centrifugation at 10 000 *g* for 15 min, the supernatant was collected for protein determination (Bradford, 1976) and subsequent analysis. All procedures were performed at 4 °C.

CAT activity was studied by monitoring the decomposition of H_2O_2 at 240 nm, according to the method described by Beers and Sizer (1952).

For superoxide dismutase (SOD) activity, muscles were homogenized on ice in 5–10 mL of cold buffer (20 mM HEPES, pH 7.2, containing 1 mM EDTA, 210 mM mannitol, and 70 mM sucrose) per gram tissue. After centrifugation at 15000 *g* for 5 min, the supernatant was collected for protein determination (Bradford, 1976) and subsequent analysis. All procedures were performed at 4 °C.

SOD activity was assayed by measuring the rate of inhibition of cytochrome c reduction by superoxide anions generated by a xanthine/xanthine oxidase system (Flohé & Ötting, 1984).

For citrate synthase (CS) activity, a small portion of muscle (10 mg) from quadriceps muscle was used. Total CS activity was measured in Tris–HCl buffer (50 mM Tris–HCl, 2 mM EDTA, and 250 µM NADH, pH 7.0) and 0.04% Triton-X. The CS reaction was started by the addition of 10 mM oxaloacetate, and activity was measured spectrophotometrically by measuring the disappearance of NADH at 412 nm. Total protein content of all homogenates was determined by the method of Bradford (1976).

Statistics

Results are presented as mean ± SD. Homocedasticity and normality was tested by Levenne's and Kolmogorov–Smirnov tests, respectively. Results were analyzed using two-factor ANOVA (with/without quercetin and with/without exercise). If this analysis revealed a significant interaction, specific differences between mean values were located using Student's *t*-test for independent samples. *t*-Test for independent samples was performed to analyze final weight, distance run and time to exhaustion. The level of significance was considered at $P < 0.05$. All analyses were performed using the Statistical Package for Social Sciences (SPSS, version 19.0 for Windows; SPSS, Inc., Chicago, Illinois, USA).

Results

During the 6-week training period, the average running distance was similar between the exercised groups (Table 1). Likewise, the final weight was similar between groups. Time to exhaustion was ~2.4-fold higher ($P < 0.001$) in the exercised groups compared with the sedentary groups, but no effect of quercetin supplementation was detected.

Quercetin supplementation increased oxidative damage by inducing higher ($P < 0.001$) amounts of PCC (Fig. 1). The increase in PCC in sedentary rats supplemented with quercetin was higher ($P < 0.001$) than in non-supplemented sedentary rats. Likewise, PCC was also higher ($P < 0.001$) in quercetin supplemented and exercised rats than in non-supplemented and exercised rats. However, exercise enhance muscle redox status by reducing TBARS ($P < 0.05$) and PCC ($P = 0.014$).

Quercetin supplementation caused higher CAT ($P < 0.001$) and tSOD ($P < 0.05$) activity in the non-exercised animals. However, CAT and tSOD activities were similar in the supplemented and non-supplemented exercising animals (Fig. 2).

Both exercise ($P < 0.05$) and quercetin ($P < 0.001$) supplementation increased PGC-1α mRNA levels (Fig. 3). Importantly, the combination of exercise and quercetin supplementation resulted in a 128% increase in PGC-1α mRNA levels compared with the exercised

Table 1. Exercise training, final weight, and final endurance test capacity data

	Exercised		Sedentary	
	QEX	NQEX	QSED	NQSED
Distance run Average per day (m)	1040 ± 83	1021 ± 60	_	_
Final weight (g)	352.89 ± 31.25	367.25 ± 24.41	379.25 ± 52.91	366.63 ± 8.97
Run to exhaustion (min)	179.4 ± 19.3	169 ± 25.4	73 ± 15.8	69.8 ± 12.8

Values are means ± SE. *t*-Test for independent samples showed no difference ($P > 0.05$) between quercetin groups and non quercetin groups in either exercised and sedentary rats.

g, grams; m, meters; min, minutes; NQSED, no-quercetin sedentary; NQEX, no-quercetin exercised; QEX, quercetin exercised; QSED, quercetin sedentary.

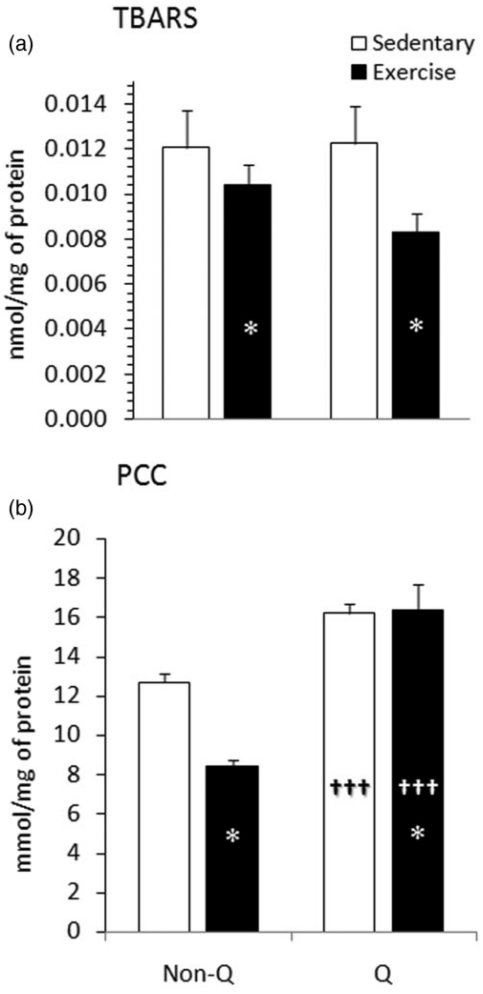

Fig. 1. Effects of quercetin supplementation and exercise training on (a) thiobarbituric acid reactive substances (TBARS) and (b) protein carbonyl content (PCC) in muscle. Values are mean ± SE; sample size for each variable ranged from $n = 8$ to 9 for all groups. Results from two-way ANOVA, and *t*-test when interaction was significant. †††$P < 0.001$, main effect for quercetin. *$P < 0.05$, main effect for exercise. PCC quercetin × exercise interaction ($P = 0.008$) are described in the Results section.

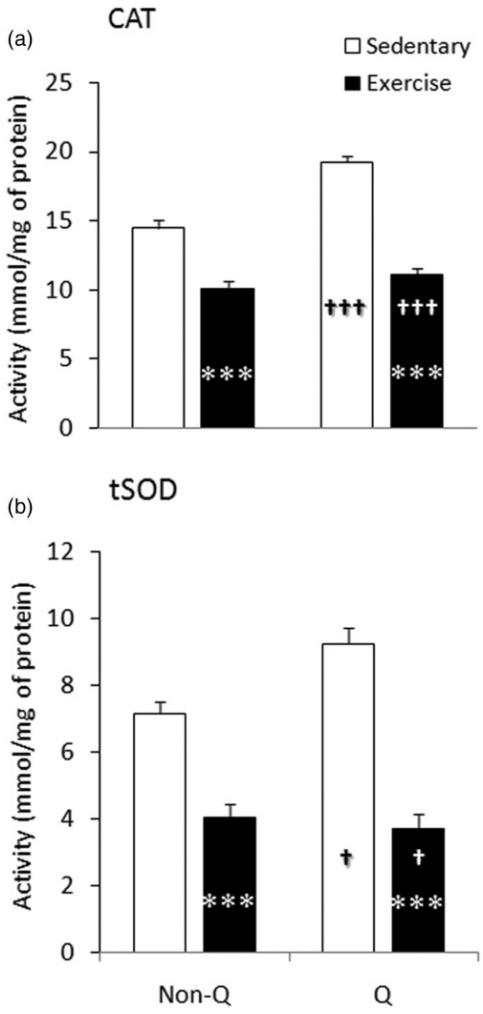

Fig. 2. Effects of quercetin supplementation and exercise training on (a) muscle catalase (CAT) and (b) total superoxide dismutase (tSOD) enzyme activity. Values are mean ± SE; sample size for each variable ranged from $n = 8$ to 9 for all groups. Results from two-way ANOVA, and *t*-test when quercetin × exercise interaction was significant. †$P < 0.05$, main effect for quercetin. †††$P < 0.001$, main effect for quercetin. ***$P < 0.001$, main effect for exercise. Quercetin × exercise interaction for CAT ($P = 0.001$) and tSOD ($P = 0.008$) are described in the text.

group, but no interactions were found for PGC-1α ($P > 0.05$). SIRT1 mRNA levels were similar before and after exercise training in the non-supplemented group (Fig. 3). Quercetin supplementation during exercise abolished ($P < 0.001$) the quercetin-induced increase ($P < 0.001$) in SIRT1 mRNA expression. Moreover, SIRT1 levels were similar between the exercised supplemented group and the non-supplemented groups.

Neither quercetin nor exercise increased mtDNA (Fig. 4a). However, the combination of exercise and quercetin supplementation caused lower ($P < 0.05$) mtDNA content when compared with exercise alone. Results for CS activity are presented in Fig. 4b. Exercise significantly increased CS activity ($P = 0.01$; main effect), quercetin did not increase the activity of CS, but a tendency was reported ($P = 0.151$; main effect). Interaction analysis revealed that in the quercetin and exercised groups, CS activity tended to increase when compared with the quercetin and sedentary groups ($P = 0.105$). In the non-quercetin groups, exercise increased CS activity ($P < 0.001$). However, if quercetin is supplemented during exercise, CS activity is diminished when compared with exercise alone ($P = 0.023$).

Discussion

For 6 weeks, rats were given 25 mg/kg of quercetin or placebo in both exercised and sedentary condition. We found that exercise acts as an antioxidant in quadriceps muscle by reducing TBARS and PCC, which are markers of oxidative damage. However, quercetin

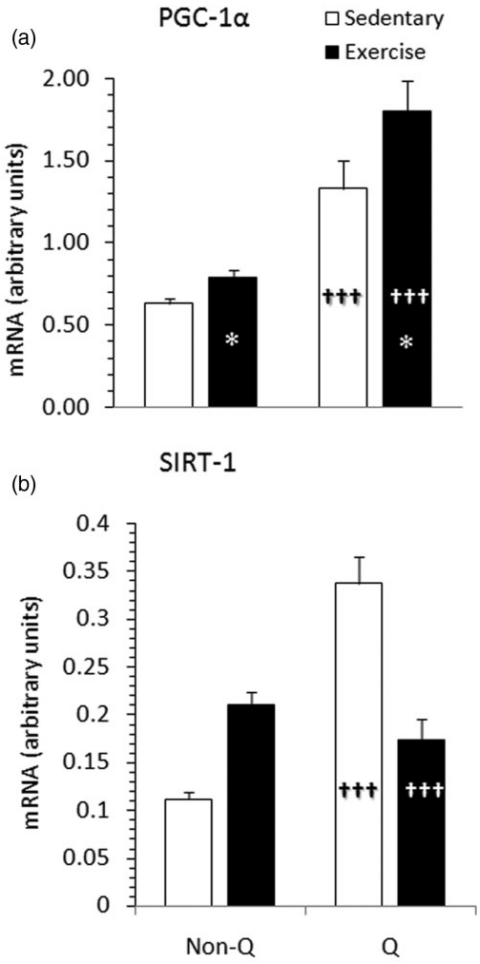

Fig. 3. Effects of quercetin supplementation and exercise training on (a) peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and (b) sirtuin 1 (SIRT-1) mRNA expression in muscle. Values are mean ± SE; sample size for each variable ranged from $n = 8$ to 9 for all groups. Results from two-way ANOVA, and *t*-test when interaction was significant. †††$P < 0.05$, main effect for quercetin. *$P < 0.01$, main effect for exercise. SIRT1 interactions ($P < 0.001$) are described in the Results section.

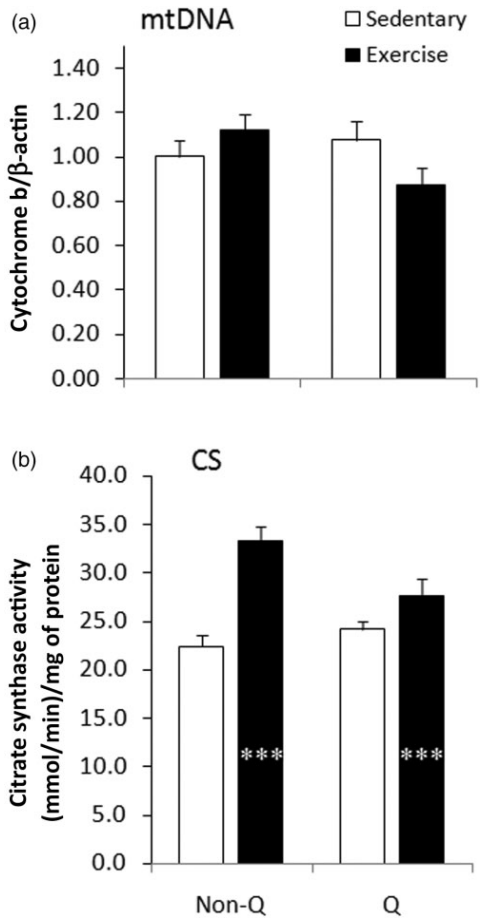

Fig. 4. Effects of quercetin supplementation and exercise training on quadriceps (a) mtDNA and citrate synthase (CS) activity. Values are mean ± SE; sample size for each variable ranged from $n = 8$ to 9 for all groups. Results from two-way ANOVA, and *t*-test when interaction was significant. Interactions are further described in the results section. ***$P < 0.001$ main effect for exercise.

supplementation hampered exercise-induced adaptation to oxidative stress because it increased PCC levels. In the sedentary condition, quercetin increased the activity of some antioxidant enzymes, whereas this effect is prevented if quercetin is supplemented during exercise. We have found a main effect of quercetin and exercise toward an increase in PGC-1α and SIRT1 mRNA in quadriceps muscle; however, quercetin supplementation during exercise abolished the quercetin-induced increase in SIRT1 mRNA in quadriceps muscle. Quercetin did not alter endurance performance but possibly compromises an exercise training effect on mitochondrial content.

The present findings provide added information to the growing evidence in literature supporting that quercetin is not an ergogenic supplement (Cheuvront et al., 2009; Cureton et al., 2009; Dumke et al., 2009; Nieman et al., 2009, 2010; Biegelman et al., 2010; Ganio et al., 2010; Pelletier et al., 2013). In fact, results from a previous study developed by our research team showed that quercetin is not ergogenic neither in sedentary nor in exercised rats (Casuso et al., 2013), but results presented here extend these results to a muscle physiological level. We choose a 25 mg/kg intake every 2 days, which is a similar intake to the dosage used in most of the quercetin studies, i.e., < 1000 mg/day. In humans, this dosage entails a rise in plasma quercetin of < 200% (Cureton et al., 2009; Nieman et al. 2009; 2010), which is close to the plasmatic levels found in our preliminary studies, when the supplementation and the exercise protocol was tested (data not shown). Nevertheless, this dosage cannot be achieved by a common diet because an 80-k subject should eat daily 1 kg of the richest food sources of quercetin (onions, curly, broccoli, and blueberries).

We have investigated the SIRT1-PGC-1α pathway in quadriceps muscle. This pathway is known to be activated in situations of energy/nutrient stress, such as exercise or caloric restriction. The NAD+-induced metabolic

sensor SIRT1 is thought to be one of the most important regulators of PGC-1α mRNA (Gerhart-Hines et al., 2007; Cantó et al., 2009). This is of importance because PGC-1α is nowadays considered as a key regulator of the mitochondrial biogenesis process (Handschin & Spiegelman, 2008; Fernandez-Marcos & Auwerx, 2011; Scarpulla et al., 2012). PGC-1α, moreover, is related to exercise-induced protection against metabolic and neurodegenerative diseases (Handschin & Spiegelman, 2008). Thus, strategies to induce PGC-1α expression, which may mimic exercise induced mitochondrial biogenesis, has been a focus of numerous research in the past years.

Quercetin is thought to increase mitochondrial content by activating the SIRT1–PGC-1α pathway, involving higher endurance performance (Davis et al., 2009). Therefore, a diet–exercise–gene expression interaction has been proposed. Our results are opposite, at least in part, than those which have been reported by Davis et al. (2009). Similarly, we have found an upregulation of the SIRT1–PGC-1α pathway in sedentary rodents; however, we have reported only a tendency toward an increase in CS activity induced by quercetin. There is one potential reason for the discrepancy between Davis et al.'s (2009) results and ours. Their results were from soleus muscle a well-known oxidative muscle with a high composition of slow-twitch muscle fibers. PGC-1α expression is enriched in tissues with high-capacity mitochondrial systems because it drives the formation of slow-twitch muscle fibers (Lin et al., 2002). Thus, quercetin-induced effect on PGC-1α may have a larger effect in predominately oxidative muscles such as soleus rather than in quadriceps muscle, which is composed of a mixed-fiber population. However, when quercetin effects on skeletal muscle adaptations were assessed in humans, a portion of m. vastus lateralis was analyzed (Nieman et al., 2009; 2010), which is composed of a mixed-fiber population broadly termed type I and type II fibers. Thus, results from the rats whole quadriceps muscle may be better to be compared with results found in human muscle.

Results presented here also report that quercetin increase the expression of both PGC-1α and SIRT1 in a sedentary condition, but when quercetin is supplemented during exercise, SIRT1 expression is hampered. SIRT1 is supposed to be critical in PGC-1α-increased mitochondrial and fatty acid utilization gene expression (Gerhart-Hines et al., 2007; Rodgers et al., 2008). In agreement, it is reasonable to suggest a critical role of SIRT1 in order to induce mitochondrial biogenesis when quercetin is supplemented. Thus, it is possible that quercetin supplementation during exercise compromises exercise-induced mitochondrial biogenesis by a downregulation of the *SIRT1* gene, in spite of the fact that PGC-1α is upregulated. However, it does not lead to mitochondrial pathology/dysfunction sufficiently severe to limit muscle fatigue during exercise. In fact, the tendency toward an increased CS activity when quercetin is supplemented during exercise when compared with sedentary groups may explain the longer running time to fatigue in the Q-exercised group.

Quercetin supplementation was previously described to provide a disadvantage for adaptive responses to stress, such as exercise on a heat environment (Kuennen et al., 2011). Exercise itself, in fact, is known to be a stressor agent because skeletal muscle fibers continually generate reactive oxygen species at a slow rate that increases during muscle contraction (Reid, 2008). Our data show that both proteins (PCC) and lipids (TBARS) oxidative damage products are reduced following training period. In addition, there is a decrease in tSOD and CAT enzymatic activity in skeletal muscle, adaptation that was previously described for exercise (Laughlin et al., 1990). Taken together, these data are in agreement with the studies that support a model whereby exercise itself acts as an antioxidant (Gomez-Cabrera et al., 2008; Ristow et al., 2009). When quercetin is administered during exercise, an antioxidant effect is not found. In fact, some studies have failed to find any antioxidant effect of quercetin during endurance exercise (McAnulty et al., 2008; Quindry et al., 2008), but it should be noted that in these studies, muscle was not analyzed and only blood antioxidant capacity was assessed. Regarding the activities of some antioxidant enzymes, there is an upregulation induced by quercetin. Thus, we suggest that quercetin moderate muscle antioxidant enzymatic activity. This effect was, however, hampered by simultaneous exercise training. In fact, when quercetin is ingested during exercise, enzymatic activities are similar than in the only exercised group, which implies that in the combination of exercise and quercetin, CAT and tSOD activities are regulated in a similar way to which it is done only with exercise (Laughlin et al., 1990). Nevertheless, a selectively oxidative damage to proteins is induced by quercetin after 6 weeks of quercetin intake.

Although quercetin is often proposed as a powerful antioxidant (Middleton et al., 2000), our results show a prooxidant effect of quercetin. As a result of its antioxidant protection, quercetin metabolites produced may turn into prooxidant agents; this paradoxical effect is called as the Quercetin Paradox (Boots et al., 2005). Moreover, the Quercetin Paradox is supposed to be produced in two steps (Boots et al., 2007): first, at the early stage of the supplementation quercetin may exert an antioxidant effect. Then, quercetin metabolites that are produced during its antioxidant effect promote a prooxidant state. It is possible therefore that the increased mitochondrial biogenesis and performance shown in mice after short-term quercetin supplementation (Davis et al., 2009) is due to an acute response. What is more, it may not be induced if quercetin is chronically supplemented due to the switch to prooxidant agents of quercetin metabolites described above. Notably, quercetin is abundant in many commonly consumed fruits and vegetables, particularly

apples, cranberries, blueberries, and onions (Manach et al., 2004). This could prevent any acute response to short-term ingestion in humans, and may also explain why researchers have failed the attempt to demonstrate any quercetin-ergogenic effect in humans (Cheuvront et al., 2009; Cureton et al., 2009; Nieman et al., 2009, 2010; Biegelman et al., 2010; Ganio et al., 2010; Pelletier et al., 2013).

Perspectives

Results presented here suggest that quercetin supplementation during exercise provide a disadvantage to exercise-induced adaptations on skeletal muscle. This is of importance because a recent review concluded that quercetin supplementation might be beneficial for athletes (Kressler et al., 2011) even when ergogenic effect has not been proved yet. Exercise-induced resistance against oxidative stress and also mitochondrial biogenesis are compromised when quercetin is supplemented during exercise. This is of importance because it may explain the null results obtained when muscle physiology and performance is assessed in trained subject supplemented with quercetin (Dumke et al., 2009; Nieman et al., 2009; Biegelman et al., 2010). Thus, further human research should be carried out in order to clarify if quercetin supplementation in trained people hampers exercise-related improvements on muscle adaptations.

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Key words: Flavonoids, SIRT1, PGC-1α, training, oxidative damage.

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V

ORAL QUERCETIN SUPPLEMENTATION HAMPERS BRAIN ADAPTATIONS IN RESPONSE TO EXERCISE TRAINING

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Submitted

Oral quercetin supplementation hampers brain adaptations in response to exercise training

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ABSTRACT

We aimed to investigate quercetin effects on brain plasticity when it is supplemented during exercise. Four groups of rats were tested: quercetin-sedentary (Q-sedentary); quercetin-exercised (Q-exercised); no-quercetin-sedentary (NQ-sedentary); and no-quercetin-exercised (NQ-exercised). Treadmill exercise training took place 5 days a week for 6 weeks. Quercetin groups were supplemented with quercetin throughout the experimental period. Sirtuin 1, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) mRNA levels and markers of mitochondrial content were measured on brain. Redox status was also quantified by measuring enzymatic activity and oxidative damage. Activities of catalase and superoxide dismutase were upregulated in the Q-sedentary but not in the Q-exercised. Exercise reduced oxidative damage in brain but this effect was hampered in the Q-exercised. Both exercise and quercetin supplementation induced an upregulation of the PGC-1α gene but also SIRT1 was upregulated in NQ-exercised and Q-sedentary. In the Q-exercised, however, exercise effects on SIRT1 and PGC-1α were abolished. This effect induced lower mitochondrial markers when compared to the NQ-exercised and the Q-exercised. Thus, exercise and quercetin promote brain adaptation but this effect is hampered in the Q-exercised group.

KEY WORDS: Quercetin, exercise, brain, mitochondria, redox status

INTRODUCTION

Regular exercise is carried out in order to evoke adaptations, which include a wide range of beneficial effects, such as maintained brain health, improved cognitive processes and increased resistance to brain injury[1,2]. In fact, there are several clinical consequences of inactivity regarding to neurological disorders[3]. During last years it has been argued that exercise may counteract neurological disorders such as neurodegeneration by an upregulation of the PGC-1α gene [3,4]. PGC-1α (peroxisome proliferator-activated receptor γ coactivator-1α) is a transcriptor factor which is considered as the master regulator of the mitochondrial biogenesis [5-7]. Mitochondrial biogenesis is a complex process in which a small amount of nuclear transcriptional factors, such as PGC-1α, coordinate the expression of a high amount of nuclear and respiratory proteins [8]. In situations of energy/nutrient stress, postrascriptional activation of PGC-1α is mainly induced by sirtuin 1 (SIRT1), a metabolic sensor regulated by NAD+, which in turn, induces PGC-1α activation by deacetylation [9,10]. PGC-1α deacetylation by SIRT1 is also described for brain tissue[11]

In fact, brain and skeletal muscle seems to have similar responses to exercise training. Moderate intensity exercise training for 8 weeks promote similar mitochondrial adaptations in both tissues in mice [12]. Thus, given that mitochondria is suggested to exert a critical role in neurodegenerative diseases[3,13,14] but also in the adaptations related to exercise training in rats brain[12]. It is of importance to test strategies that may mimic exercise induced mitochondrial biogenesis in brain tissue. Dietary flavonoids induce mitochondrial biogenesis by activating the SIRT1-PGC-1α pathway at the transcriptional level[15-17]. Quercetin (3,3´,4´,5,7-Pentahydroxyflavone) is a natural polyphenolic flavonoid present in significant amounts in onions, garlic, leeks, cabbages, apples, blueberries, tea and red wine[18]. It has been proposed that quercetin may mimic exercise-induced mitochondrial adaptations on brain of sedentary mice[17].

However, recent researches have reported that quercetin is not ergogenic neither in humans[19] nor in rats[20]. What is more, quercetin has been proposed to provide a disadvantage for exercise in the heat[21]. This can be explained because during the biological effects of quercetin some metabolites produced change their activity to prooxidants[22.23]. In fact, we have previously reported, in a rat model, that after 6 weeks of quercetin supplementation during exercise both mitochondrial biogenesis and resistance against oxidative stress are compromised in skeletal muscle[24]. Given that at the early stage of exercise training, before homeostasis is achieved, exercise acts as an oxidative stressor in a similar way in muscle[25] and brain tissue[26]. It is of importance to test the hypothesis that quercetin would not provide any advantage to adaptive response of brain tissue to endurance exercise training.

Thus, the purpose of the present study was to test the role of long-term (6 weeks) supplementation of quercetin during exercise training on the SIRT1-PGC-1α pathway and mitochondrial content in rat´s brain. The second goal of the present study was to evaluate effects of quercetin on brain redox status following an exercise period.

EXPERIMENTAL METHODS

Animals

This study was performed on male Wistar rats (6 weeks old). The animals were maintained for 8 weeks in individual cages under standard conditions of light and temperature and allowed ad libitum access to food (Harlan 2014, maintenance chow) and water. All experiments were approved by the Committee for Ethics of the University of Jaén (Spain).

Exercise and supplementation

Rats were randomly assigned to quercetin (Q; n = 17) and no-quercetin (NQ; n = 16) groups. Both groups were further divided into Q-exercised (n = 9), Q-sedentary (n = 8), NQ-exercised (n = 8), NQ-sedentary (n = 8). All rats were acclimated to experimental conditions for two weeks and 3 days before the 6-week training period trained groups were acclimated to the treadmill. Treadmill training took place 5 days a week for 6 weeks (5 rats Panlab Treadmills for LE 8710R). The rats ran at a constant speed of 44cm / s at an angle of 10 °. The rats ran for 20 minutes during the first two days and for 25 minutes on the third day. Training duration increased by 5 min every two days.

The rats ran for 80 min on the last day of the fifth week and also throughout the last week of training[20]. The rats in the supplemented groups were supplemented with quercetin,via gavage, (QU995; Quercegen Pharma, Newton, MA) on alternate days throughout the experimental period. A dose of 25mg/kg diluted in a 1% solution of methylcellulose was used. Quercetin dosage and length was chosen because in a preliminary study we found a ~2.5-fold increase in plasma quercetin levels. The no-quercetin groups were also supplemented, using the gavage procedure, with the vehicle (1% solution of methylcellulose).

Tissue collection

All rats were anesthetized with pentobarbital and were bled by cannulation of the aorta 48h after the last exercise. Brains were immediately collected, rinsed in saline solution, frozen in liquid nitrogen, and stored at 80°C until their further analysis.

Plasmatic quercetin

Quercetin and it methylated form isoharmetin were measured in plasma after 6 weeks. Two aliquots of plasma were acidified with acetic acid (0.583 mol/L) to a final pH of 5. 1 of the 2 plasma

samples was treated with b-glucuronidase/sulfatase. All samples were incubated for 60 min at 37°C. Further treatment of samples and analysis was performed by HPLC.

Quantitative RT-PCR

Gene expression of different genes (peroxisome proliferator-activated receptor γ coactivator 1, PGC-1; NAD(+)-dependent histone deacetylases, SIRT-1) was quantitatively assessed by real-time PCR using β-actin as the normalizing gene. Total RNA was isolated from cell extracts using Trizol reagent (Invitrogen) according to the manufacturer's instructions. After treatment with DNase,cDNA was synthesised from 1.5 μg total RNA using reverse transcriptase (SuperscriptTM III RT, Invitrogen) with oligo-(dT) 15 primers (Promega). Real-time PCR was performed on the Stratagene MxPro 3005P qPCR system using the Brilliant II SyBR Green QPCR Master Mix (STRATAGENE, La Jolla, CA, USA). The following primer pairs were used: PGC-1, 5′-GCGGACAGAACTGAGAGACC-3′ and 5′-CGACCTGCGTAAAGTATATCCA-3′; SIRT-1, 5`-CCTGACTTCAGATCAAGAGATGGTA-3′ and 5′-CTGATTAAAAATATCTCCACGAACAG-3′; β-actin, 5′ CTTAGAAGCATTTGCGGTGCCGATG-3′ and 5′-TCATGAAGTGTGACGTTGCATCCGT-3′. Experiments were performed with triplicates, and the relative quantities of target genes corrected with the normalizing gene, β-actin, were calculated using the STRATAGENE MxProTM QPCR Software. Quantification of mRNA expression of PGC-1 and SIRT-1 was calculated using the ΔΔCT method as previously described[27].

Mitochondrial DNA quantification

DNA (mitochondrial and nuclear) was extracted from quadriceps muscle samples using a QIAamp DNA minikit (QIAGEN, Chatworth, CA), and the concentration of each sample was determined spectrophotometrically at 260 nm. Real-time PCR was performed on the Stratagene MxPro 3005P qPCR system using the Brilliant II SyBR Green QPCR Master Mix (STRATAGENE, La Jolla, CA, USA). Mitochondrial content was estimated as the ratio between copy numbers of mtDNA (cytochrome b; forward, 5′-AAAGCCACCTTGACCCGATT-3′; reverse, 5′-GATTCGTAGGGCCGCGATA-3′; probe, 5′-CGCTTTCCACTTCATCTTACCATT-3′) versus nuclear DNA (β-actin).

Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS), major indicators of oxidative stress, were determined in mice brain following the instructions of the Oxikek TBARS Assay Kit (ZeptoMetrix Corp.). Final values were referred to the total protein concentration in the initial extracts.

Protein carbonyls

Determination of the protein carbonyl contents, an indicator of oxidative stress, were analyzed by 2,4- dinitrophenylhydrazine method as described by Levine *et al*. [28]. Results were expressed as mmol/mg protein.

Enzyme assays

For catalase (CAT) activity, the cerebral cortex from animals was dissected, rinsed in saline solution and stored at -80º C until used. Cerebral cortices were homogenized on ice in 5-10 ml of cold buffer (50mM potassium phosphate, pH 7.0, containing 1mM EDTA) per gram of tissue. After centrifugation at 10,000 x g for 15 min, the supernatant were collected for protein determination[29] and subsequent analysis. All the procedures were performed at 4º C. CAT activity was studied by monitoring the decomposition of H2O2 at 240 nm, according to the method described by Beers and Sizer[30]. For superoxide dismutase (SOD) activity, the cerebral cortices were homogenized on ice in 5-10 ml of cold buffer (20mM HEPES, pH 7.2, containing 1mM EGTA, 210 mM mannitol, and 70 mM sucrose) per gram tissue. After centrifugation at 15000 x g for 5 min, the supernatant were collected for protein determination (Bradford, 1976) and subsequent analysis. All the procedures were performed at 4º C. SOD activity was assayed by measuring the rate of inhibition of cytochrome c reduction by superoxide anions generated by a xanthine/xanthine oxidase system[31].

Total citrate synthase (CS) activity was measured in Tris·HCl buffer (50 mM Tris·HCl, 2 mM EDTA, and 250 μM NADH pH 7.0) and 0.04% Triton-X. The CS reaction was started by the addition of 10 mM oxaloacetate, and activity was measured spectrophotometrically by measuring the disappearance of NADH at 412 nm. Total protein content of all homogenates was determined by the method of Bradford[29].

Statistics

Results are presented as mean ± SD. Homoscedasticity and normality was tested by Levene and Kolmogorov-Smirnov test respectively. Results were analyzed using two-factor ANOVA (with/ without quercetin and with/without exercise). If this analysis revealed a significant interaction, specific differences between mean values were located using Student's t test for independent samples. t Test for independent samples was performed to analyze final weight, distance run and food intake. The level of significance was considered at $P<0.05$. All the analyses were performed using the Statistical Package for Social Sciences (SPSS, version 19.0 for Windows; SPSS, Inc., Chicago, IL, USA).

RESULTS

Exercise training, food intake, and body weight (table 1).

No quercetin effect was found on the average meters run over the trained period when both trained groups were compared ($P>0.05$). No difference was also observed in final weight between groups ($P>0.05$). Food intake average during the experimental period did not change significantly when quercetin is supplemented, although exercised groups increased food intake ($P<0.001$).

Effect of quercetin and exercise on brain CAT and SOD activity.

Quercetin seems to modulate brain antioxidant activity, in fact, there was a 65% increase in CAT ($P<0.001$) and a 66% increase in SOD ($P<0.01$) in Q-sedentary compared with NQ-sedentary rats (Fig.1). However, this effect is abolished when quercetin is supplemented during exercise ($P<0.001$). Exercise decreased CAT and SOD activities ($P<0.001$, main effect for exercise)

Effect of exercise and quercetin on brain lipid and protein oxidative damage.

Exercise increase antioxidant protection in brain, in fact, exercise decreased thiobarbituric acid reactive substances (TBARS) in brain ($P=0.001$; main effect for exercise; figure 2A). Moreover exercise reduces the amount of protein carbonyls content (PCC) when NQ-sedentary was compared with NQ-exercised ($P<0.05$). When looking at PCC, quercetin x exercise interaction was significant ($P=0.020$; figure 2B). Quercetin seems to act as a prooxidant, there was, in fact, a 23% increase ($P=0.042$) in PCC in the Q-sedentary group (22.3±4.6) compared with NQ sedentary (17.7±3.6). Furthermore, there was a 83% increase ($P<0.001$) in the Q-exercised group (25.4±4.3) compared with NQ-exercised group (13.9±3.2).

Effect of exercise and quercetin on brain SIRT1 and PGC-1α mRNA gene expression.

Interaction quercetin x exercise ($P<0.001$) showed that exercise is a powerful tool to upregulate both PGC-1α ($P<0.01$) and SIRT1 ($P<0.001$) at the mRNA level in brain tissue (Fig.3). Likewise, quercetin seems to mimic exercise effects on the SIRT1-PGC-1α pathway. In fact, regarding to the Q-sedentary group there was a 49% increase ($P=0.03$) in PGC-1α and a 194% increase ($P<0.001$) in SIRT1 when compared to NQ-sedentary. However there was a disruptive effect in the SIRT1-PGC-1α pathway in brain when quercetin is supplemented during exercise. There was a 64% decrease ($P<0.01$) in PGC-1α and a 360% decrease in SIRT1 ($P<0.01$) when the Q-exercised group is compared with the Q-sedentary group. In addition, when quercetin was supplemented during exercise the exercise effect on the SIRT1-PGC-1α pathway was abolished. There was a 98% decrease ($P=0.003$) in PGC-1α and a 194% decrease in SIRT1 ($P<0.01$) when the Q-exercised group was compared with the NQ-exercised.

Effect of exercise and quercetin on brain mitochondrial DNA content and CS activity

Exercise training for 6 weeks increased mitochondrial content in brain (Fig.4), in fact, our results showed a higher ($P<0.001$) mtDNA content when NQ-exercised is compared with NQ-sedentary. Likewise, quercetin acts in a similar way because Q-sedentary showed a higher mtDNA content ($P<0.001$) when compared with NQ-sedentary. Importantly, quercetin supplementation during exercise may compromise both exercise and quercetin induced mitochondrial biogenesis. In fact, the Q-exercised group showed a lower mtDNA content when compared with both the NQ-exercised ($P<0.001$) and the Q-sedentary ($P<0.001$). Exercise training increased CS activity in brain tissue ($P<0.05$; main effect). What is more, there was a significant increase in NQ-exercised ($P<0.001$) and Q-sedentary ($P<0.01$) when compared with the NQ-sedentary group. What is more, when compared with NQ-exercised ($P<0.01$) and Q-sedentary ($P<0.05$) the effects on CS activity were abolished when quercetin was supplemented during exercise.

DISCUSSION

In the present study we used a rat model to study both exercise and quercetin effects on brain redox status and mitochondrial content. Following 6-week´s of exercise training and/or quercetin supplementation (25mg/kg every other day), we found that quercetin acts as a prooxidant agent in brain. Quercetin, however, seems to upregulate antioxidant enzymatic activity in the sedentary condition, nevertheless this effect is hampered in the Q-exercised group. An interesting finding is that quercetin seems to mimic exercise-induced effect on the SIRT1-PGC-1α pathway and the mitochondrial genesis in brain tissue. Importantly, in the Q-exercised group there is a decrease in the transcription of SIRT1 and PGC-1α which may explain the lower mitochondrial content found.

Brain is usually a forgotten organ when exercise adaptations are studied. Inactivity, however, is a risk factor for many chronic disorders, regardless of age, gender, race and health status. In fact, regarding to neurological disorders, possible clinical consequences of inactivity are: Learning and memory impairment, cognitive dysfunction, dementia, depression, mood and anxiety disorders and neurodegeneration[3]. Some of these neurological disorders, if not all, can be counteracted by regular moderate intensity exercise by an upregulation of the PGC-1α gene[3]. In fact, a recent study have proved that endurance exercise increase brain mitochondrial biogenesis through the activation of the SIRT1-PGC-1α pathway at the transcriptional level[12]. Accordingly, results reported here suggest that 6 weeks of exercise training increase brain mitochondrial content through the transcription of both SIRT1 and PGC-1α.

Quercetin, a natural polyphenolic flavonoid present in a variety of plant foods[18], is supposed to mimic exercise induced upregulation of the SIRT1-PGC-1α pathway and mitochondrial biogenesis in brain[17]. Our data support this statement, because we have found that quercetin induce brain mitochondrial biogenesis through the activation of the SIRT1-PGC-1α pathway. PGC-1α is considered the main regulator of mitochondrial biogenesis, because it interacts and coactivate some transcription factors such as peroxisome-proliferator-activated receptors (PPARs), estrogen-related receptors (ERRs), and nuclear respiratory factors, NRF-1 and NRF-2, that govern most of mitochondrial functions and biogenesis[8]. In brain, activation of PGC-1α in response to exercise may be due to the activation of SIRT1[12]. The SIRT1-PGC-1α relationship is well described in scientific literature, SIRT1 is a metabolic sensor induced by NAD+ in situations of energy/nutrient stress[32]. It is an important functional regulator of PGC-1α by deacetylation in skeletal muscle[9] and, similarly, in brain tissue[11]. Studies on mice treated with resveratrol (a polyphenol with a similar structure than quercetin) showed an expression of SIRT1, which correlated with deacetylation of PGC-1α, in the hippocampus[15,33]. Accordingly our results indicate that long-term quercetin supplementation mimic exercise-induced mitochondrial biogenesis in brain tissue. Moreover, this effect is supposed to be by activating the SIRT1-PGC-1α pathway at the transcriptional level.

However, quercetin supplementation during exercise compromise both exercise and quercetin effects on brain mitochondrial content by disrupting the SIRT1-PGC-1α pathway. This is in accordance with previous results by our research team which concluded that quercetin supplementation during exercise abolish exercise-induced effects on mitochondrial content by decreasing SIRT1 transcription[24]. Moreover, a recent study demonstrated that quercetin supplementation provide a disadvantage for adaptive response to stress[21]. Given that physical activity is a brain stressor at least at the early stage of exercise training, when high amounts of ROS are produced and the adaptation has not been achieved yet[26]. Given that oxidative stress is thought to link metabolic health and mitochondrial physiology[34]. It is possible that the mitochondrial disfunction found when quercetin is supplemented during exercise may induce an unadaptive response to exercise-induced resistance against oxidative challenge.

Similar to the conclusion of an elegant review[26], exercise training likely increases brain resistance and tolerance against oxidative challenge. Both TBARS and PCC are decreased after 6 weeks of exercise training in brain tissue. Quercetin, however, induce higher amounts of proteins carbonyls, suggesting brain oxidative damage promoted by quercetin. The prooxidant effect of quercetin was previously named as the “Quercetin Paradox”[22] because during its antioxidant effect, some quercetin metabolites formed turn to prooxidant agents[23]. Thus, the oxidative damage induced

by quercetin in brain might be a long-term effect of quercetin supplementation, as previously described in skeletal muscle by our research group[24]. It should be noted that in the Q-sedentary group there is an increased activity of some antioxidant enzymes which suggest a self protection effect against oxidative damage. However, this enzymatic upregulation is hampered in the Q-exercised group. Taken together, long-term quercetin supplementation induce oxidative damage in brain which, in the sedentary condition, may be counteracted by increasing the activity of some antioxidant enzymes. However, in the Q-exercised group brain oxidative resistance seems to be knocked out.

The primary conclusion of the present study is that quercetin supplementation in exercised rats compromise brain mitochondrial biogenesis by disrupting the SIRT1-PGC-1α pathway. Moreover, resistance against oxidative damage achieved after exercise training is abolished when quercetin is supplemented during exercise. These results are in accordance with previous studies developed by our research team on rats skeletal muscle. In fact, we found that quercetin supplementation during exercise compromise both mitochondrial content and resistance against oxidative stress after a 6 week intervention period[24]. It should be noted, however, that in skeletal muscle only SIRT1 transcription was knocked out, while PGC-1α reminded upregulated. Thus, although the physiological effect is the same (i.e. lower mitochondrial content when quercetin is supplemented during exercise), the underlying pathway may be different depending on the tissue. Future research should assess neurodegenerative diseases when quercetin is supplemented and go in depth study of the physiological pathway in both brain an muscle tissues.

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The authors declare no conflict of interest

Authorship:

RAC was involved in designed and conducted research, wrote the paper, and analyzed the data. EM-L was involved in designed and conducted research, and analyzed data. FH-C was involved in designed, and analyzed data. RM-R and AC were involved in provided essential reagents and materials and analyzed data. AM-A was involved in designed research and had primary responsibility for final content. All authors have read and approved the final manuscript.

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Table 1. Exercise training, final weight and food intake during experimental period.

	Exercised				Sedentary			
	Quercetin		No-quercetin		Quercetin		No-Quercetin	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Distance run. Average per day (m)	1040	83	1021	60	–	–	–	–
Final weight (g)	352. 89	31. 25	367. 25	24. 41	379. 25	52. 91	366. 63	8. 97
Food intake. Average (g)	22.41*	2. 05	22.17*	1. 22	18. 47	1. 44	17. 28	1. 46
Plasmatic quercetin (µmol/L)	3.56#	0. 4	0. 51	0. 01	3.57#	0. 5	0. 45	0. 08

t Test for independent samples showed no difference (P>0.05) between quercetin groups and non quercetin groups in either exercised and sedentary rats. m = meters; g = grams. *P<0.001 Between exercised and sedentary groups. #P<0.001 between quercetin and no-quercetin.

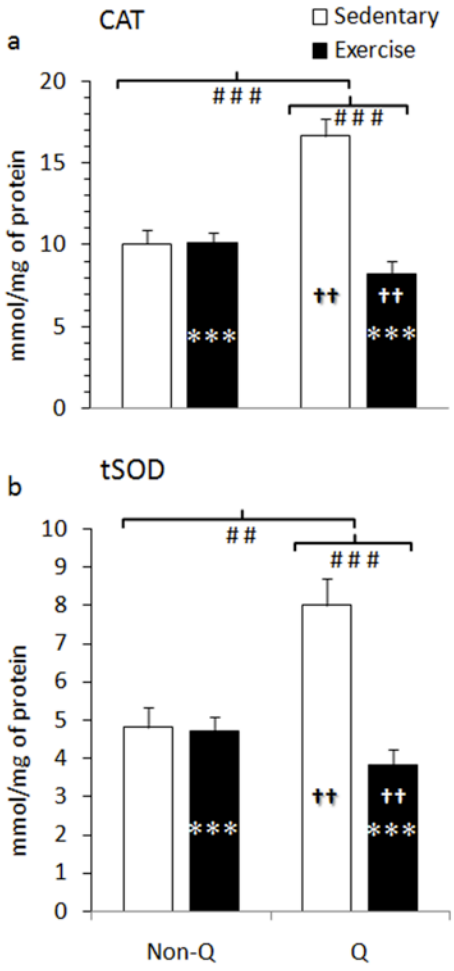

Figure 1. Effects of quercetin supplementation and exercise on CAT (A) and total SOD (B) activity in brain. Values are means ± SD; sample sizes for each variable ranged from n = 8 to 9 for all groups. Results for two-factor ANOVA analysis (with/without quercetin and with/without exercise) and student's t test for independent sample, when interaction was significant. ††P<0.01, main effect for quercetin. ***P<0.001, main effect for exercise. ##P<0.01 and ###P<0.001 for quercetin x exercise interaction.

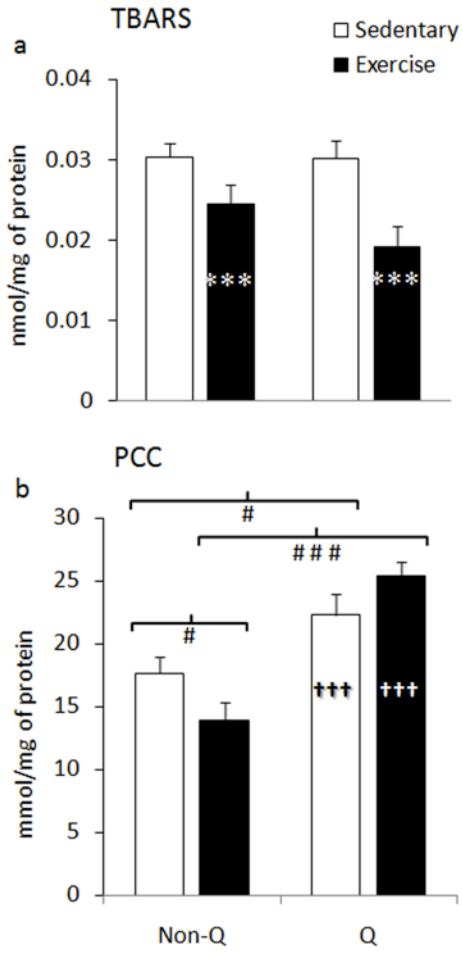

Figure 2. Effects of quercetin supplementation and exercise on TBARS (A) and protein carbonyls content (PCC) (B) in brain. Values are means ± SD. Results for two-way ANOVA analysis (with/ without quercetin and with/without exercise) and student's t test for independent sample, when interaction was significant. †††P<0.001, main effect for quercetin. ***P<0.001, main effect for exercise. #P<0.05 and ###P<0.001 for quercetin x exercise interaction.

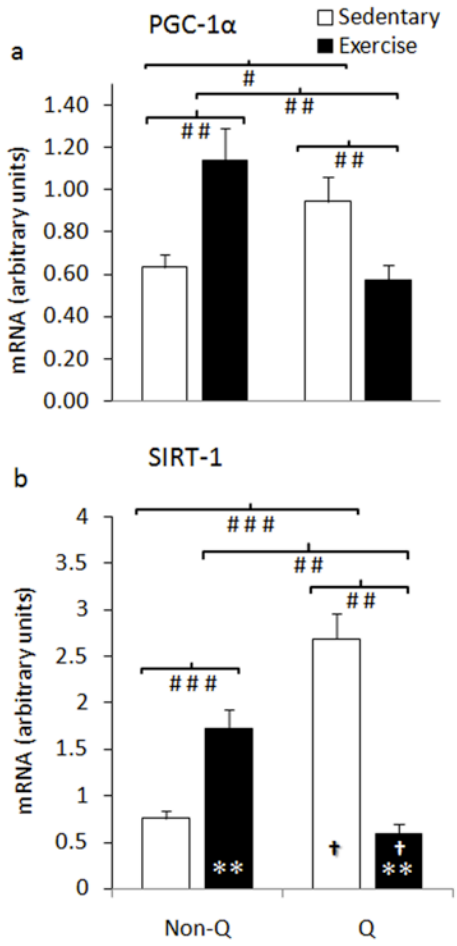

Figure 3. Effects of quercetin supplementation and exercise on PGC-1α and SIRT1 expression in brain. Values are means ± SD. Results for two-way ANOVA analysis (with/without quercetin and with/without exercise) and student's t test for independent sample, when interaction was significant. †P<0.05, main effect for quercetin. **P<0.01, main effect for exercise. #P<0.05, ##P<0.01, and ###P<0.001 for quercetin x exercise interaction.

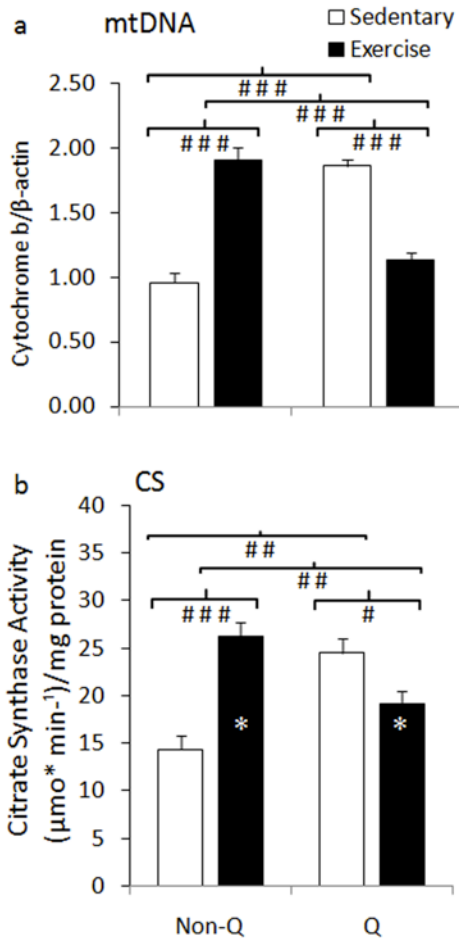

Figure 4. Effects of quercetin supplementation and exercise on mitochondrial DNA content and CS activity. Values are means ± SD. Results for two-way ANOVA analysis (with/without quercetin and with/without exercise) and student's t test for independent sample, when interaction was significant. * P<0.05, main effect for exercise. #P<0.05, ##P<0.01, and ###P<0.001 for quercetin x exercise interaction.

VI

QUERCETIN AND EXERCISE INDUCED ADAPTATIONS IN RATS CEREBELLUM ARE HAMPERED WHEN QUERCETIN IS SUPPLEMENTED DURING EXERCISE

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Submitted

Quercetin and exercise induced-adaptations in rats cerbellum are hampered when quercetin is supplemented during exercise

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Abbreviations

PCC: Proteins carbonyls content

TBARS: Thiobarbituric acid reactive substances

CAT: Catalase activity

tSOD: Total superoxide dismutase activity

SIRT1: Sirtuin 1

PGC-1: Peroxisome proliferator-activated receptor γ coactivator 1

mtDNA: mitochondrial DNA

CS: Citrate synthase

Abstract

The aim of the present study was to assess the effects of quercetin on rat's cerebellum during exercise. Six weeks old Wistar rats were randomly allocated into quercetin exercised (Q-Ex; n= 9); quercetin sedentary (Q-Sed; n=9); no quercetin exercised (NQ-Ex; n=9) and no quercetin sedentary (NQ-Sed; n=8). After 6 weeks of quercetin supplementation and/or exercise training cerebellum was collected. Protein carbonyl content (PCC), SIRT1 (Sirtuin 1) and PGC-1α (peroxisome proliferator-activated receptor γ coactivator 1) mRNA levels and CS (citrate synthase) and mtDNA (mitochondrial DNA) were measured. When Q-Sed was compared with NQ-Sed PCC ($P<0.005$) was diminished, and PGC-1α, SIRT1 (both, $P<0.01$), mtDNA ($P<0.001$) and CS ($P<0.01$) were increased. Nevertheless, when Q-Ex was compared with Q-Sed PCC was increased ($P<0.001$) and CS was lowered ($P<0.01$). What is more, in the NQ-Ex there was an increase in PGC-1α mRNA levels when compared with NQ-Sed ($P>0.01$). This effect was, however, abolished when NQ-Ex and NQ-Sed were compared ($P<0.05$). Thus, both quercetin and exercise promote cerebellar plasticity, however these adaptations may not be achieved when quercetin is supplemented during exercise.

Keywords: Flavonoids, training, oxidative stress, PGC-1α, SIRT1

1. Introduction

Quercetin (3,3´,4´,5,7-Pentahydroxyflavone) is a natural polyphenolic flavonoid presented in large amounts in onions, garlic, leeks, cabbages, apples, blueberries, tea and red wine [1]. Despite quite slow absorption rate [2], quercetin is thought to be accumulated significantly in lung, liver, and kidney but it is found in most of tissues [3,4]. In fact, quercetin triggers mitochondrial genesis in brain and skeletal muscle [5]. What is more, polyphenols share a common pathway to activate mitochondrial genesis, by means of the increase in the transcription of both SIRT1 and PGC-1α genes [5,6,7]. Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is described in current literature as the master regulator of mitochondrial biogenesis. PGC-1α co-activate and augment the expression and activity of several transcription factors that in turn bind to the promoters of distinct sets of nuclear-encoded mitochondrial genes [8,9,10]. In terms of energy/ nutrient stress, such as exercise, postrascriptional activation of PGC-1α is mainly induced by SIRT1, a metabolic sensor regulated by NAD+, which in turn, induces PGC-1α activation by deacetylation [11,12,13].

Quercetin induce the transcription of the PGC-1α-SIRT1 axis, which in turn increase the mitochondrial content, in response to 1wk supplementation in sedentary rats [5]. Nevertheless, these effects were compromised, in exercised rats supplemented with quercetin for 6wks probably because SIRT1 transcription was abolished [14]. Previous studies have described the harmful effects of quercetin. [15,16]. The authors proposed that quercetin metabolites may change to prooxidants agents, which attack to protein structures. In fact, *in vivo* results showed that quercetin increase the oxidative damage to proteins, which is faced by increasing the activity of catalase (CAT) and superoxide dismutase (SOD), nevertheless when quercetin is supplemented during exercise no antioxidant modulation is found [14].

Cerebellum is a key target for quercetin, however its effects seem to be toxic due to an increased oxidative damage [17]. Quercetin supplementation is recommended for athletes in order to improve exercise performance [18]. Exercise training evokes similar adaptations in skeletal muscle and cerebellum, in fact, it increases the mitochondrial genesis in a similar way [19]. In addition, exercise is known to reduce oxidative damage in cerebellar tissue [20]. Given that mitochondrial dysfunction and oxidative damage is presented in ataxia disease [21] where cerebellum has a primary role [22]. It is of importance to test if, quercetin supplementation during exercise could hampers exercise effects on mitochondrial content and oxidative status in rat's cerebellum. Thus, the aim of the present study was to assess the SIRT1 and PGC-1α transcription,

mitochondrial content and redox status in the cerebellum of trained rats supplemented with quercetin.

2. Methods

2.1. Animals

This study was performed on male Wistar rats (6 weeks old). The animals were maintained for 8 weeks in individual cages under standard conditions of light and temperature and allowed ad libitum access to food (Harlan 2014, maintenance chow) and water. All experiments were approved by the Committee for Ethics of the University of Jaén (Spain).

2.2. Exercise and supplementation

Rats were randomly assigned to quercetin (Q; n = 17) and no-quercetin (NQ; n = 16) groups. Both groups were further divided into Q-exercised (n = 9), Q-sedentary (n = 8), NQ-exercised (n = 8), NQ-sedentary (n = 8). The animals were acclimated to experimental conditions for two weeks and adapted to the treadmill. Treadmill training took place 5 days a week for 6 weeks (5 rats Panlab Treadmills for LE 8710R). The rats ran at a constant speed of 44cm/s at an angle of 10°. The rats ran for 20 minutes during the first two days and for 25 minutes on the third day. Training duration increased by 5 min every two days.

The animals ran for 80 min on the last day of the fifth week and also throughout the last week of training [14]. The rats in the quercetin groups were supplemented via gavage, (QU995; Quercegen Pharma, Newton, MA) on alternate days throughout the experimental period. A dose of 25mg/kg diluted in a 1% solution of methylcellulose was used. Quercetin dosage and length was chosen because in a preliminary study we found a ~2.5-fold increase in plasma quercetin levels. The no-quercetin groups were also supplemented, using the gavage procedure, with the vehicle (1% solution of methylcellulose).

2.3.Tissue collection

All rats were anesthetized with pentobarbital and were bled by cannulation of the aorta 48h after the last exercise. Brains were immediately collected, rinsed in saline solution, frozen in liquid nitrogen, and stored at 80°C until their further analysis.

2.4. Quantitative Real Time Polymerase Chain Reaction (RT-PCR)

Gene expression of different genes (peroxisome proliferator-activated receptor γ coactivator 1, PGC-1; NAD(+)-dependent histone deacetylases, SIRT-1) was quantitatively assessed by real-time PCR using β-actin as the normalizing gene. Total RNA was isolated from cell extracts using Trizol reagent (Invitrogen) according to the manufacturer's instructions. After treatment with

DNase, cDNA was synthesised from 1.5 μg total RNA using reverse transcriptase (SuperscriptTM III RT, Invitrogen) with oligo-(dT) 15 primers (Promega). Real-time PCR was performed on the Stratagene MxPro 3005P qPCR system using the Brilliant II SyBR Green QPCR Master Mix (STRATAGENE, La Jolla, CA, USA). The following primer pairs were used: PGC-1, 5′-GCGGACAGAACTGAGAGACC-3′ and 5′-CGACCTGCGTAAAGTATATCCA-3′; SIRT-1, 5`-CCTGACTTCAGATCAAGAGATGGTA-3′ and 5′-CTGATTAAAAATATCTCCACGAACAG-3′; β-actin, 5′ CTTAGAAGCATTTGCGGTGCCGATG-3′ and 5′-TCATGAAGTGTGACGTTGCATCCGT-3′. Experiments were performed in triplicates, and the relative quantities of target genes corrected with the normalizing gene, β-actin, were calculated using the STRATAGENE MxProTM QPCR Software. Quantification of mRNA expression of PGC-1 and SIRT-1 was calculated using the ΔΔCT method as previously described [23].

2.5. Mitochondrial DNA quantification

DNA (mitochondrial and nuclear) was extracted from quadriceps muscle samples using a QIAamp DNA minikit (QIAGEN, Chatworth, CA), and the concentration of each sample was determined spectrophotometrically at 260 nm. Real-time PCR was performed on the Stratagene MxPro 3005P qPCR system using the Brilliant II SyBR Green QPCR Master Mix (STRATAGENE, La Jolla, CA, USA). Mitochondrial content was estimated as the ratio between copy numbers of mtDNA (cytochrome b; forward, 5′-AAAGCCACCTTGACCCGATT-3′; reverse, 5′-GATTCGTAGGGCCGCGATA-3′; probe, 5′-CGCTTTCCACTTCATCTTACCATT-3′) versus nuclear DNA (β-actin).

2.6. Thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (TBARS), major indicators of oxidative stress, were determined in mice brain following the instructions of the Oxikek TBARS Assay Kit (ZeptoMetrix Corp.). Final values were referred to the total protein concentration in the initial extracts (nmol/mg of protein).

2.7. Protein carbonyls

Determination of the protein carbonyl contents, an indicator of oxidative stress, were analyzed by 2,4- dinitrophenylhydrazine method as described by Levine et al. [24]. Results were expressed as mmol/mg protein.

2.6. Enzyme assays

For catalase (CAT) activity, the cerebral cortex from animals was dissected, rinsed in saline solution and stored at -80º C until used. Cerebral cortices were homogenized on ice in 5-10 ml of

cold buffer (50mM potassium phosphate, pH 7.0, containing 1mM EDTA) per gram of tissue. After centrifugation at 10,000 x g for 15 min, the supernatant were collected for protein determination [25] and subsequent analysis. All the procedures were performed at 4º C. Catalase activity was studied by monitoring the decomposition of H2O2 at 240 nm, according to the method described by Beers and Sizer [26]. For superoxide dismutase (SOD) activity, the cerebral cortices were homogenized on ice in 5-10 ml of cold buffer (20mM HEPES, pH 7.2, containing 1mM EGTA, 210 mM mannitol, and 70 mM sucrose) per gram tissue. After centrifugation at 15000 x g for 5 min, the supernatant were collected for protein determination [25] and subsequent analysis. All the procedures were performed at 4º C. SOD activity was assayed by measuring the rate of inhibition of cytochrome c reduction by superoxide anions generated by a xanthine/xanthine oxidase system [27].

Total citrate synthase (CS) activity was measured in Tris·HCl buffer (50 mM Tris·HCl, 2 mM EDTA, and 250 μM NADH pH 7.0) and 0.04% Triton-X. The CS reaction was started by the addition of 10 mM oxaloacetate, and activity was measured spectrophotometrically by measuring the disappearance of NADH at 412 nm. Total protein content of all homogenates was determined by the method of Bradford [25].

2.7. Statistics

Results are presented as mean ± SD. Homoscedasticity and normality was tested by Levene and Kolmogorov-Smirnov test, respectively. Results were analyzed using two-factor ANOVA (with/ without quercetin and with/without exercise). If this analysis revealed a significant interaction, specific differences between mean values were located using Student's t test for independent samples. The level of significance was considered at $P<0.05$. All the analyses were performed using the Statistical Package for Social Sciences (IBM-SPSS, version 22.0 for Windows).

3. Results

Figure 1 shows the effect of exercise training and quercetin on the cerebellum oxidative damage. There was a main effect both for quercetin and exercise towards a decrease in TBARS content (both, $P<0.001$). However, no further interactions were found for TBARS ($P=0.156$). When looking at protein carbonyl content (PCC), there was a main effect of exercise towards a decrease in PCC ($P=0.001$). What is more, when quercetin is supplemented during exercise there was an increased PCC in rat's cerebellum when compared with NQ-Ex and Q-Sed (both, $P<0.001$). However, in the Q-Sed group there was a decrease in PCC when compared to NQ-sed ($P<0.05$).

Total SOD and CAT antioxidant activities are represented in Figure 2. tSOD and CAT activities were lower in exercised groups compared to sedentary groups(P=0.01 and P=0.006, respectively). No main effects were found for quercetin nor any statistical interactions between groups.

Transcription of PGC-1α and SIRT1 are showed in Figure 3. Statistical analysis revealed a main effect in quercetin groups towards an upregulation of the SIRT1 transcription (P=0.002). No main effect was described for exercise training, but interaction analysis showed that SIRT1 mRNA levels were higher in Q-Sed compared with NQ-Sed (P=0.001). However, quercetin supplementation during exercise strongly tended to hamper this quercetin-induced adaptation (P=0.052). In regard to PGC-1α, exercise training increased its transcription (P=0.001). No differences were found for quercetin groups. However, interaction analysis revealed that when compared to NQ-Sed both quercetin and exercised groups increased PGC-1α transcription in rat's cerebellum (P=0.009 and P=0.001, respectively). What is more, exercise-induced effect on PGC-1α was abolished when quercetin is supplemented during exercise (P=0.043).

Markers of mitochondrial content are shown in Figure 4. Quercetin increased mtDNA content (P=0.007 main effect for quercetin). Interaction analysis also revealed that NQ-Ex increased mtDNA content when compared with NQ-Sed (P=0.002). Q-Sed group also showed a greater mtDNA content when compared with NQ-Sed (P<0.001). In regards to CS quercetin also showed an increased activity (P<0.001; main effect for quercetin). Interaction analysis showed an increased CS activity in both Q-Sed and NQ-Ex (both, P<0.001) compared to NQ-Sed. What is more, quercetin-induced increased in CS activity is abolished when quercetin is supplemented during exercise (P<0.01).

4. Discussion

Results described above suggest that when quercetin is supplemented during exercise it acts as an oxidative agent to the proteins of the rat´s cerebellum. Importantly, quercetin supplementation increases the transcription of some key coactivator of the mitochondrial biogenesis process such as SIRT1 and PGC-1α. Likewise, exercise induces the transcription of PGC-1α, however, this effect is hampered in the Q-Ex group. Both exercise and quercetin increase mitochondrial content in cerebellar tissue, however, the increased CS activity in response to quercetin supplementation is compromised if it is supplemented during exercise. Quercetin seems to regulate cerebellar mitochondrial content by increasing the transcription of both SIRT1 and PGC-1α while exercise targets only on the PGC-1α transcription.

Quercetin is the main representative of the flavonoids group called flavonols, and it is presented at relatively low concentrations of 15– 30 mg/kg fresh wt. The richest sources are onions (up to 1.2 g/kg fresh wt), curly kale, leeks, broccoli, and blueberries, nevertheless, it is presented in all plant foods [1]. Quercetin has a quite extensive metabolism, it can be absorbed by the stomach [28], but most quercetin is absorbed from the small intestine. Conjugation of quercetin occurs in the small intestine, and then in liver, after incorporating in portal circulation [29]. The major circulating compounds in plasma were identified as quercetin 3-O-glucuronide, 3'-O-methylquercetin 3-O-glucuronide, and quercetin 3'-O- sulfate [30]. In fact we have found a 2.7 increase in plasma quercetin after 6 weeks of every other day supplementation suggesting that dosage used in the present study is appropriated to increase plasmatic quercetin (unpublished data). Once quercetin metabolites are in the blood flow they are able to transverse the blood-brain barrier [29]. It may be useful to measure cerebellar concentration of quercetin, but it is difficult to find it on brain tissue [4], probably because it is rapidly metabolized.

Exercise training is thought to increase mitochondrial biogenesis in cerebellum in a similar way than in skeletal muscle [19]. However, after 8 weeks of exercise training relative induction of PGC-1α is more increased than SIRT1 in cerebellar tissue [19], thus, it may be supposed that exercise has a greater effect on the transcription of the PGC-1α rather than the SIRT1. In fact, our data support this statement because SIRT1 remains unchanged when NQ-Sed and NQ-Ex are compared. However, mitochondrial content is increased after exercise training, which might be explained by the fact that exercise target on the PGC-1α and not on its upstream regulator SIRT1. In fact, PGC-1α is the main ragulator of the mitochondrial genesis process [8,9,10]. PGC-1α is thought to be regulated by some metabolic energy sensors such as AMPK, however this activation is though to occur in a SIRT1 dependent manner [14]. It is possible, however, that preexercise SIRT1 content are enough to deacetilate PGC-1α and thus, trigger the mitochondrial genesis process. This statement is supported by the fact that mitochondrial content is increased after an increased SIRT1 activity despite a decrease in SIRT1 protein content [31]

Similarly to the data previously reported for brain tissue [5] quercetin seems to increase mitochondrial content in cerebellum by activating the transcription of the SIRT1-PGC-1α pathway. What is more poliphenols such as isoflavones [7], resveratrol [6] and quercetin [5] are thought to target to SIRT1 at the transcriptional level which may increase PGC-1α mRNA levels. In fact, in a previous study we found that despite an increased PGC-1α mRNA level in skeletal muscle, mitochondrial content was not higher in exercised rats supplemented with quercetin, probably because SIRT1 transcription was not activated [14]. The present study reveals that quercetin

supplementation during exercise also inhibits cellular adaptations related to mitochondrial genesis in rats cerebellum.

Toxicity of quercetin supplementation was previously described for rats cerebellum [17]. Probably due to the fact that antioxidant effects of quercetin are thought to occur at the first stage of the supplementation, later, quercetin metabolites change to prooxidant agents [15,16]. This prooxidant effect was previously described in vivo for skeletal muscle in exercised rats [14] and results derived from the present study extend this effect to the cerebellar tissue. In fact the increased PCC showed in the Q-Ex is in accordance with the "Quercetin Paradox" showing selective damage to proteins induced by quercetin´s metabolites [15,16]. However, quercetin´s effect on the activity of some antioxidants enzymes differs between cerebellar and muscle tissues. We described previously that quercetin may modulate antioxidant enzymatic activity in order to counteract oxidative damage in skeletal muscle [14]. Probably because of differences on quercetin distribution after long therm supplementation [3,4]. Thus, different metabolites and concentration may induce different effects.

Although it is likely that this study is the first in which redox status and mitochondrial genesis is assessed in the cerebellum of exercised rats supplemented with quercetin, a few limitations should be mentioned. First, gavage can be a stressor procedure, so we choose an every other day supplementation. Owing to limited funding, SIRT1 and PGC-1α protein expression were not measured, however, it was not the aim of the study. Nevertheless, given that SIRT1 [32] and PGC-1α [33] exert a critical role in neuroprotection, further research is guaranteed.

In conclusion long-term quercetin supplementation during exercise training acts as an oxidative stressor by damaging proteins structures from rats cerebellum. What is more, both exercise and quercetin increase mitochondrial content in rats cerebellum. These effects, however, are abolished when quercetin is supplemented during exercise probably because the transcription of some key genes are downregulated.

5. Acknowledgment

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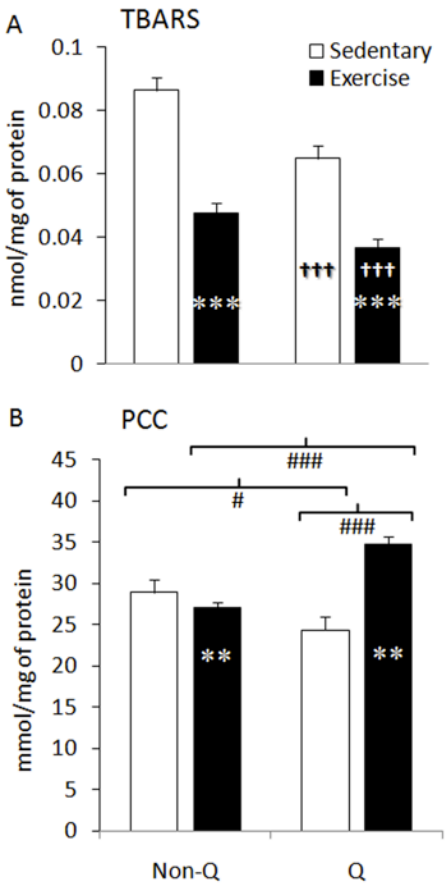

Figure 1. Effects of quercetin supplementation and exercise on TBARS (A) and protein carbonyls content (PCC) (B) in cerebellum. Values are means ± SD. Results for two-way ANOVA analysis (with/without quercetin and with/without exercise) and student's t test for independent sample, when interaction was significant. †††P<0.001, main effect for quercetin. ***P<0.001, main effect for exercise. #P<0.05 and ###P<0.001 for quercetin x exercise interaction.

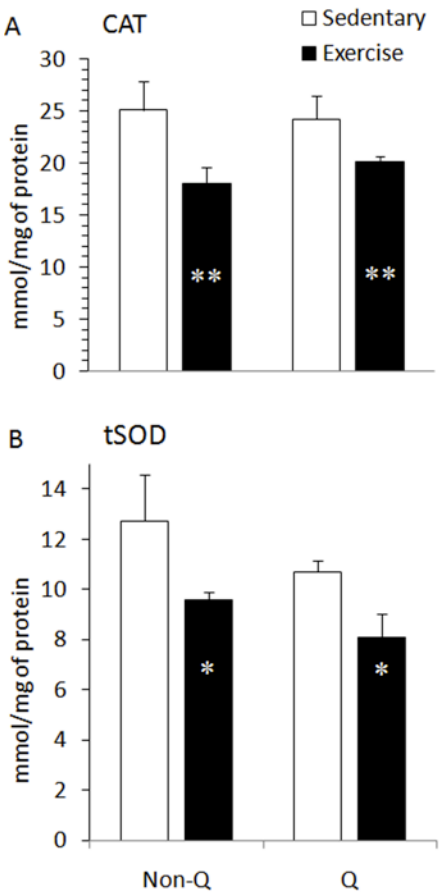

Figure 2. Effects of quercetin supplementation and exercise on CAT (A) and total SOD (B) activity in cerebellum. Values are means ± SD; sample sizes for each variable ranged from n = 8 to 9 for all groups. Results for two-factor ANOVA analysis (with/without quercetin and with/without exercise) and student's t test for independent sample, when interaction was significant. ††P<0.01, main effect for quercetin. *P<0.05, **P<0.01, main effect for exercise.

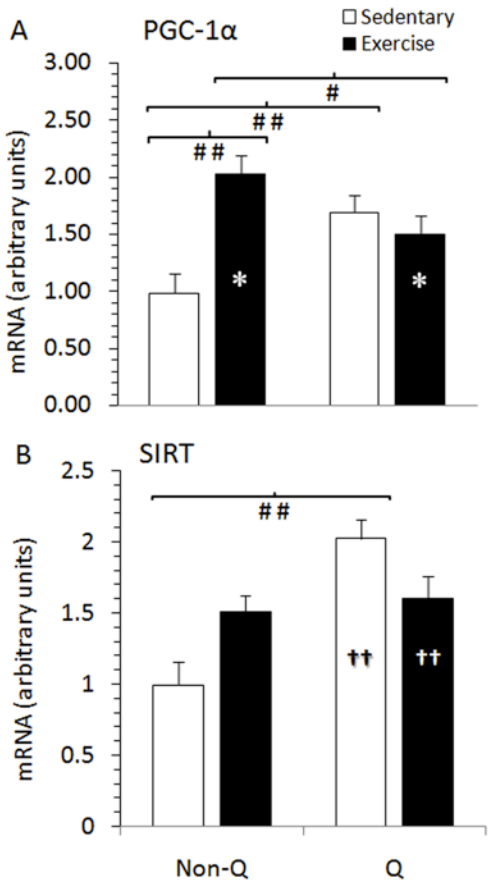

Figure 3. Effects of quercetin supplementation and exercise on PGC-1α and SIRT1 expression in cerebellum. Values are means ± SD. Results for two-way ANOVA analysis (with/without quercetin and with/without exercise) and student's t test for independent sample, when interaction was significant. ††P<0.01, main effect for quercetin. *P<0.05, main effect for exercise. #P<0.05, ##P<0.01 for quercetin x exercise interaction.

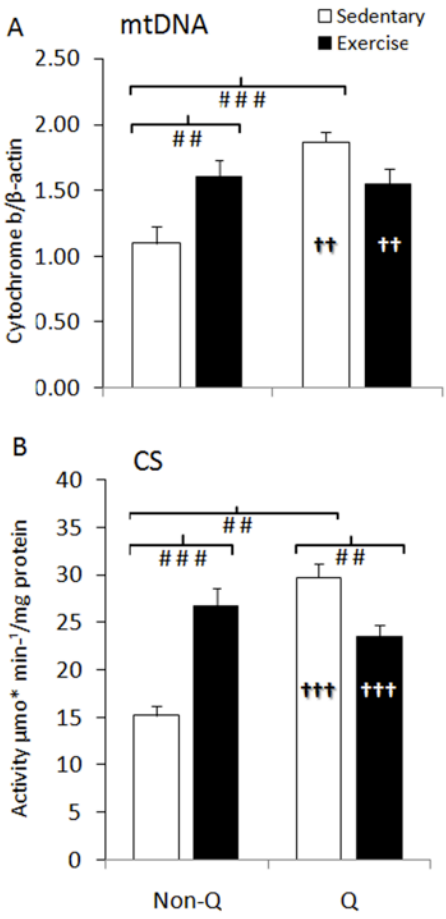

Figure 4. Effects of quercetin supplementation and exercise on mitochondrial DNA content and CS activity. Values are means ± SD. Results for two-way ANOVA analysis (with/without quercetin and with/without exercise) and student's t test for independent sample, when interaction was significant. ††P<0.01, and ††† P<0.001, main effect for quercetin. ##P<0.01, and ###P<0.001 for quercetin x exercise interaction.

CONCLUSIONES

- La suplementación con quercetina es incapaz de mejorar el rendimiento tanto en ratas sedentarias como en ratas ejercitadas.
- La quercetina no parece ejercer efecto alguno en la ganancia de peso.
- Cuando se suplementa durante el ejercicio, la quercetina incrementa el RDW de una forma dependiente al NO.
- La suplementación de larga duración con quercetina incrementa el daño oxidativo en todos los tejidos analizados. Este efecto es más importante si la quercetina se suplementa durante el ejercicio ya que la actividad antioxidante se encuentra noqueada.
- La quercetina parece mimetizar las adaptaciones mitocondriales inducidas por el ejercicio, especialmente en cerebro y cerebelo, sin embargo, si la quercetina se suplementa durante el ejercicio estas adaptaciones son impedidas.
- La quercetina actúa sobre la transcripción de SIRT1, lo que sugiere que las adaptaciones mitocondriales alcanzadas con la quercetina son debidas a los efectos de la transcripción de dicho gen. Cuando la quercetina se suplementa durante el ejercicio la disminución de las adaptaciones mitocondriales son debidas a la disminución de la transcripción de SIRT1.

Conclusión general:

Los resultados de la presente tesis no entran en confrontación con el hecho de que una dieta rica en vegetales y frutas, con una mezcla de polifenoles de distinto tipo es beneficiosa para la salud. Sin embargo, la quercetina suplementada de forma aislada no proporciona ningún beneficio para las adaptaciones relacionadas con el ejercicio y la plasticidad de músculo esquelético y cerebro.

CONCLUSIONS

- Quercetin supplementation is unable to enhance exercise performance both in sedentary and exercised rats.
- Quercetin do not has any effect on weight gain.
- When it is supplemented during exercise, quercetin increase blood RDW in a NO-dependent manner.
- Long-term quercetin supplementation increase oxidative damage in the analyzed tissues. This effect is more pronounced if quercetin is supplemented during exercise probably because antioxidant activity is knocked out.
- Quercetin seems to mimic exercise-induced mitochondrial biogenesis, specially in brain an cerebellum, however, if quercetin is supplemented during exercise these adaptations are hampered.
- Quercetin activate SIRT1 transcription, thus, quercetin-induce mitochondrial biogenesis seem to be due to the transcription of this gene. What is more, if quercetin is supplemented during exercise impaired mitochondrial biogenesis is due to the lower SIRT1 transcription.

Overall conclusion:

Our results do not disagree with the fact that fruits and vegetables rich diet with mixed type of polyphenols is useful for health improvement. Nevertheless, the results from the present Thesis highlight that supplementation of isolated quercetin does not provide any advantage for exercise-induced adaptations and the plasticity of skeletal muscle and brain.

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"Con fuerza de voluntad todo se puede lograr"

A mi madre, por su eterna paciencia conmigo hasta en los momentos de máximo estrés, siempre mostrándome su apoyo, ayuda y comprensión.

"Beatus ille quem vivere in locus amoenus et carpe diem"

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“Felicidad no es hacer lo que uno quiere sino querer lo que uno hace”