Tesis Doctoral Internacional / International Doctoral Thesis

VALIDACIÓN DE LA α-ACTINA COMO MARCADOR DE DAÑO MUSCULAR EN DIFERENTES DISCIPLINAS DEPORTIVAS

VALIDATION OF α-ACTIN AS A MARKER OF MUSCLE DAMAGE IN DIFFERENT SPORTS

Programa de Doctorado de Nutrición Humana

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Dedicado a mis padres, mi familia y mis amigos,

porque pase lo que pase, siempre están ahí cuando más lo necesito.

“La gota de agua perfora la roca… no por su fuerza, sino por su constancia.”

Bruce Lee

CONTENIDOS

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PUBLICACIONES ORIGINALES

La presente tesis ha dado lugar a las siguientes publicaciones.

I. Barranco-Ruiz Y, **Aragón-Vela J**, Casals C, Martínez-Amat A, Villa-González E, Huertas JR. Lifelong amateur endurance practice attenuates oxidative stress and prevents muscle wasting in senior adults. Journal of Sports Medicine and Physical Fitness. 2017; 57: 670-677.

II. Casuso RA, **Aragón-Vela J**, López-Contreras G, Gomes SN, Casals C, Barranco-Ruiz Y, Mercadé JJ, Huertas JR. Does Swimming at a Moderate Altitude Favor a Lower Oxidative Stress in an Intensity-Dependent Manner? Role of Nonenzymatic Antioxidants. High Altitude Medicine and Biology. 2017; 18:46-55.

III. Barranco-Ruiz Y, **Aragón-Vela J**, Casals C, Martínez-Amat A, Casuso RA, Huertas JR. Control of antioxidant supplementation through interview is not appropriate in oxidative-stress sport studies: Analytical confirmation should be required. Nutrition. 2017; 33:278-284.

IV. **Aragón-Vela J;** Barranco-Ruiz Y; Casals C; Plaza-Díaz J; Casuso RA; Huertas JR. A novel approach to estimate electromyography fatigue threshold in maximum incremental strength tests. Control Motor. 2017 (Segunda revisión).

V. **Aragón-Vela J**; Barranco-Ruiz Y; Casals C; Casuso RA; Fontana L; Huertas JR. Cardiac and skeletal muscle damage in bench press versus half-squat exercises: are they heart-healthy sporting activities? Journal of strength and conditioning research. 2017 (Tercera revisión).

VI. **Aragón-Vela J**; Fontana L; Barranco-Ruiz Y; Casals C; Casuso RA; Huertas JR. Sex differences in muscle and oxidative damage after a maximal strength exercise. Research Quarterly for Exercise and Sport. 2017 (enviado).

ABREVIATURAS

AD: Deltoides anterior

ADP: Adenosin difosfato

AMP: Adenosin monofostato

ANOVA: Análisis de Varianza

aRMS: Area sobre el RMS

ATP: Adenosin trifosfato

CK-MB: Creatina quinasa isoenzima cardiaca

EGF: Factor de crecimiento epidérmico

EMG: Electromiografía de superficie

FGF: Factor de crecimiento de fibroblastos

HGF: Factor de crecimiento de hepatocitos

IGF: Factor de crecimiento insulínico

IMC: Índice de masa corporal

IL: Interleuquina

ISAK: Sociedad internacional en el Avance de la Cineantropometría

LDH: Lactato deshidrogenasa

LD: Dorsal Ancho

LIF: Factor inhibidor de leucemia

NADPH: Nicotinamida adenina dinucleótido fosfato

PM: Pectoral Mayor

RF: Recto Femoral

RMS: Raíz cuadrada de la media de los cuadrados

ROS: Especies reactivas del oxígeno

TB: Triceps braquial

TNF-α: Factor de necrosis tumoral alfa

TFG-β: Factor de crecimiento transformante beta

VL: Vasto Lateral

VM: Vasto Medial

1RM: Una repetición máxima

RESUMEN GENERAL

Los fisiólogos deportivos requieren marcadores séricos de daño muscular sarcomérico que proporcionen información para discriminar entre síndrome de sobreentrenamiento y microtraumas adaptativos, así como identificar si el origen del daño es metabólico o mecánico. En la actualidad, los marcadores más empleados para evaluar el daño muscular son la creatina quinasa y la lactato deshidrogenasa, pero no son de origen sarcomérico sino citosólico. En el presente trabajo investigamos la utilidad de la α-actina, proteína sarcomérica del músculo esquelético, para tal fin dada su implicación en la contracción muscular, y ha dado lugar a 6 artículos en revistas internacionales.

En el **primer artículo** de esta tesis doctoral comparamos tres grupos de sujetos que siguieron sendas pautas de entrenamiento semanal, comprobando que la concentración de α-actina en suero, y por tanto de daño sarcomérico, es directamente proporcional a las horas de entrenamiento.

En el **segundo artículo** comprobamos que la práctica de actividad física en un ambiente hipóxico no alteró la liberación de α-actina post-esfuerzo. Doce nadadores realizaron tres tipos de entrenamiento con intensidad incremental tanto en normoxia como en hipoxia, mostrando una liberación similar de α-actina en sangre en ambas situaciones. Esto sugiere que el incremento metabólico por el estrés de catecolaminas en el entrenamiento en ambiente hipóxico agudo no tiene efectos sobre el daño sarcomérico.

En el **tercer artículo** comparamos el daño muscular basal de un grupo de deportistas que recibieron un suplemento de α-tocoferol con un grupo control que no recibió suplemento. La suplementación con α-tocoferol no afectó a la liberación de α-actina en sangre.

En el **cuarto artículo** diseñamos un test de fuerza máxima incremental basado en movimientos explosivos de corta duración (media sentadilla), con el objetivo de identificar el umbral de fatiga en músculos individuales. Estudiamos variables fisiológicas clásicas, como la frecuencia cardiaca y la concentración de lactato, pero no fueron válidas para tal fin. En cambio, la regresión/correlación entre cargas crecientes y sus correspondientes registros electromiográficos se ajustaron a una curva exponencial que nos permitió identificar el umbral como un punto de inflexión.

En el **quinto artículo** utilizamos el test descrito en el artículo anterior y comprobamos que dos ejercicios tan diferentes como *press* banca y media sentadilla generaron valores similares de α-actina, lo que sugiere que no se afectó la estructura sarcomérica en ninguno de los dos. En cambio, marcadores citosólicos como la creatina quinasa isoenzima MB (CK-MB) y la lactato deshidrogenasa (LDH) fueron mayores tras el ejercicio de *press* banca, probablemente porque en la media sentadilla la carga se distribuye en más masa muscular y, en consecuencia, el daño muscular fue menor.

Por último, en el **sexto artículo** sometimos deportistas de ambos sexos a un esfuerzo incremental de media sentadilla. Varones y mujeres tuvieron valores similares

de α-actina, LDH y CK-MB. Sin embargo, cuando normalizamos estos marcadores por la volemia sí apreciamos valores significativamente mayores de α-actina en los varones, probablemente debido a su mayor masa muscular.

SUMMARY

Sport physiologists require serum markers of sarcomeric damage that provide information to discriminate between overtraining syndrome and adaptative microtraumas, and to identify whether the origin of the damage is metabolic or mechanical. The markers most frequently used to evaluate muscular damage nowadays are creatine kinase and lactate dehydrogenase, but they both are cytosolic, not of sarcomeric origin. In the present work we investigated the usefulness of α-actin, sarcomeric protein of the skeletal muscle, for this purpose given its role in muscle contraction. Our results translated into 6 articles published in international journals.

In the **first paper** we compared three groups of subjects that adhered to different weekly training schedules. We found that α-actin concentration in serum, and therefore sarcomeric injury, is directly proportional to the hours of training.

In the **second paper** we proved that the practice of physical activity in a hypoxic environment does not alter the release of α-actin post-exercise. Twelve swimmers performed three types of training with incremental intensities in both normoxia and hypoxia, showing similar values of α-actin in serum regardless of the situation. This finding suggests that sarcomeric damage is not affected by the catecholamine-induced metabolic stimulation that takes place during training in a hypoxic environment.

In the **third paper** we compared the basal muscular damage of a group of athletes that received a supplement of α-tocopherol with a control group that did not receive the supplement. Supplementation did not affect the release of α-actin into blood.

In the **fourth paper** we designed a test of incremental strength based on explosive and short-duration movements (half-squat), with the goal of identifying the fatigue threshold of single muscles. Classic physiological variables such as heart rate and lactate concentration were investigated, but they were found to be not suitable for this purpose. In contrast, regression/correlation between increasing weight loads and their respective electromyographic values fitted an exponential curve that allowed us to pinpoint the aforementioned threshold as an inflection point.

In the **fifth paper** we used the test mentioned above and demonstrated that two exercises as different as bench press and half-squat induced the release of similar values of α-actin, which suggests that the structure of the sarcomere was not affected in either exercise. In contrast to this finding, the cytosolic markers creatine kinase isoenzyme MB (CK-MB) and lactate dehydrogenase increased after the bench press exercise, probably because the load is distributed throughout a larger muscular mass and, therefore, the damage was less severe.

Lastly, in the **sixth paper** both male and female athletes were enrolled to perform an incremental strength half-squat exercise. Males and females exhibited similar serum values of α-actin, LDH and CK-MB. However, significantly greater α-

actin values were obtained in males when concentration of this protein was normalized by the volemia, probably due to the larger muscle mass of males.

INTRODUCCIÓN

Generalidades del daño muscular

El daño muscular puede ser generado tanto por factores directos como indirectos. Entre los factores directos podemos identificar las lesiones por aplastamiento, electrocución, traumatismos y por la práctica de ejercicio físico intenso [1]. Entre los factores indirectos podemos destacar la rabdomiólisis que es la descomposición del tejido muscular ocasionada por drogas, toxinas, alteraciones electrolíticas, infecciones virales, etc [2]. Dentro del marco teórico de esta tesis doctoral nos vamos a centrar en el daño provocado por factores directos, ya que serían los responsable del daño muscular a nivel sarcomérico durante la actividad física. Cuando se ha producido este tipo de deterioro, los síntomas más frecuente suelen ser dolor muscular, espasmos e inflamación local. Este daño muscular se manifiesta con la degeneración sarcomérica a partir de la fragmentación del disco Z, localizado en los márgenes del sarcómero, y haciendo de unión con el siguiente sarcómero. Está formada por numerosas proteínas, entre las que destaca la desmina y la alfa-actinina [3]. Otras proteínas sarcoméricas también se ven afectada por el daño muscular, la titina, que conecta la línea Z con la miosina; la nebulina, que mantiene alineados los filamentos de actina o la proteína c, que mantiene perpendiculares los filamentos de miosina y actina con respecto al disco Z [4]. Esta destrucción de las estructuras sarcoméricas genera un sistema de alarma en nuestro organismo, con el fin de dar solución a este problema, comenzando con un proceso de inflamación local.

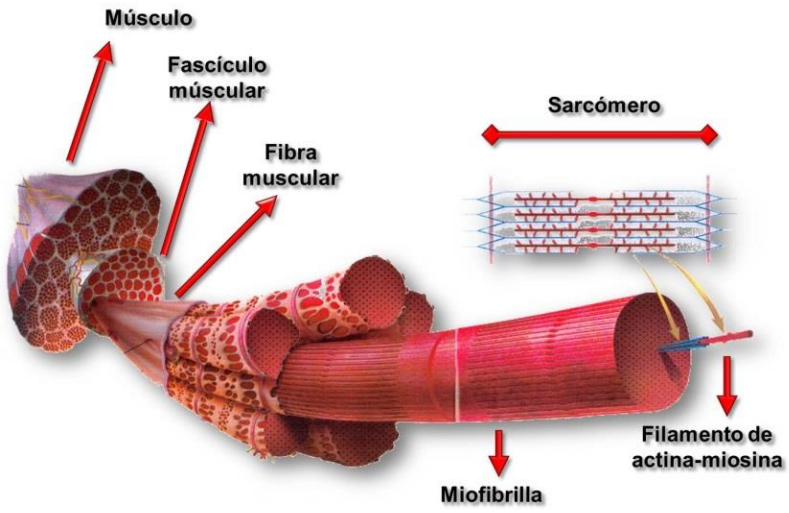

Figura 1. Estructura del Sarcómero.

En este proceso inflamatorio entra en juego un tipo de célula que reside entre la lámina basal y el sarcolema de las fibras musculares, llamadas células satélites [5]. La señal que activa estas células requiere la influencia de diversos factores, siendo los más influyente FGF, HGF, IGF-I, IGF-II, TGF-β, Macrofagos, IL-6, LIF, extractos derivados de plaquetas y EGF [6, 7]. Una vez activadas generan células precursoras (mioblastos) que proliferan y, finalmente, se fusionan con las células musculares para culminar con la reparación y posterior regeneración del tejido muscular [8].

El daño muscular generado en la actividad física

El daño muscular inducido por la actividad física es el trauma a nivel esquelético más común en las distintas disciplinas deportivas existentes, que puede ser producido por una intensa o incluso moderada actividad física, especialmente en la ejecución de ejercicios excéntricos [9]. Se cree que los efectos dañinos del ejercicio excéntrico están relacionados con la disrupción mecánica de los enlaces de actina-miosina, en oposición al desprendimiento dependiente del adenosín trifosfato (ATP), lo que coloca un mayor grado de estrés y tensión en las estructuras involucradas, en comparación con otras acciones musculares [10]. A su vez la severidad de la lesión dependerá de la duración y las características del ejercicio, la época de entrenamiento y la posible existencia de un desequilibrio muscular entre los músculos agonista con sus músculos antagonista [11]. Estos daños pueden ser específicos de solo algunas macromoléculas del tejido muscular, o también puede ser debido a grandes desgarros en el sarcolema, en la lámina basal o en el tejido conectivo de apoyo, induciendo lesiones a los elementos contráctiles y al citoesqueleto [12] y provocando una disminución de la tensión de las fibras e incluso la muerte de la fibra muscular [13].

Sin embargo, a pesar de las consecuencias que puede generar una lesión muscular severa, el abandono del entrenamiento o la pérdida de tiempo por la necesidad de una recuperación correcta en deportistas profesionales, el daño muscular a menor escala, conocido como “microtrauma adaptativo”, es fundamental para conseguir mejoras a nivel metabólico y muscular [14]. Estos microtraumas son de vital importancia porque gracias a ellos se consigue un fortalecimiento del tejido, protección del músculo contra posibles lesiones e incremento de la fibra muscular (hipertrofia muscular) [10], siendo las responsables de este incremento muscular las células satélites mencionadas con anterioridad.

El daño muscular inducido por el ejercicio físico disminuye con una práctica continua del mismo tipo de ejercicio a la misma intensidad de trabajo [15]. Este fenómeno, denominado "efecto de supercompensación", se ha atribuido al fortalecimiento del tejido conectivo, a una mayor eficiencia en el reclutamiento de unidades motrices, a una mayor sincronización de la unidad motora, a una distribución más uniforme de la carga de trabajo entre las fibras, una mayor contribución de los músculos sinérgicos y la remodelación muscular por sustitución de isoformas de proteínas sarcoméricas más eficientes. [16–18]. Las adaptaciones pueden durar incluso hasta varios meses, aunque no se realice ningún tipo de entrenamiento.

El proceso de daño muscular inducido por la actividad física está dividido en dos fases generales. Una inicial o daño primario, que ocurre durante el ejercicio y un daño secundario que se propaga a través de un proceso asociado con la respuesta inflamatoria [19]. El inicio del daño muscular que se produce durante la actividad física puede dividirse a su vez en dos posibles vías: metabólica y mecánica [20], que se explica a continuación.

Daño muscular metabólico por consumo de oxígeno

El daño muscular a nivel metabólico suele estar generado por la práctica deportiva de carácter aeróbico, la cual se caracteriza por una excesiva producción de ROS en el proceso de obtención de energía a través de la cadena de transporte de electrones mitocondrial [21]. Muller y col. han identificado que los complejos I y III de esta cadena son los principales lugares de producción del radical superóxido, considerado como la principal especie reactiva de oxigeno [22], por su capacidad de interaccionar con otras moléculas y generar especies reactivas secundarias con mayor poder de agresión [23].

Tradicionalmente, las mitocondrias han sido consideradas como la principal fuente intracelular de ROS, sin embargo existen otros sistemas enzimáticos, tales como la Nicotinamida adenina dinucleótido fosfato (NADPH) oxidasa, citocromo P-450, aldehido oxidasa u óxido nítrico sintasa, que también contribuyen a la producción de ROS [24]. A día de hoy, la enzima NADPH oxidasa, es considerada como la mayor fuente de ROS inducido por el ejercicio físico [25]. Esta enzima se localiza en la membrana plasmática de la fibra muscular [26], en el retículo sarcoplasmático [27], y en los túbulos T [28], y está caracterizada por la producción de anión superóxido que según diferentes autores, influye en la liberación de calcio por el retículo sarcoplasmático a través de la oxidación del receptor de rianodina [29]. Además, se ha descrito que la NADPH oxidasa del sarcolema podría liberar aniones superóxido tanto al citoplasma como al espacio extracelular, lo cual implicaría no solo la activación de dianas sensibles a la oxidación de la propia fibra muscular, sino que tendría importantes consecuencias a nivel biológico [24].

Esta excesiva producción de ROS es debida a la gran demanda de energía requerida cuando realizamos una actividad física aeróbica durante un largo periodo de tiempo, que puede alcanzar valores aproximadamente 100 veces superiores a los de reposo [30]. Esta producción excesiva de ROS interfiere en el equilibrio pro-oxidante y antioxidante intracelular, desencadenando numerosas patologías incluyendo cáncer, diabetes y enfermedades neurológicas [31, 32]. Sin embargo, niveles bajos o moderados de oxidantes, juegan un papel importante como reguladores en la modulación de la producción de fuerza muscular, en el control de las vías de señalización celular y en la regulación de la expresión génica [33, 34].

Daño muscular metabólico por isquemia/reperfusión

El daño muscular metabólico generado por isquemia/reperfusión [24, 35] es la segunda fuente de producción de especies reactivas de oxigeno (ROS) inducida por el ejercicio detrás de la cadena de transporte de electrones mitocondrial [36]. Esta producción de ROS es debida a la disminución del suministro de oxígeno y al agotamiento de las reservas de energía [37]. Este tipo de daño suele ocurrir en

ejercicios de alta intensidad, donde la mayoría de los tejidos y órganos implicados son sometidos a periodos repetidos de hipoxia [38].

Esta disminución de oxígeno en situaciones con grandes demandas de ATP genera un acúmulo de adenosín difosfato (ADP) y adenosín monofosfato (AMP), y un aumento del calcio intracelular por encima de los niveles de normalidad [39]. Esto lleva a una pérdida de la integridad de la membrana del retículo sarcoplasmático y un mayor flujo de Ca^{2+} hacia el citoplasma, lo que genera una activación de las proteasas dependientes del calcio (calpaína) que actúan sobre la xantina deshidrogenasa reconvirtiéndola a xantina oxidasa, que cataliza la oxidación de hipoxantina a xantina [40, 41].

La xantina oxidasa genera dos aniones superóxido, propiciando el ataque de los ácidos grasos poliinsaturados de las membranas celulares [42]. Esto conlleva la pérdida de la integridad de estas membranas, permitiendo la "fuga" de proteínas intramusculares, tanto estructurales como citosólicas, que se pueden cuantificar en sangre tras varias horas y días post-ejercicio [1], como por ejemplo CK, mioglobina y LDH.

Daño muscular mecánico

El daño muscular generado por la propia acción mecánica de la actividad física se produce como consecuencia de una sobrecarga sobre las miofibrillas, siendo mayor en ejercicios excéntricos que en ejercicios concéntricos e isométricos [33]. Factores mecánicos tales como la longitud del músculo, la fuerza y la velocidad parecen jugar un papel importante en la gravedad del daño muscular generado por contracciones excéntricas [43]. Esta sobrecarga mecánica genera una contracción que se caracteriza por un alargamiento no uniforme de los sarcómeros, dando lugar a que algunos miofilamentos se estiren y ya no puedan superponerse dentro del sarcómero [34]. Por lo tanto cuando los filamentos se estiran más allá del punto de solapamiento, las estructuras pasivas (desmina, sinemina y titina) suman más tensión, provocando un fallo de la estructura (disco Z) y reduciendo la capacidad del músculo para generar fuerza [35].

Este daño en los discos Z induce cambios citoesqueléticos que pueden atribuirse a proteínas tales como la α-actinina, vimentina y desmina. Sin embargo, este efecto mecánico que inicia el daño muscular que más tarde desencadenará la inflamación local necesita un evento intermedio [44]. Probablemente, este evento intermedio que propicia el proceso inflamatorio es el incremento de calcio a nivel intracelular [45], que activa endógenamente proteasas (calpaína), causando un deterioro muscular. Curiosamente algunos autores muestran cómo la calpaína no afecta a proteínas citoesqueléticas como la actina y la miosina; sin embargo la α-actinina y desmina sí son alteradas con facilidad [44].

En resumen, el daño muscular generado por isquemia/reperfusión, la excesiva producción de ROS y el daño muscular a nivel sarcomérico generado por la propia acción mecánica de la actividad física, genera una pérdida en la integridad de las membranas celulares, provocando la liberación de enzimas tales como CK, LDH, mioglobina y α-actina. El análisis de estos parámetros post-esfuerzo son buenos marcadores de daño muscular, pero la α-actina siempre estará asociada a un daño más severo puesto que afecta a estructuras sarcoméricas.

Proceso Inflamatorio asociado a ambos tipos de estrés: metabólico y mecánico

La excesiva producción de ROS, que suele desbordar a los mecanismos de defensa antioxidante, desencadena en nuestro organismo una respuesta inmune proinflamatoria debido al daño muscular inducido por la actividad física, que moviliza un gran número de leucocitos entre los que se encuentran los neutrófilos, los linfocitos T y los macrófagos (monocitos) al tejido muscular dañado, con el fin de eliminar los productos de desecho y reparar los tejidos afectados [46].

Esta movilización de células fagocíticas puede perdurar horas después del ejercicio y además puede generar un daño adicional en el tejido no dañado, debido a que su movilización produce una liberación añadida de ROS [41]. Los primeros en llegar al tejido dañado son los neutrófilos localizados en capilares sanguíneos del tejido muscular entre 1 a 24 horas después del ejercicio [47], siendo limitada su acumulación después de este tiempo [48]. Sin embargo, los monocitos/macrófagos son más consistentes en el musculo esquelético y se mantienen durante más tiempo, entre 48 horas y 7 días, en función de la lesión muscular [49].

Se ha demostrado que los neutrófilos y los macrófagos contribuyen a la degradación del músculo dañado por la liberación de especies reactivas del oxígeno y de nitrógeno [50] y a su vez pueden producir citoquinas pro- y anti-inflamatorias tales como interleuquina (IL)-1, IL-6, IL-8, IL-10 y el factor de necrosis tumoral (TNF)-α, desempeñando un papel importante en el inicio de la regeneración del musculo esquelético ya que están asociadas a la activación de las células satélite y a la fagocitosis de la lesión y de las miofibrillas necróticas [51]. Los macrófagos junto con las células satélites trabajan conjuntamente para

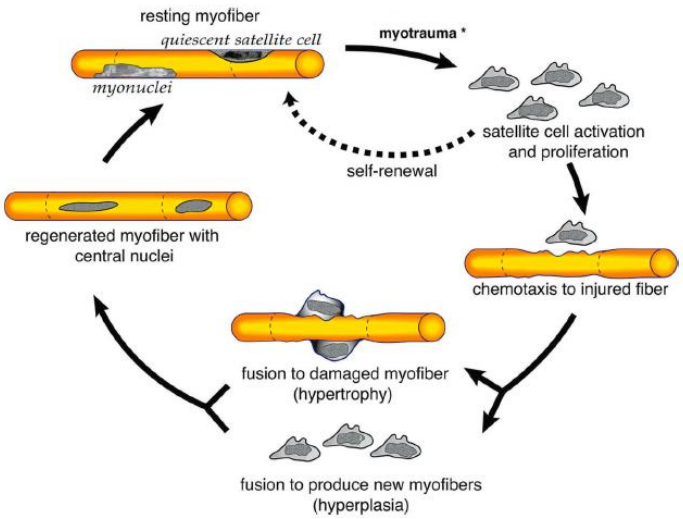

Figura 2. Activación, proliferación, y acción de las células satélites. Tomado de Hawke TJ & Garry DJ, 2001 (6).

reparar el daño generado [52].

En la fase inicial, los macrófagos atenúan la inflamación, activan a las células satélites y estimulan su proliferación, promoviendo la regeneración y el crecimiento del tejido muscular [46]. Mientras no son activadas a través de la respuesta asociada al daño muscular, estas células se mantienen en estado quiescente [53]. La gran mayoría de estas células satélite se origina en el ciclo celular y se comprometen con la diferenciación miogénicas terminal, fusionándose con los miocitos para formar miotubos [54, 55]. Una vez que las miofibrillas han crecido lo suficiente y el músculo ha regresado a su homeostasis, el número de macrófagos disminuye a un nivel basal por el drenaje a los ganglios linfáticos [56].

INTRODUCTION

Muscular damage generalities

Muscular damage may be caused by both direct and indirect factors. Among the direct factors we can identify crush injuries, electrical injury, direct hits and strenuous physical exercise [1]. The main indirect factor is rhabdomyolysis, which consists in the breakdown of muscle tissue caused by drugs, toxins, electrolyte alterations, viral infections, etc. [2]. In the theoretical framework of this doctoral thesis we will focus on the damage caused by direct factors, since they are the responsible ones for muscular damage at the sarcomere level during physical activity. When this kind of damage occurs, the most frequent symptoms are muscular pain, spasms and local swelling. Muscle damage expresses through sarcomere disruption after the fragmentation of the Z-disc, which is located on the sarcomere sides and constitutes the link to the adjacent sarcomere. It consists of numerous proteins, including desmin and alpha-actinin [3]. Other sarcomeric proteins are also affected by muscle damage, such as titin, which connects the Z-line with myosin; nebulin, which keeps actin filaments aligned; or protein c, which keeps myosin and actin filaments perpendicular to the Z-disc [4]. The breaking down of the sarcomeric structures generates an alarm situation in our body, triggering a local swelling process with the purpose of finding a solution to this problem.

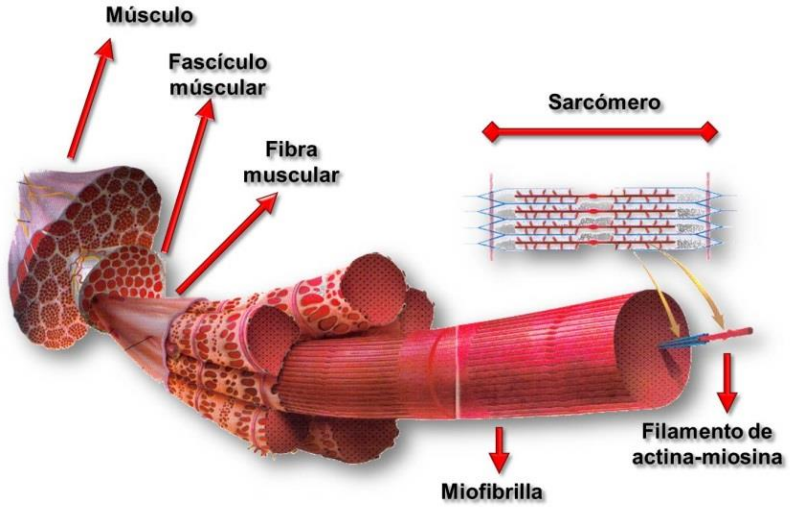

Figure 1. Structure of Sarcomere.

Another type of cell which resides between the basal lamina and the sarcolemma of the muscle fibres is involved in this swelling process: the satellite cells [5]. In order to activate these cells, the modulation by growth factors is needed. The ones with a strongest effect are FGF, HGF, IGG-I, IGF-II, TGF-β, macrophages, IL-6, LIF, platelet-derived growth factor and EGF [6, 7]. Once they become activated, they create precursor cells (myoblasts) which proliferate and finally fuse to the muscle fibres, to finish with the repair and regeneration of the muscle tissue [8].

Exercise-induced muscular damage

Exercise-induced muscular damage is the most common skeletal trauma in the different sport modalities. It may be produced by intense or even moderate physical activity, especially during eccentric exercises [9]. The damaging effects of eccentric

exercise are believed to be related to the mechanical disruption of the actomyosin bonds, as opposed to adenosine triphosphate (ATP)–dependent detachment, which places a higher degree of stress and strain on the involved structures, compared with other muscle actions [10]. The injury severity depends on the duration and characteristics of the exercise, the training stage and the possible presence of muscular imbalance between the agonist and antagonist muscles [11]. The damage may be specific of a few macromolecules of the muscle tissue, or may be due to large tears in the sarcolemma, basal lamina and supportive connective tissue, inducing damage within the contractile elements and the cytoskeleton [12], and leading to decreased fibre tension and even death of the muscle fibre [13].

Nevertheless, despite the consequences that may derive from a severe muscle injury in professional athletes, such as refraining from training or the time "wasted" to achieve a full rehabilitation, smaller-scale muscular damage, known as "adaptive microtrauma", is essential to obtain metabolic and muscular improvements [14]. These microtraumas are of paramount importance, since they allow for tissue strengthening, muscle protection against potential injuries and growth of the muscle fibre (muscle hypertrophy) [10]. The aforementioned satellite cells are responsible for this muscle growth.

Exercise-induced muscle damage decreases with the repeated practice of the same type of exercise at the same training intensity [15]. This phenomenon, called "supercompensation effect", is attributed to strengthening of the connective tissue, higher efficiency in the recruitment of motor units, higher synchronisation of the motor unit, more uniform distribution of the training load among fibres, larger contribution of synergistic muscles and muscular remodelling through the substitution of sarcomeric proteins by more efficient isoforms [16–18]. The adaptations can last up to a few months, even though no training at all is performed.

The muscular damage process induced by physical activity is divided into two general phases: the first phase or primary damage, which occurs during exercise, and the secondary damage, which spreads during a process associated with the inflammatory response [19]. The initial muscle damage produced during physical activity may be divided into two routes: metabolic and mechanic [20], which will be explained below.

Metabolic muscular damage induced by oxygen consumption

Metabolic muscular damage is usually induced by aerobic sport practice, characterised by an excessive production of reactive oxygen species (ROS) in the energy-obtaining process of the mitochondrial electron transport chain [21]. Muller et al. identified complexes I and III of this chain as the main sites of superoxide production, which is considered to be the principal reactive oxygen species [22], due to its capacity to interact with other molecules to generate secondary reactive species, which are more aggressive [23].

Historically, mitochondria have been considered as the main intracellular ROS source; however, other enzymatic systems, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cytochrome P-450, aldehyde oxidase or nitric oxide synthase also contribute to ROS production [24]. Nowadays, the enzyme NADPH oxidase is considered as the major source of exercise-induced ROS [25]. This enzyme is located in the plasma membrane of the muscle fibre [26], in the sarcoplasmic reticulum [27] and in the T-tubules [28], and it is characterised by the production of superoxide anion, which, according to several authors, affects the calcium release from the sarcoplasmic reticulum through the oxidation of the ryanodine receptor [29]. Furthermore, it has been described that the sarcolemmal NADPH oxidase might release superoxide anions both to the cytoplasm and to the outside of the sarcolemma, which could not only activate many redox-sensitive targets in the muscle fibre, but also have significant biological consequences [24].

This excessive ROS production is due to the large amount of energy that is required when any aerobic physical activity is performed for a long period of time, which may increase by approximately 100-fold above resting values [30]. The excessive ROS production interferes with the intracellular pro-oxidant/antioxidant balance, causing numerous pathologies, including cancer, diabetes and neurological disorders [31, 32]. Nevertheless, low or moderate oxidant levels play an important regulatory role in the modulation of muscle strength production, in the control of cellular signalling pathways and in gene expression [33, 34].

Metabolic muscular damage induced by ischemia/reperfusion

Metabolic muscle damage produced by ischemia/reperfusion [24, 35] is the second source of exercise-induced production of reactive oxygen species (ROS) after the mitochondrial electron transport chain [36]. This ROS production is due to the decrease in oxygen supply and the depletion of energy reserves [37]. This type of damage usually occurs during high-intensity exercises, where most of the tissues and organs involved are exposed to repeated periods of hypoxia [38].

This oxygen decrease in situations with high ATP demands generates an accumulation of adenosine diphosphate (ADP) and adenosine monophosphate (AMP), as well as an increase in intracellular calcium above normality levels [39]. This leads to loss of integrity in the sarcoplasmic reticulum membrane and a larger flux of Ca^{2+} into the cytoplasm, activating calcium-dependent proteases (calpain), which act on xanthine dehydrogenase turning it into xanthine oxidase, which catalyses the oxidation of hypoxanthine to xanthine [40, 41].

Xanthine oxidase produces two superoxide anions, fostering the attack to the polyunsaturated fatty acids of cellular membranes [42]. This entails loss of integrity of these membranes, allowing for the “leak” of both structural and cytosolic intramuscular

proteins, which may be quantified in blood several hours or days after exercise [1], such as, for example, CK, myoglobin or LDH.

Mechanical muscular damage

Muscular damage generated by physical activity mechanical action is produced as a consequence of an overload on the myofibres, it being higher in eccentric than in concentric or isometric exercises [33]. Mechanical factors such as muscle length, strength and velocity seem to play an important role in the severity of the muscular damage provoked by eccentric contractions [43]. Such mechanical overload produces a contraction characterised by a non-uniform sarcomere lengthening, making some myofilaments stretch, so that they can no longer overlap within the sarcomere [34]. Therefore, as the filaments stretch further than their overlapping point, the passive structures (desmin, sinemin and titin) increase their tension, leading to a structural failure (Z-disc) and reducing the muscle capacity to generate force [35].

Z-discs damage induces cytoskeletal changes which may be attributed to proteins such as α-actinin, vimentin and desmin. Nonetheless, this mechanical effect, which initiates the muscle damage that will afterwards trigger the local swelling, needs an intermediate event [44]. The intermediate event that induces the inflammatory process is probably the increase of intracellular calcium [45], which activates proteases (calpain) endogenously, producing muscle damage. Curiously, some authors show how calpain does not affect cytoskeletal proteins such as actin and myosin; however, α-actinin and desmin are easily affected [44].

In summary, the muscular damage induced by ischemia/reperfusion, the excessive ROS production and the muscular damage at the sarcomere level produced by physical activity mechanical action generate loss of cellular integrity, inducing the release of enzymes like CK, LDH, myoglobin or α-actin. These post-effort parameters are good markers of muscle damage, but α-actin is associated with more severe damage, since it affects the sarcomeric structures.

Inflammatory process associated with both types of stress: metabolic and mechanical

Excessive ROS production, which usually exceeds the antioxidant defence mechanisms, triggers a proinflammatory response in our body due to exercise-induced muscle damage which mobilises a large number of leukocytes, including neutrophils, T lymphocytes and macrophages (monocytes) to the damaged muscle tissue, with the purpose of removing waste products and repairing the affected tissue [46].

This mobilisation of phagocytic cells may last for hours after exercise and may also produce additional damage in the non-damaged tissue, since its mobilisation induces greater ROS release [41]. The first to arrive at the damaged tissue are the

neutrophils located in muscle tissue capillaries, which occurs between 1 - 24 hours after exercise [47]. After this period of time, their accumulation is limited [48]. Nonetheless, monocytes/macrophages in skeletal muscle are more consistent and last longer, between 48 hours and 7 days, depending on the muscle injury [49].

Neutrophils and macrophages have proved to contribute to the degradation of the muscle damaged by the release of reactive oxygen species and nitrogen [50]. They are also able to produce pro- and anti-inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-8, IL-10 and tumour necrosis factor (TNF)-α, which play an important role in the beginning of skeletal muscle regeneration, for they are associated with satellite cell activation and with damage and necrotic myofibre phagocytosis [51]. Macrophagues and satellite cells work jointly to repair the damage [52].

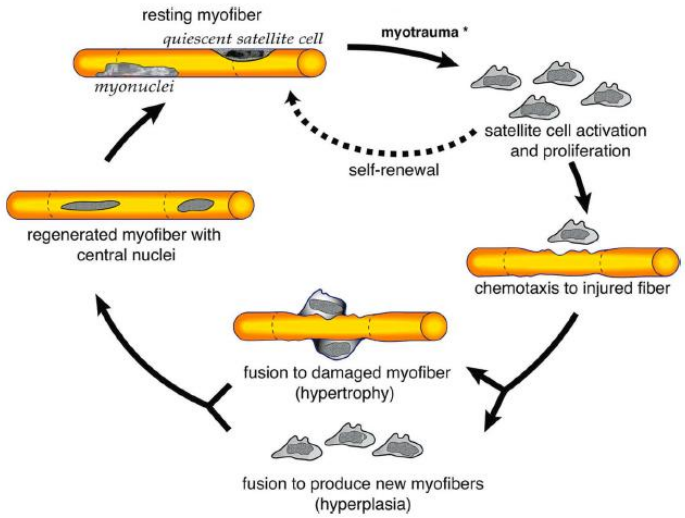

Figure 2. Activation, proliferation, and action of satellite cells. Hawke TJ & Garry DJ, 2001 (6).

In the initial phase, macrophagues attenuate the inflammation, activate the satellite cells and stimulate their proliferation, promoting the regeneration and growth of the muscle tissue [46]. Unless they are activated by the response associated to muscle damage, these cells remain in a quiescent state [53]. The vast majority of these satellite cells originate in the cell cycle and commit to terminal myogenic differentiation, fusing with myocytes to form myotubes [54, 55]. Once myofibres have grown sufficiently and muscle has returned to its homeostasis, the number of macrophagues decreases to a steady state level due to drainage into lymph nodes [56].

JUSTIFICACIÓN

La actividad física supone un elevado trabajo, tanto mecánico como metabólico, en el que se reclutan de forma ajustada una serie de unidades motoras involucradas en el gesto deportivo. Cuando la actividad física se hace de forma crónica, competitiva, con macro y microciclos de entrenamiento, se producen una serie de adaptaciones sistémicas que maximizan el rendimiento [57, 58]. No obstante, un exceso de sesiones de entrenamiento y/o una disminución de las fases de recuperación pueden generar en el organismo un síndrome de fatiga crónica o de sobreentrenamiento, que puede ser nefasto no solo en términos de rendimiento sino también para la salud de los atletas [59]. Por ello, en el ámbito de la actividad física se emplean **marcadores séricos de daño muscular** para predecir y diagnosticar lesiones.

En la actualidad, el marcador más empleado para detectar daño muscular es la **creatina quinasa** (CK), pero existe controversia a la hora de establecer el tiempo en el que aparecen los valores máximos en el suero [60], ya que algunos autores lo detectan dentro de las primeras 24 horas post-ejercicio [61, 62] y otros observan más de un pico máximo entre las 24-72 horas [63, 64]. A pesar de estas y otras discrepancias, la CK se utiliza habitualmente como marcador de daño celular de origen citosólico.

Otra enzima muy empleada como marcador de daño muscular es la **lactato deshidrogenasa** (LDH). Suele emplearse junto con la CK; sin embargo, ambas son marcadores inespecíficos debido a su amplia distribución tisular [65]. Así, se han descrito valores altos de LDH y CK en deportistas con un daño severo hepático pero no muscular [66].

La **mioglobina** es otro marcador de daño muscular muy empleado en el ámbito del alto rendimiento, aunque también presenta inconvenientes: al igual que la CK y la LDH, es citosólica y su aparición en sangre puede deberse a lesión de los músculos esquelético y cardiaco o de otros tejidos (hígado, riñón, etc.) [67]; existe una gran variabilidad entre sujetos en lo que se refiere a su liberación; sus valores son independientes de la intensidad de la actividad física y, además, mayores en actividades físicas continuas (natación, atletismo o ciclismo), en comparación con actividades discontinuas (fútbol, tenis o voleibol) [68].

Existe, por tanto, la necesidad de encontrar marcadores específicos de origen sarcomérico. Diferentes autores han centrado su atención en la **α-actina**, proteína que representa el 20% de las proteínas celulares. Comenzó empleándose como marcador de angina inestable [69]; sin embargo, en 2005 se empezó a utilizar en el ámbito de la actividad física como marcador de daño muscular sarcomérico. Los primeros trabajos en los que se ha determinado α-actina en suero fueron los de Martínez-Amat y cols. [70], quienes analizaron esta proteína en sujetos con daño muscular esquelético severo causado por traumatismos graves. Estos autores detectaron cambios significativos en la α-actina pero no en otros marcadores habituales como los comentados anteriormente, y por tanto sugirieron que la α-actina es un marcador fiable y específico de daño sarcomérico.

Los profesionales del deporte (médicos, fisioterapeutas, entrenadores y deportistas) necesitan marcadores sanguíneos de daño muscular que permitan prever e identificar lesiones y, por tanto, establecer pautas de entrenamiento, tratamiento y rehabilitación. Entre los marcadores utilizados actualmente se encuentran las actividades CK y LDH y la concentración de mioglobina. Sin embargo, son inespecíficos y de origen citosólico, por lo que resulta crucial disponer de marcadores específicos de daño sarcomérico. Un candidato que cumple estos requisitos es la α-actina. Nuestra **hipótesis** se basa en que esta proteína podría ser un marcador válido, específico y fiable de daño muscular sarcomérico para distintas actividades deportivas, y que permitiría distinguir daños de distinta intensidad.

Por lo tanto, el **objetivo** de esta tesis fue verificar si la α-actina puede ser de utilidad como marcador de daño muscular sarcomérico y permita discriminar niveles de daño menos intensos. Para ello, se han realizado estudios en deportistas de diversas disciplinas (carrera, ciclismo, natación y *fitness*) sometidos a distintas situaciones fisiológicas (hipoxia y normoxia).

JUSTIFICATION

Physical activity implies a hard work, both mechanical and metabolic, in which motor units are recruited in a fine-tuned fashion. When physical activity is carried out chronically and competitively, with macro and microcycles of training, systemic adaptations that maximize performance occur [57, 58]. However, an excess of training and/or a decrease in the period of recovery after exercise may induce a syndrome of fatigue or overtraining, which in turn may result disastrous for the performance, or even for the health, of the athlete [59]. Therefore, **serum markers of muscle damage** are used in the physical activity field to predict and diagnose injuries.

Nowadays, one of the markers most frequently used for the detection of muscular injury is the enzyme **creatine kinase** (CK). However, controversy exists with regards to the timing when maximum values are found in serum [60]. Some authors detect the highest values within the first 24 hours after exercise [61, 62], whereas others describe more than a peak between 24 and 72 hours [63, 64]. Despite the discrepancies, CK activity is customarily used as a marker of cytosolic cell damage.

Another enzyme frequently used as a marker of muscular damage is **lactate dehydrogenase** (LDH). LDH activity is usually measured together with CK even though they both are nonspecific markers given their widespread tissue distribution [65]. In fact, high values of LDH and CK have been reported in athletes with severe liver, but not muscular, damage [66].

Myoglobin is also used often as a marker of muscular harm in the high performance sport area, but it has disadvantages also: As was the case for CK and LDH, myoglobin is cytosolic and its presence in serum may be due to damage in either the heart, the skeletal muscle or other tissues (liver, kidney, ...) [67]; a wide variation among individuals regarding mioglobin release to serum has been described; its values in serum are independent of the intensity of physical activity, higher after long but sustained activities (swimming, cycling or running) and lower in intermittent sports (soccer, tennis or volleyball) [68].

Hence, there is a necessity of specific markers of sarcomeric origin. Various authors have focused their attention on **α-actin**, which stands for 20% of cellular proteins. Although initially used as a marker of unstable angina [69], α-actin was later on used in the physical activity field as an index of sarcomeric damage. Martínez-Amat et al. [70] were the first to determine the concentration of this protein in the serum of patients with muscular damage induced by severe traumatism, finding statistically significant changes in α-actin but not in other customary markers such as the ones mentioned above, and consequently suggested that α-actin is a reliable and specific marker of sarcomeric injury.

Sport professionals (physicians, physiotherapists, coaches and athletes) have a need for blood markers of muscular damage to foresee and recognize injuries and, consequently, to schedule training, treatment and rehabilitation guidelines. Among the markers currently used are CK, LDH and myoglobin, but all three are nonspecific and

of cytosolic origin. Therefore, it is crucial to have specific markers of sarcomeric damage. One candidate that meets these requirements is α-actin. Our **hypothesis** was that this protein could be a valid, specific and reliable marker of sarcomeric damage for different sport activities, and as such could allow to discriminate injuries of varying intensities.

Accordingly, the **aim** of this research was to verify whether α-actin may be useful or not as a marker of sarcomeric damage and allows for the discrimination of more gentle injuries. For this purpose, we carried out six studies with athletes practicing different sport activities (running, cycling, swimming and fitness) and under two physiological situations (hypoxia and normoxia).

MÉTODOS

Sujetos experimentales

En el primer estudio participaron 23 varones divididos en tres grupos experimentales en función de la práctica deportiva, predominantemente aeróbica, que habían realizado a lo largo de su vida. En el segundo participaron 12 nadadores, con más de 12 años de experiencia y 9 años de participación en alta competición a nivel nacional. En el tercer estudio se reclutaron a 94 varones bien entrenados en disciplinas deportivas de corta y media distancia, predominantemente aeróbica, divididos en base al consumo de vitamina E. Y por último, un cuarto estudio en el que participaron 20 sujetos sometidos a un programa de entrenamiento de fuerza.

Para poder formar parte de los cuatro estudios, los sujetos no debían consumir ningún tipo suplemento ergogénico, drogas o medicamentos, ni presentar síntomas clínicos de infección y de alteraciones cardiovasculares y/o metabólicas. Todos los participantes estuvieron al margen de cualquier tipo de actividad física 48 horas antes de empezar el experimento. Los participantes fueron informados de forma escrita y verbal, de la propuesta del estudio y de sus posibles riesgos y firmaron su consentimiento informado por escrito. El protocolo fue seguido de acuerdo con la Declaración de Helsinki (2000) y aprobado por el comité de ética de la Universidad de Granada.

Diseño experimental

El diseño experimental llevado a cabo en los diferentes estudios que completan esta tesis doctoral fue intergrupo, transversal y descriptivo.

Análisis corporal. Valoración antropométrica

Todos los participantes fueron sometidos a una evaluación antropométrica siguiendo el protocolo sugerido por la International Society for Advanced of Kinanthropometry (ISAK) [71]. Se obtuvieron las siguientes variables antropométricas: índice de masa corporal (IMC), peso, altura, 7 pliegues cutáneos (bíceps, tríceps, subscapular, supraespinal, abdominal, muslo y pantorrilla media), 3 circunferencias (bíceps contraído, muslo y pantorrilla media) y tres amplitudes óseas (epicóndilos humeral y femoral y muñeca). La masa grasa fue calculada según la fórmula para deportistas, descrita por Carter JEL [72], mientras que para la masa muscular se utilizó la de Matiegka [73].

Electromiografía.

La activación eléctrica de los músculos se monitorizó usando electromiografía de superficie (EMG). Las señales electromiográfica obtenidas durante las acciones de

extensión y flexión de los músculos del tren inferior del lado derecho se registraron en el vasto lateral (VL), vasto medial (VM), recto femoral (RF) y bíceps femoral (BF). Los valores de los músculos del tren superior del lado derecho se registraron a partir del pectoral mayor derecho (PM, aproximadamente 4 cm medial respecto al pliegue axilar [74], deltoides anteriores (AD, 1,5 cm distal y anterior al acromion), dorsal ancho (LD) y tríceps braquial (TB, cabeza larga, aproximadamente 3 cm medial y en el 50% en la línea entre acromion y olecrán) [75], en press banca. La contracción muscular fue realizada en condiciones dinámicas.

La superficie de la piel se rasuró cuidadosamente y se limpió con alcohol antes de la aplicación de los electrodos electromiográficos para reducir la impedancia de la piel. Los electrodos diferenciales bipolares se colocaron longitudinalmente sobre los músculos siguiendo las recomendaciones de SENIAM (Electromiografía de superficie para la evaluación no invasiva de los músculos) [76], y se fijaron a la piel para minimizar los artefactos debido al movimiento. El electrodo de referencia se colocó en la piel sobre el acromion. La posición de los electrodos se marcó en la piel con tinta indeleble y los electrodos se mantuvieron fijos a lo largo de la prueba.

Las señales electromiográficas se adquirieron utilizando un sistema de registro de 4 canales (Myomonitor IV, Delsys Inc., Boston, EE.UU) a una frecuencia de muestreo de 1000 Hz usando electrodos de superficie bipolares de forma rectangular (19,8 mm de ancho y 35 mm de largo) con 1 x 10 Mm 99,9% de conductores Ag, y con una distancia entre conductores de 10 mm (DE-2,3 Delsys Inc.). Se utilizó una tarjeta de adquisición de datos analógico-digital de 12 bits BNC-2090 (National Instruments Corporation, Austin, TX, EE.UU.) para muestrear la señal a 4000 Hz. La señal electromiográfica correspondiente a cada contracción muscular fue adquirida y analizada utilizando un programa específico (EMGworks 4.1.7 Analysis, Delsys, Inc. Boston, EE.UU.). Después de una inspección visual, las grabaciones electromiográficas fueron filtradas usando un filtro de clase Butterworth con paso alto de 100 Hz y un paso bajo de 450 Hz (IIR Filter, Delsys, Inc. Boston, EE.UU.). Se calculó el cuadrado medio de la raíz (RMS) para cada contracción y, por último, se determinó el área sobre RMS (aRMS) de cada contracción.

Se calculó el promedio de aRMS de la segunda, tercera y cuarta repetición de las intensidades del 20% al 60% para asegurar que cada análisis se realizó con el mismo ritmo y con la técnica correcta. A intensidades de 20%, 40% y 60%, la primera y la última repetición siempre fueron excluidas del cálculo para evitar registrar movimientos derivados de la retirada y colocación de la barra en la máquina de Smith.

Análisis de parámetros bioquímicos

Determinación de marcadores de peroxidación lipídica: sustancias reactivas al ácido tiobarbitúrico e hidroperóxidos

Como marcador directo de peroxidación lipídica se determinó la concentración de hidroperóxidos utilizando un kit comercial Sigma PD1 kit (Saint Louis, MO, EE.UU.) y espectrofometría con una lectura a 560 nm [77]. Además, se analizaron las sustancias reactivas al ácido tiobarbitúrico siguiendo el método descrito por Orrenious et al., con una lectura a 532 nm [78].

Actividad en suero de creatina quinasa isoforma MB y lactato deshidrogenasa

Las actividades CK-MB y LDH se midieron por espectrofotometría mediante kits comerciales de la casa Spinreact SA (LDH ref: 41223) (CK-MB ref: TK41255). Los resultados fueron expresados como U/L, siguiendo las indicaciones del fabricante.

Cuantificación de la concentración de α-actina en suero

La α-actina sarcomérica liberada al suero se detectó mediante Western blot y se cuantificó utilizando una curva patrón realizada con concentraciones crecientes de la proteína pura (Sigma-Aldrich Química, S. L., Madrid), tal como han descrito Casuso y cols. [58].

Las muestras de suero (5 µL) de cada sujeto se diluyeron con tampón de carga 3X (100 mM Tris-HCl pH 6,8, 10% 2-mercaptoetanol, 25% SDS, 2% glicerol y 0.4% azul de bromofenol) y se hirvieron durante 10 minutos. A continuación, las proteínas se separaron mediante electroforesis en gel de poliacrilamida utilizando una cubeta Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, California, EE. UU.) y geles TGX Any kD (Bio-Rad Laboratories). Finalizada la electroforesis, las proteínas se transfirieron a membranas de nitrocelulosa (Trans-Blot transfer pack, Bio-Rad Laboratories) en un sistema de transferencia Trans-Blot Turbo (Bio-Rad Laboratories) durante 7 minutos a 25 V y 1,3 A. Seguidamente, las membranas se incubaron durante 1 hora con solución de bloqueo TBS-T (5 % leche en polvo desnatada, 1% Tween-20, 20 mM Tris, 0,9 % NaCl).

Finalizado el bloqueo, las membranas se incubaron toda la noche a 4 ºC con una dilución 1:1000 del anticuerpo primario anti-actina (C-11): sc-1615 (Santa Cruz Biotechnology, Dallas, EE. UU.) en una solución de leche desnatada al 5% en TBS-T. Al día siguiente, las membranas se lavaron tres veces durante 15 minutos con TBS-T. Por último, las membranas se incubaron con una dilución 1:5000 del anticuerpo

secundario (bovine anti-goat IgG-HRP: sc-2350, Santa Cruz Biotechnology) en una solución de leche desnatada al 5% en TBS-T durante 1 hora a temperatura ambiente, y se volvieron a lavar 3 veces durante 15 minutos en TBS-T.

Las bandas correspondientes a α-actina se detectaron mediante quimioluminiscencia utilizando el kit Super-Signal West Dura Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, EE.UU.), se fotografiaron y analizaron con el programa ImageJ (National Institutes of Health, Bethesda, EE.UU.). Para cuantificarlas, se compararon con los distintos puntos de la curva patrón.

Análisis estadístico

Todas las determinaciones se hicieron por duplicado. Los datos se expresan como media ± error estándar de la media. La distribución normal de las variables fue comprobada con un test de Kolmogorov-Smirnov y la homogeneidad de la varianza por el método de Levene. En algunos casos fue empleado una T de Student (Manuscrito III, IV y VI). En otros estudios fue utilizada una ANOVA de una vía (Manuscrito I, IV,). En un estudio fue necesario el empleo de una ANOVA de dos vías (Manuscrito II). Y por último en algunos trabajo fue necesario una ANOVA mixto de medidas repetidas (Manuscrito V, VI) para comparar las medias en el tiempo y entre los diferentes grupos experimentales.

Las diferencias entre grupos se realizaron usando un test “a posteriori” (post-hoc) por Bonferroni, se consideró diferencias significativas para el valor de $p<0.05$. El tratamiento estadístico se realizó con el paquete informático SPSS para Windows, versión 22.0, en español.

CONCLUSIONES

1. La liberación de α-actina a la sangre depende de las horas de entrenamiento y no se ve alterada cuando este último tiene lugar en ambientes hipóxicos moderados.

2. La suplementación dietética con dosis elevadas de α-tocoferol no afecta al daño muscular.

3. Se ha desarrollado un test basado en registros electromiográficos que permite identificar el umbral de fatiga en músculos individuales y es aplicable a ejercicios con movimientos explosivos de corta duración.

4. La α-actina sérica es un marcador valido y fiable para identificar el daño muscular sarcomérico en deportes como natación, ciclismo y *running*, y también en ejercicios de fuerza como la media sentadilla y *press* banca.

5. Los ejercicios de *press* banca y media sentadilla generan valores similares de α-actina en suero tras un test de fuerza máxima incremental. Sin embargo, los marcadores citosólicos CK-MB y LDH son significativamente más elevados en *press* banca, probablemente porque en la media sentadilla el daño se distribuye en una masa muscular mayor.

6. Ejercicios incrementales hasta 1RM, tanto en media sentadilla como en *press* banca, no generan cambios en los marcadores de peroxidación lipídica, lo que sugiere que en el daño muscular predominan los factores mecánicos sobre los metabólicos.

7. Aparentemente, hombres y mujeres poseen los mismos valores séricos de α-actina, LDH y CK-MB tras un esfuerzo máximo incremental en el ejercicio de media sentadilla. Sin embargo, la concentración de α-actina es significativamente mayor en varones cuando este marcador se normaliza con la volemia, probablemente debido a su mayor masa muscular.

CONCLUSIONS

1. Release of α-actin to blood depends on the hours of training, except when the latter takes place in moderately hypoxic environments.

2. Muscular damage is not affected by dietary supplementation with great doses of α-tocopherol.

3. We have developed an electromyograhy-based test to identify the fatigue threshold in single muscles, which is applicable to explosive exercises of short duration.

4. Serum α-actin is a valid and reliable marker of sarcomeric muscular damage for sports such as swimming, running and cycling, as well as for strength exercises such as half-squat and bench press.

5. Bench press and half-squat exercises generate similar values of α-actin in serum after an incremental strength test. However, the cytosolic markers CK-MB and LDH reach higher values in the bench press exercise, likely because the damage is distributed through a larger muscle mass in the half-squat exercise.

6. Incremental exercises up to 1RM in either bench press or half-squat do not induce changes in markers of lipid peroxidation, which suggests that mechanical factors prevail over metabolic ones in the muscular damage.

7. In the half-squat exercise, males and females apparently exhibit similar values of α-actin, LDH and CK-MB in serum after an incremental effort. However, α-actin concentration is found to be significantly greater in males compared with females when values are normalized by the volemia, probably due to the larger muscular mass of the former.

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APÉNDICES-ESTUDIOS I-VI

I

LIFELONG AMATEUR ENDURANCE PRACTICE ATTENUATES OXIDATIVE STRESS AND PREVENTS MUSCLE WASTING IN SENIOR ADULTS

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ORIGINAL ARTICLE

Lifelong amateur endurance practice attenuates oxidative stress and prevents muscle wasting in senior adults

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ABSTRACT

BACKGROUND: The aim of the study was to investigate oxidative stress, muscle damage, enzymatic antioxidant defense and body composition in senior adults who have performed different lifelong physical activity practices.

METHODS: Twenty-three healthy senior men (60±1.88 years old) were divided into three groups according to their lifelong physical activity practice as follows: A) sedentary (N.=7); B) recreational (N.=9); and C) amateur (N.=7). Blood sampling was performed at rest to analyze plasma malondialdehyde (MDA) by TBARs-assay, nuclear DNA-damage in peripheral lymphocytes using Comet-Assay, the plasma enzymatic activity of glutathione peroxidase by spectrophotometry and serum alpha-actin release as skeletal muscle damage marker through western blot. Body composition was evaluated using anthropometric assessments by the ISAK protocol through skinfold thickness.

RESULTS: The lowest value of MDA was shown in the amateur group. Nuclear DNA-damage was significantly lower in the recreational group than in sedentary and amateur groups (MD=5.53±1.70; P=0.013. MD=5.61±1.62; P=0.008), respectively. The amateur group showed trends toward higher glutathione peroxidase enzymatic activity than recreational and sedentary groups. Alpha-actin levels were significantly higher in the amateur compared with recreational (MD=4.34±0.46; P<0.001) and sedentary groups (MD=4.89±0.46; P<0.001). The sedentary group showed significantly lower muscle mass (MD=3.67±1.10; P=0.011) and higher fat mass (MD=4.19±0.98; P=0.001) than amateur group.

CONCLUSIONS: The results described above suggest that the lifelong amateur endurance practice seems to improve oxidative stress response and strengthens hypertrophy mechanisms that might preserve muscle mass in senior adults.

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Key words: Exercise - Oxidative stress - Physical endurance.

Senescence or biological aging is an age-dependent functional loss, where the organism fail to respond to external environmental changes as a consequence of a multifactorial process related to genetic factors, nutrition, and lifestyle.[1] Aging is associated with damage in several molecules (protein, DNA, lipid) by the action of Reactive Oxygen Species (ROS).[2] However, ROS are also essential signaling molecules to balance muscle cell homeostasis and gene expression for preventing oxidative damage.[3] Moreover, during exercise, the muscle contraction generates an increase in ROS, which in excess could lead to damage several tissues such as skeletal muscle cells and generate dysfunction.[4]

Furthermore, a strength and muscle mass decline of 3-8% is observed after 30 years, and this rhythm decline increases to 12-15% from 50 years old,[5] which is associated with the risk of morbidity and mortality at older ages.[6] Many factors are the responsible for this weak-

ening in muscle force and mass. Aging mainly affect to type II fibres, since these kind of fibres have lower resistance to the denervation and oxidative stress, which could explain the hormonal deficit in old people.[7] In addition, the reduction in muscle mass could be due to a decline in the synthesis of protein, and repair mechanisms of muscle fibers, lower activity of satellite cells, higher chronic inflammation, all of this due to a cumulative oxidative stress which damages the mitochondrial-DNA, with a consequently mitochondrial dysfunction in the skeletal muscle cell. Thus, a ROS overproduction seems to be one of the most prominent causes.[8] Physical activity is suggested as a preventive treatment, since a combination of resistance and endurance exercise conserves the strength and protects against the age-related changes in muscle fibers.[9] It also conserves the mechanisms of muscle hypertrophy through structural changes associated with exercise-induced muscle damage (EIMD).[10] Hence, sedentary lifestyle may aggravate oxidative stress production and cause harmful alterations associated with age-related sarcopenia.[11] Regular physical activity, therefore, is claimed as a preventive means against age-related oxidative stress and the loss of muscle mass.[12] Thus, the type of sport practice affects both the oxidative stress and skeletal muscle damage blood markers. Additionally, the production of ROS has been associated to loss of muscle mass with age and physical inactivity.

Therefore, we consider that a high muscle activity, as amateur endurance athletes presented, might involve an important increase of ROS as several previous studies reported,[13, 14] which could be potentially detrimental in the process of muscle hypertrophy, and consequently, could have a negative impact on the muscle growth.[15] Thereby, the purpose of this study was to investigate the oxidative stress, muscle damage and body composition in senior adults who had different lifelong physical activity practices.

Materials and methods

This study was conducted in accordance with The Helsinki Declaration (2000). Approval from the ethical committee of the University of Granada was obtained before starting the study (President: Maria Dolores Suarez Ortega. Date of approval: 3/10/2010. Number case/417). All participants received detailed information about the study and their informed consent was obtained at study onset.

Participants

A total of 23 healthy senior adults (60±1.88 years old) voluntarily participated in the present study. The participants were divided into three groups according to their lifelong physical activity practice: A) sedentary, physically inactive, performing only routine daily activities (N.=7); B) recreational, participants physically active for three to six times a week (N.=9); and (C) amateur, who routinely practiced in cycling competition who usually trained more than eight times a week (N.=7). All participants declared having had a lifelong (30 years) history of the same physical activity practice. Amateur group should have conducted at least 30 years of high intensity training and have participated routinely in competitions. All of them were healthy and declared not to have any specific physical/disease issues or the intake of any supplementation (protein/antioxidant). Consequently, all participants met the study inclusion criteria (Table I).

Design

This is a cross-sectional study where the selection of participants was performed in an initial interview.

TABLE I.—*Characteristic of the participants.*

Parameters	Sedentary	Recreational	Amateur
Main grouping characteristics			
Age (years)	58.5± 1.6	60.8± 2.3	60.7± 1.7
Physiscal activity (times/week)	0.3± 0.2	5.2± 0.4	9.1± 1.1
Health assessment			
SBP (mmHg)	125.0± 4.7	119.4± 3.9	124.3± 3.7
DBP (mmHg)	83.3± 3.3	73.9± 3.5	79.3± 3.2
Glycaemia (mg/dL)	100.3± 9.0	86.9± 4.1	84.4± 3.3
Cholesterol (mg/dL)	219.3± 4.9	208.3± 14.0	176.6± 13.9
Uric acid (mg/dL)	4.58± 0.49	4.86± 0.36	5.60± 0.65
Creatinine (mg/dL)	0.94± 0.09	1.01± 0.06	0.94± 0.06
Total protein (mg/dL)	7.12± 0.11	6.89± 0.23	6.43± 0.17
Body composition			
BMI (kg/m^2)	27.4± 1.0	26.4± 0.6	27.2± 0.4
Fat mass (%)	22.8± 1.0	20.0± 0.6	19.4± 0.6
Muscle mass (%)	43.6± 1.2	45.5± 0.6	46.6± 0.7
Bone mass (%)	9.4± 0.4	10.4± 0.4	10.0± 0.2
N	7	9	7

Data are expressed mean (M)±standard error of the mean (SEM).
*Statistical differences between amateur and sedentary; $ statistical differences between amateur and recreational; # statistical differences between all groups; Bonferroni Test at P=0.016.

They were allocated to the study groups according to the lifelong practice of physical activity that they had performed. The study participants were evaluated the same day in the Physiology Exercise Laboratory at the Faculty of Physical Activity and Sport Science (University of Granada). All measurements were performed in fasting condition (anthropometry and blood samples). No exercise was performed 48 hours before the beginning of the study. The last competition of amateur group was performed three days before evaluations.

Experimental procedures

ANTHROPOMETRY

All participants underwent an anthropometric assessment by skinfold thickness. A total of 17 anthropometric variables (height [m] and body mass [kg]; skinfolds [mm]: biceps, triceps, subscapularis, suprailiac, abdominal, thigh, calf; body circumferences [cm]: relaxed biceps, contracted biceps, waist, hip, thigh and calf; Bone diameters [mm]: humerus, wrist and femur) were measured using (anthropometric set, Harpenden) the protocol developed by the International Society for Advanced Kinanthropometry (ISAK).[16] The evaluator presented a variation of less than 2.29% between measurements, with reproducibility determined by an intra-class correlation coefficient of 0.987 within the assessment performance period. Body Mass Index (BMI) was calculated as body mass (kg)/height (m)^2. The Body fat mass was calculated by the Faulkner equation (1968). From this former equation, the other body compartments (muscle, bone and residual masses) were obtained using the methodology of four compartments described by Rose and Guimaraes derived from Matiegka equation (1980). All the final measures were expressed in relative terms of (%).

BLOOD SAMPLING

Blood samples (3 mL) were drawn from the antecubital vein using vacutainers. Qualified nursing staff was in charge of the procedure. The samples were stored in two different containers, one for whole-blood analysis and the other for serum. Samples were transported at -4 °C to the Biomedical Research Center of the University of Granada. Plasma, serum and lymphocytes were obtained through gradient centrifugation (Eppendorf 5810 Centrifuge, Hamburg, Germany). Plasma and serum were extracted and frozen (-80 ºC) awaiting for oxidative stress and muscle damage analysis, while lymphocytes followed the procedure for the Comet Assay to evaluate the DNA-damage.

HEALTH ASSESSMENT

Systolic (SBP) and diastolic (DBP) blood pressures were recorded in both arms by using an upper arm blood pressure monitor (Omron M10-IT, Healthcare, Kyoto, Japan) which automatically takes the measurements.

Plasma biochemical parameters (glycaemia, cholesterol, uric acid, creatinine total protein) was measured spectrophotometrically in the analyser Biochemist BS-200 (chemistry analyzer, shenzhen mindray biomedical electronics CO., LTD) using commercial kits home Spinreact (Sant Esteve de Bas, Girona, Spain).

OXIDATIVE STRESS RESPONSE

Malondialdehyde by TBARs assay.—Malondialdehyde (MDA) is one of several low-molecular-weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products and is considered a sub-product of cellular oxidative stress. Plasma MDA was measured as described by Orrenius (Orrenius 1977) through the spectrophotometric measurement of substances which react with thiobarbituric acid (TBARs). The reaction of MDA-TBA leads to orange pigmentation, which was then read spectrophotometrically on a U-bottomed microplate (Synergy HT Biotek, Winooski, VT, USA) at 532 nm.

Evaluation of nDNA-damage in lymphocyte (% DNA-tail damage).—Lymphocytes were isolated by gradient centrifugation. The total sample of blood was diluted in PBS (1:1) and centrifuged at 3500 RPM (Beckman GS-6R Refrigerated Table Top Centrifuge, L'Hospitalet de Llobregat, Barcelona, Spain) for 30 minutes. After several centrifugations at 1750 RPM in RPMI histopaque, the top layer of cells (lymphocytes) was removed and diluted in 500 µl of 10 % DMSO-SBF. The mix was suspended and samples were divided into aliquots of 200 µl for subsequent storage in cryogenic boxes (Nalgene® 5100-0001 Cryo 1°C Freezing Container Rack, New York, NY; USA). Then, lymphocytes (50 µl) were embedded in a thin agarose gel on a microscope slide.

After lysing and electrophoresis treatments (horizontal electrophoresis. Model HE 03, Labolan, Navarra, Spain) fragments of damaged DNA migrated away from the nucleus. After staining with a DNA-specific fluorescent, the gel was read in a UV microscope (DMLS, Leica, Wetzlar, Germany) through specific software (Komet 5.5, Belfast, UK). The extent of DNA liberated from the head of the comet was directly proportional to the amount of DNA damage.[17]

Enzymatic antioxidant defense.—Enzymatic antioxidant activity of glutathione peroxidase (GPx) was measured in erythrocytes by spectrophotometry (Synergy HT Biotek) as described by Ochoa *et al.*[18]

Skeletal muscle damage

Skeletal muscle damage was determined by serum α-actin quantification as a specific mucle skeletal damage biomarker through Western Blot and Densitmetric analysis as described by Martínez-Amat *et al.*[19]

Western blot.—A sample of serum (5 µl) from every study subject was dissolved in Laemmli's sample buffer (62.76 mm Tris-HCl pH 6.8, 1% 2- mercaptoethanol, 1% sodium dodecyl sulphate, 10% glycerol and 0.01% bromophenol blue) at a 1:5 ratio, boiled for 5 min, microfuged for 1 min and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a Mini Protean II cell (Bio-Rad, Hercules, CA, USA) at 60 mA for 1 h at ambient temperature. Gels with samples of serum were run in duplicate in all cases. Gels for immunoblot analyses were separated electrophoretically and transferred to a nitrocellulose membrane by applying a current of 20 V at ambient temperature for 30 min. Blots were treated with blocking solution (20 mM Tris, 0.9 NaCl, 10% non-fat milk) for 3 h at ambient temperature and then reacted with a 1:2000 dilution of anti-sarcomeric actin monoclonal antibody (Alpha-Sr-1 Clones, Dako, Glostrup, Denmark). Primary antibodies were incubated overnight at 4 °C. Membranes were washed (15 min in 5% Tris-buffered saline with Tween) and incubated with horseradish peroxidase conjugated goat anti-mouse IgM (whole molecule) conjugated with type VI horseradish peroxidase (1:1000, Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at ambient temperature, followed by additional washes (15 min in 5% Trisbuffered saline with Tween). Proteins were visualized by enhanced chemiluminescence (Bonus, Amersham, Little Chalfont, UK).

Densitometric analysis.—It was performed by scanning radiographic images of membranes. Image resolution was 100 points per inch. Image treatment software (Adobe Photoshop 5.0, Adobe Systems, San Diego, CA, USA) was used to treat images, which were stored in TIF format to allow them to be accessed by the software for quantification. This method yielded digital images of 10 wells: one for positive control (pure actin), another for molecular weight (Prestained SDS-PAGE Standard, Low Range, Bio-Rad Laboratories, Hercules, CA, USA) and the remaining eight for the study samples. Quantity One 1-D Analysis Software for densitometric image analysis (Bio-Rad) was used to compare the different wells with the positive control. This method was used to calculate absolute a-actin values in all study samples.

Statistical analysis

Data are expressed as mean (M)±standard error of mean (SEM) in tables and figures, whereas mean of the difference (MD) and standard error of the difference (SED) were used in the results text. A one-way ANOVA test was used to compare the mean values of oxidative stress, antioxidant defense, muscle damage, and body composition between the three experimental groups. *Post-hoc* contrasts were performed by the Bonferroni Test at P=0.016 significance level. All statistical procedures were carried out using PASW V.22 (SPSS for Windows, Chicago, IL, USA).

Results

Oxidative stress response and muscle damage

MDA concentrations and nDNA-damage of the peripheral lymphocytes as biomarkers of oxidative stress response induced by lifelong physical activity practice were presented in Figure 1. No statistical differences were shown in MDA concentrations between the three experimental groups. However, the amateur group presented the lowest values without statistical differences. Nuclear-DNA-damage expressed as % Tail DNA-damage was significantly lower in the recreational group than in the sedentary group (MD=5.53±1.70, P=0.013),

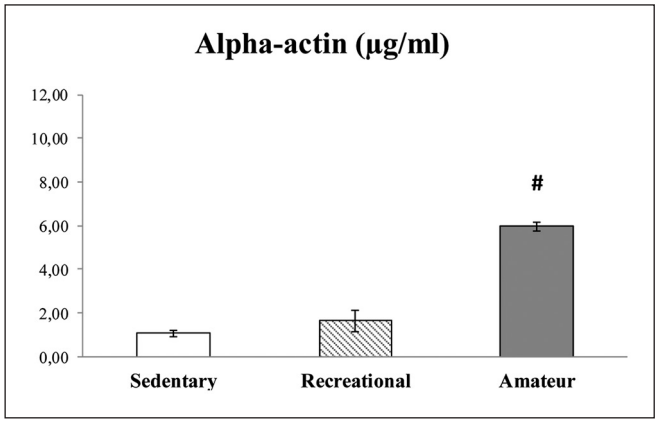

Figure 1.—Oxidative stress response represented by the levels of MDA, nDNA-damage (% tail DNA-damage) and enzymatic activity of GPx in the different groups.
Data are expressed mean (M)±standard error of the mean (SEM).
*Statistical differences between amateur and sedentary; $ statistical differences between amateur and recreational; # statistical differences between all groups. Bonferroni Test at P=0.016

and Amateur group (MD=5.61±1.62, P=0.008). The amateur group presented trends toward to higher GPx antioxidant activity than in the rest of groups analyzed (sedentary group: MD=8.63±3.53, P=0.075; recreational group: MD=7.81±3.04, P=0.058). Serum alpha-actin release as a specific sarcomeric damage marker was represented in Figure 2. The amateur group showed statistical higher values than the recreational (MD=4.34±0.46, P<0.001) and sedentary groups (MD=4.89±0.46, P<0.001) respectively.

Body composition

Descriptive characteristics of the sample, BMI, fat mass, muscle mass and bone mass are shown in Table I. BMI and bone mass (%) values were similar between groups. However, statistical differences were observed related to fat mass (%) and muscle mass (%). The sedentary group presented statistically higher fat mass than the amateur group (MD=4.19±0.98, P=0.001). The sedentary group showed lower muscle mass than the amateur group (MD=3.67±1.10, P=0.011).

Discussion

Previous evidence has demonstrated that ROS production is associated with loss of muscle mass with age. Thus, this study shows results about how the type of

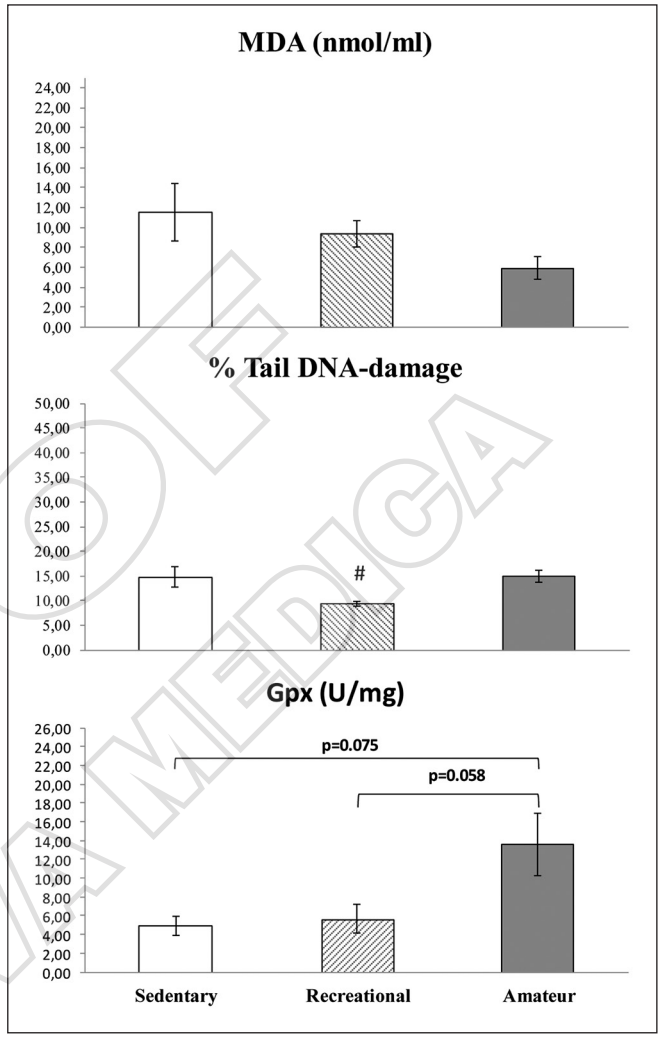

Figure 2.—Skeletal muscle damage represented by the levels of serum alpha-actin release o the different groups. Data are expressed mean (M)±standard error of the mean (SEM).
*Statistical differences between amateur and sedentary; $ statistical differences between amateur and recreational; # statistical differences between all groups. Bonferroni Test at P=0.016.

habitual physical activity for at least 30 years affects the production of blood markers oxidative stress and muscle damage, and the loss of muscle mass in senior adults. In the present study, the MDA concentrations, nDNA-damage, and alpha-actin release were analyzed as biomarkers, which could be associated with body composition in healthy senior adults who have performed different lifelong physical activity practice. The main findings revealed was that cycling amateur lifelong sport practice could attenuate MDA production,

improve enzymatic antioxidant activity, and maintain higher alpha-actin liberation compared with lifelong recreational practice or a sedentary lifestyle. In addition, an improved muscle mass was evident in the Amateur group compared to the Sedentary group.

Regarding oxidative stress markers, in our study, MDA levels showed greater attenuation in the amateur athletes than in the rest of the study groups. Accordingly, several studies support that an optimal physical fitness in adult and older ages resulted in a decreased in lipid peroxidation levels.[20, 21] Moreover, sedentary lifestyle have been associated to a major vulnerability of the body to oxidative stress.[22] In fact, in our study, the sedentary group showed higher values of MDA than recreational and amateur groups. A recent study [23] showed similar results concluding that a lifelong endurance training attenuated the age-related elevation in oxidative stress levels compared with untrained. In the study mentioned above, a similar group of amateur cyclists (>55 years old) was evaluated, although the biomarkers to analyze oxidative stress response were different from our study.

Another oxidative stress biomarker analyzed in this study was n-DNA-damage, which was significantly lower in the Recreational group than in Amateur and Sedentary groups. According to this point, previous studies evidenced that chronic moderate-intensity exercise could improve the up-regulation of antioxidant genes with a corresponding decrease of DNA-damage.[24] However, the Amateur group showed higher n-DNA-damage levels than the Recreational group and similar with respect to the Sedentary group. In respect to this, the n-DNA-damage is usually greater in individuals with an optimal aerobic physical fitness such as Amateur cyclist due to the acute effects of daily physical exercise.[25] Additionally, the endurance exercise high intense bouts, such as amateur competitions, induce DNA-damage[26], with a decrease of this damage after 24 hours,[27] or even in more days.[28] Other studies reported that an increase in DNA-damage markers could be associated with a signaling pathway to modulate the DNA repair gene expression, and the improvement of antioxidant defenses at nuclear level induced by chronic endurance training.[29] All these studies could explain the high n-DNA-damage level in the Amateur group. However, the greater nDNA-damage in the Sedentary group could be a persistent damage due to a weak antioxidant response derived from physical inactivity.[30]

Regarding to the antioxidant response, the GPx antioxidant activity was higher in the Amateur cyclist's group than in the other groups. According to this, several studies have demonstrated that aerobic training improves antioxidant defenses.[31, 32] Likewise, a recent study where 3 groups with different physical fitness level were compared (unfit group, low physical fitness level group and high physical fitness level group) concluded with a greater GPx activity at basal conditions in the high physical fitness group who were represented by members of a Federation of Cyclotourisme with more than 10 hour of training per week similar to the Amateur group in our study.[33] Thereby, in our study, the high enzymatic activity of GPx in the Amateur group may be linked to the level of training and the adaptation to chronic exercise at this level.

Regarding to alpha-actin, the Amateurs showed significantly higher levels of this skeletal muscle marker than Recreational and Sedentary groups. In this sense, as it has been demonstrated by Martinez-Amat *et al.*,[19] this is a peculiar muscle damage of myofibrils due to an exercise adaptation without being regarded as muscle damage typical of a sport injury. Skeletal muscle damage in sport injury is considered by these authors above 10.49 μgr/mL of serum alpha-actin, and in our study, the average of serum alpha-actin release in amateur athletes is below this concentration (M=5.96±0.21). Therefore, the significant higher levels of alpha-actin in the Amateur than in the Recreational and in Sedentary groups could be due to a hypertrophic response depending on the level of physical activity performed,[34] since repetitive contractions in a high demand sport activity lead to the break of sarcomeres generating adaptive training micro-traumas.[35]

As it was expected, body composition data revealed that amateur athletes showed a significant higher muscle mass than the sedentary group. In addition, fat mass was lower in the amateurs than in the sedentary group. All these findings about body composition could be associated with the physical activity practice done along the life, since it has been clearly established that increased ROS production induced by inactivity significantly contributes to muscle atrophy.[36] Moreover, there is an important mechanism for muscle-specific adaptations induced by chronic endurance training.[37] Habitual

endurance training could protect skeletal muscle from chronic damage to macromolecules, and may preserve muscle and force elderly.[38] In addition, a recent review affirmed that aerobic exercise such as cycling training preserves the hypertrophy mechanisms in seniors sportsmen.[39] Thereby, all these previous studies could explain the more proper response to oxidative stress, muscle hypertrophy mechanisms, and muscle mass revealed by amateur athletes in our study compared with recreational and sedentary people. Hence, in the present study we believe that the low muscle mass showed in the sedentary group could be related to the lower physical activity levels, higher levels of oxidative stress, lower antioxidant enzymatic activity, and low release of alpha-actin reported by this group.

Limitations of the study

The present study had some limitations. This is a cross-sectional study with a small sample, although interesting differences were found. Notwithstanding, the characteristics of the participants provide quality to the study and reply to the goal. The sample size is limited due to the characteristics of the participants: people over 55 years old who had performed the same the lifelong practice of physical activity (30 years), and they did not present any specific physical/disease issues or taking antioxidants supplements. Additionally, the sample size of our study had sufficient statistical power to show significant differences in the Bonferroni test adjusted at significance level less or equal to 0.0016 P value. Likewise, the present study encompasses some strengths, such as that various biomarkers of cellular oxidative response (oxidative stress and enzymatic antioxidant defense) were analyzed together with a specific muscle skeletal damage marker such as alpha-actin. This is a specific protein of sarcomeric origin,[19] which could demonstrate the real function of muscle sarcomeres that are very important to consider during the hypertrophy process, and therefore, in the maintenance of muscle mass.

Conclusions

In summary, a lifelong amateur endurance practice, that implies a potential physiological demand, could attenuate lipid peroxidation, increase antioxidant defense, and maintain optimal levels of rupture of sarcomeres (muscle microtraumas), which are characteristic of the process of muscle hypertrophy, and thus prevent muscle loss associated with aging and physical inactivity at long-term. These results can lead to new support to lifelong amateur endurance training since it may improve age-related oxidative stress response and preserve optimal hypertrophy mechanisms to keep the ideal muscle mass at advanced adult ages.

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Conflicts of interest.—The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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II

DOES SWIMMING AT A MODERATE ALTITUDE FAVOR A LOWER OXIDATIVE STRESS IN AN INTENSITY-DEPENDENT MANNER?

Casuso RA, Aragón-Vela J, López-Contreras G, Gomes SN, Casals C, Barranco-Ruiz Y, Mercadé JJ, Huertas JR

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Does Swimming at a Moderate Altitude Favor a Lower Oxidative Stress in an Intensity-Dependent Manner? Role of Nonenzymatic Antioxidants

Rafael A. Casuso,[1,*] Jerónimo Aragón-Vela,[1,*] Gracia López-Contreras,[2] Silvana N. Gomes,[1] Cristina Casals,[1] Yaira Barranco-Ruiz,[3] Jordi J. Mercadé,[4] and Jesus R. Huertas[1]

Abstract

Casuso, Rafael A., Jerónimo Aragón-Vela, Gracia López-Contreras, Silvana N. Gomes, Cristina Casals, Yaira Barranco-Ruiz, Jordi J. Mercadé, and Jesus R. Huertas. Does swimming at a moderate altitude favor a lower oxidative stress in an intensity-dependent manner? Role of nonenzymatic antioxidants. *High-Alt Med Biol.* 18:46–55, 2017.—we aimed to describe oxidative damage and enzymatic and nonenzymatic antioxidant responses to swimming at different intensities in hypoxia. We recruited 12 highly experienced swimmers who have been involved in competitive swimming for at least 9 years. They performed a total of six swimming sessions carried out at low (LOW), moderate (MOD), or high (HIGH) intensity at low altitude (630 m) and at 2320 m above sea level. Blood samples were collected before the session (Pre), after the cool down (Post), and after 15 minutes of recovery (Rec). Blood lactate (BL) and heart rate were recorded throughout the main part of the session. Average velocities did not change between hypoxia and normoxia. We found a higher BL in response to MOD intensity in hypoxia. Plasmatic hydroperoxide level decreased at all intensities when swimming in hypoxia. This effect coincided with a lower glutation peroxidase activity and a marked mobilization of the circulating levels of α-tocopherol and coenzyme Q10 in an intensity-dependent manner. Our results suggest that, regardless of the intensity, no oxidative damage is found in response to hypoxic swimming in well-trained swimmers. Indeed, swimmers show a highly efficient antioxidant system by stimulating the mobilization of nonenzymatic antioxidants.

Keywords: α-tocopherol; coenzyme Q10; exercise; hypoxia; oxidative damage

Introduction

High-altitude training is a common practice for athletes trying to improve their performance. Long-term performance enhancing effect induced by high-altitude training is mainly due to an enhanced oxygen-carrying capacity, through an increase in hemoglobin (HB) mass (Bonne et al., 2014), which typically occurs above 1800 m of altitude (Millet et al., 2010).

In addition, an increase from low to high altitude induces a number of rapid responses mediated by an enhanced sympathetic drive, which in turn induces a higher heart rate (HR) and cardiac output (Talbot et al., 2005; Siebenmann and Lundby, 2015). The activation of the sympathetic nervous system also leads to an attenuation of whole muscle fatty acid oxidation within the mitochondria while glucose uptake is maintained to support glycolytic flux (Horscroft and Murray, 2014). Thereby, aerobic capacity of the athlete is compromised when exercising in a hypoxic environment (Lundby and Robach, 2015).

Due to the critical role of mitochondria in producing reactive oxygen species (ROS) in response to skeletal muscle contractions (Jackson, 2015), ROS production is increased while exercising in hypoxia (Quindry et al., 2016). The increased shuttles of ROS within the exercise muscle in hypoxia produce oxidative stress, which is exacerbated in an intensity-dependent manner (Quindry et al., 2016). To prevent oxidative damage of biological membranes, excessive ROS production is counteracted by a number of both enzymatic and nonenzymatic antioxidants (Powers et al., 2011).

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In fact, untrained subjects increase their catalase (CAT) and superoxide dismutase (SOD) activity in response to an acute bout of hypoxic exercise (Debevec et al., 2014; Krzeszowiak et al., 2014). However, high training level increases the ability of skeletal muscle to rapidly detoxify ROS produced within the exercised muscle (Finaud et al., 2006; Brooks et al., 2008). Nevertheless, athletes differ in their redox responses to exercise in hypoxia, for example, if compared with runners, swimmers exhibit a higher antioxidant potential during exercise to exhaustion in hypoxia, due to an enhanced nonenzymatic antioxidant activity such as α-tocopherol (Pialoux et al., 2009a, 2009b). This difference can be explained by the involvement of the upper limbs during swimming, as the triceps brachii is the muscle responsible for the propulsive phase of swimming. A recent study showed that in response to a 4-week period of high-intensity training, the triceps brachii shows a greater oxidative challenge than the quadriceps (Larsen et al., 2015).

It can, therefore, be anticipated that well-trained swimmers would rapidly counteract ROS production by a highly developed antioxidant machinery when swimming in hypoxia. However, ROS-induced oxidative stress and the antioxidant response of swimmers to different intensities in hypoxia have not been tested yet.

Since managing training intensities is one of the main problems of coaches when training in hypoxia (Lundby and Robach, 2015), we aimed to describe ROS-related oxidative damage and both enzymatic and nonenzymatic antioxidant responses to swimming at different intensities in hypoxia.

Materials and Methods

Participants

For our study, we recruited 12 swimmers from the University of Granada Swimming Club (6 men and 6 women) who had more than 12 years of swimming practice and enrollment in competitive swimming events for at least 9 years. Swimmers' characteristics were 24 ± 1. 8 years old, 65.8 ± 3.25 kg of weight, 170 ± 2.5 cm of height, and 13.2% ± 1.66% of body fat. After being fully informed of all the procedures and risks of the study, the swimmers gave their written consent to participation. The protocol followed was according to the Declaration of Helsinki and approved by the Ethics Committee of the University of Granada (Granada, Spain).

Study design

Two months before the experimental period, all swimmers were informed about the need to avoid any antioxidant supplementation. Moreover, after 5 days of food recording, the swimmers' diets were evaluated by means of the Spanish food database to ensure normal ingestion of key antioxidants and macronutrients (Mataix et al., 2009). After an independent dietitian declared that all micro- and macronutrients were near nutritional recommendations, all the swimmers were asked to adopt a similar food pattern throughout the study.

The week before the experimental periods, the participants came to the Physiology Laboratory of the Faculty of Sports for anthropometric measurements. Following the guidelines provided by the International Work Group of Kinanthropometry (IWGK), we calculated the percentage of body fat by taking 19 anthropometric measurements, using the average of three measurements for calculations. Body mass index was calculated as weight (kg)/height $(m)^2$.

The experimental period consisted of six training sessions performed over 3 weeks. During the first training session, swimmers performed low-intensity swimming (LOW) and after 3 days of rest they repeated the same LOW swimming session at moderate altitude (hypoxia). The participants then rested for 7 days and repeated the same protocol but at moderate intensity (MOD), repeating the MOD session in hypoxia after 3 days of rest. Finally, after 7 days of recovery, they performed a high-intensity (HIGH) swimming session and repeated it in hypoxia after 3 days of rest. No exercise was allowed during the 3 days between normoxia and hypoxia sessions, and only light swimming was allowed within the 7-day intervals. All swimmers refrained from vigorous physical activity 48 hours before each swimming session.

Swimming sessions

All the swimming sessions were performed in a 50-m swimming pool with the front crawl technique. The normoxia sessions were performed in Granada, Spain, at 630 m above sea level. The hypoxia swimming sessions were carried out at the High Performance Altitude Training Centre of Sierra Nevada (CARD) located in Granada 2320 m above sea level. For the hypoxia training, subjects woke up early in the morning in Granada and travelled to the CARD by bus for 45 minutes. Once in the swimming pool, the participants performed a typical swimming session, which was divided into warm-up, main training, and cooldown periods of 30 minutes each.

Warm-up and cooldown periods were always similar in terms of objectives and tasks (number of sets, number of repetitions, rest, distance, swimming style, and so on) and focused especially on technical drills.

The main training period, however, was divided into three intensities: LOW, MOD, and HIGH. We followed the swimming intensities described by Mujika et al. (1995) as follows: LOW consisted of continuous swimming, maintaining a constant speed as low as possible, with a focus on a perfect technique, with a lactate accumulation around 2 mmol/L (Intensity 1). MOD consisted of 100-m repetitions interspersed with 20 seconds of recovery. The target time for MOD was obtained by halving each swimmer's personal best time of the season in the 200-m freestyle and adding 10 seconds (Intensity 3). HIGH consisted of 50-m repetitions interspersed with 20 seconds of recovery. The target time for HIGH was obtained by dividing by four each swimmer's personal best time of the season in the 200-m freestyle and adding 2 seconds (Intensity 4). During the main set, the average speed was also registered and the time used to cover each lap noted.

Blood lactate concentration and heart rate assessment

HR (Polar Team 2) and blood lactate (BL; KDK Corporation Lactate Pro System, Kyoto, Japan) were recorded before the beginning of each swimming session and during each resting period within the main training. During the LOW session subjects performed continuous swimming, but, given that 10 seconds is enough to accurately measure HR while swimming (Casuso et al., 2014), they were stopped for 10 seconds every 5 minutes for that purpose.

Blood sampling

Before each training session (Pre), immediately after the cooldown period (Post), and after 15 minutes of recovery

(Rec), blood samples (3 mL) were drawn from the antecubital vein with vacutainers. The samples were stored in different containers, one for whole-blood analysis and the other for serum. Hematocrit (HCT) was obtained by microcentrifugation for 10 minutes at 11,000 rpm (BIOCEN). HB was analyzed with Drabkin's method, whereby 20 μL samples of blood were oxidized and quantified spectrophotometrically at 540 nm. After centrifugation, serum was obtained and stored at −80°C until further analysis. All the blood parameters, unless otherwise stated, were normalized to changes in plasma volume (ΔPV). ΔPV at Post and Rec time points were calculated with the Dill and Costill (1974) equation as follows:

$$\Delta PV\ (\%) = 100 \times ([Hbpre/Hbpost] \times [100 - HCTpost]/ [100 - HCTpre] - 1)$$

where pre refers to the basal hematological data within a given intensity (i.e., LOW, MOD, or HIGH) and hypoxic condition (i.e., hypoxia and normoxia), and post refers to the post measurement (i.e., Post or Rec) under the same condition as Pre.

Nuclear DNA damage (comet assay)

We performed the Comet Assay to evaluate nuclear DNA damage (nDNA-damage) in lymphocytes (% DNA-tail damage). Lymphocytes were isolated by gradient centrifugation. The total sample of blood was diluted in phosphate-buffered saline (PBS; 1:1) and centrifuged at 3500 rpm for 30 minutes. After several centrifugations (1750 rpm in an RPMI Histopaque), the top layer of cells (lymphocytes) was removed and diluted in 500 μL of 10% dimethyl sulfoxide (DMSO)-SBF. The mixture was divided into aliquots of 200 μL for subsequent storage in cryogenic boxes. Lymphocytes (50 μL) were then embedded in a thin agarose gel on a microscope slide. Fragments of damaged DNA then migrated away from the nucleus after electrophoresis treatment. After staining with a DNA-specific fluorescent, the gel was read (Komet 5.5). The extent of DNA liberated from the head of the comet is directly proportional to the amount of nDNA damage (Collins, 2004).

Lipid peroxidation

Hydroperoxides (HPx), as a specific and direct biomarker of lipid peroxidation, were measured (Yoshida et al., 2013). Plasma HPx were determined with the Sigma PD1 kit (St Louis, MO). Absorbance changes at 560 nm were monitored spectrophotometrically.

Thiobarbituric acid assay

Plasma thiobarbituric acid (TBAR) concentration is an indirect method of estimating malondialdehyde (MDA) and was measured as described by Orrenius et al. (1976) through the spectrophotometric measurement of substances that react with TBARs. The reaction of MDA-TBA led to orange pigmentation, which was then read spectrophotometrically on a U-bottomed microplate at 532 nm.

Nonenzymatic antioxidant activity

Alpha-tocopherol and coenzyme Q10 content in plasma were quantified as described by Quiles et al. (2005). First, extraction with ethanol–petroleum ether (60:40) by high-performance liquid chromatography (HPLC) equipped with a diode array detector (Beckman Instruments, Inc., Fullerton, CA) and an HPLC column with a reverse-phase C18 Spherisorb ODS1 of 25 × 0.46-cm was performed. For chromatographic separation, a 120 μL sample (150 μL of pure ether plus the extracted antioxidant sample) was injected. The mobile phase used was a mixture of ethanol and doubly distilled water for HPLC in a ratio of 97:3 (air was removed with a vacuum pump). The flow rate was 1 mL/min, and the total duration of the method was 25 minutes. A TM Automatic Waters 717 plus Autosampler (Gen Tech Scientific) was also used. The images were analyzed by chromatograms that were quantified later.

CAT activity

CAT enzymatic activity was measured in erythrocytes by spectrophotometry (Synergy HT Biotek) as described by Ochoa et al. (2003). A sample of erythrocytes (10 μL) was used. The enzymatic activity of CAT was determined by kinetic reaction as spectrophotometrically analyzed. H_2O_2 decomposition was conducted by absorbance decrease at 240 nm. The absorbency difference by unit time was the mean of CAT activity.

Glutation peroxidase activity

Glutathione peroxidase (GPx) activity was measured in erythrocytes by spectrophotometry (Synergy HT Biotek). PBS potassium phosphate buffer (KH_2PO_4 0.1 pH 7.0; 56 μL) containing 0.1 mM EDTA, 19 μL of GSH 99% in bidistilled H_2O, 19 μL of glutathione reductase, and sample erythrocytes of 18 μL were incubated at 37°C for 10 minutes. A GEN 5 program was used in the spectrophotometer. First, a 19 μL sample of NADPH (1.5 mM in 0.1% $NaHCO_3$) was added to monitor the independent chemical reaction of HPx for 3 minutes at 340 nm; immediately a 19 μL sample of 30% H_2O_2 was added for full monitoring of the second enzymatic reaction at 340 nm for 5 minutes. The decrease in concentration of NADPH (Δ[NADPH] min) was calculated from the decrease in absorbance at 340 nm by means of the Lambert–Beer theory and molar extinction coefficient.

SOD activity

SOD activity was determined by the epinephrine method of Misra and Fridovich (1972) based on the inhibition by SOD of cytochrome C reduction. One hundred microliters lysate and 1 mL carbonate buffer were mixed, and then 100 μL of epinephrine was added. Detection was performed at 470 nm.

Skeletal muscle damage

Skeletal muscle damage was determined by serum α-actin quantification as a specific muscle skeletal damage biomarker. Western Blot and densitometric analysis were performed as described previously (Martínez-Amat et al., 2005).

A serum sample (5 μL) from every swimmer was dissolved in Laemmli's sample buffer (62.76 mm Tris-HCl pH 6.8, 1% 2-mercaptoethanol, 1% sodium dodecyl sulfate [SDS], 10% glycerol, and 0.01% bromophenol blue) in a 1:5 ratio, boiled for 5 minutes, microfuged for 1 minute, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini Protean II cell (Bio-Rad Laboratories,

Hercules, CA) at 60 mA for 1 hour at ambient temperature. Gels with samples of serum were run in duplicate in all cases. Gels for immunoblot analyses were separated electrophoretically and transferred to a nitrocellulose membrane by applying a current of 20 V at ambient temperature for 30 minutes. Blots were treated with blocking solution (20 mM Tris, 0.9 NaCl, 10% nonfat milk) for 3 hours at ambient temperature and then reacted with a 1:2000 dilution of anti-sarcomeric actin monoclonal antibody [Actin (C-11): sc-1615; Santa Cruz Biotechnology, Inc.]. Primary antibodies were incubated overnight at 4°C. Membranes were washed (15 minutes in 5% Tris-buffered saline with Tween) and incubated with bovine anti-goat IgG-HRP: sc-2350 (1:5000, Santa Cruz Biotechnology, Inc.) for 1 hour at ambient temperature, followed by additional washes (15 minutes in 5% Tris-buffered saline with Tween). Proteins were visualized by enhanced chemiluminescence (ECL; Bonus, Amersham, United Kingdom).

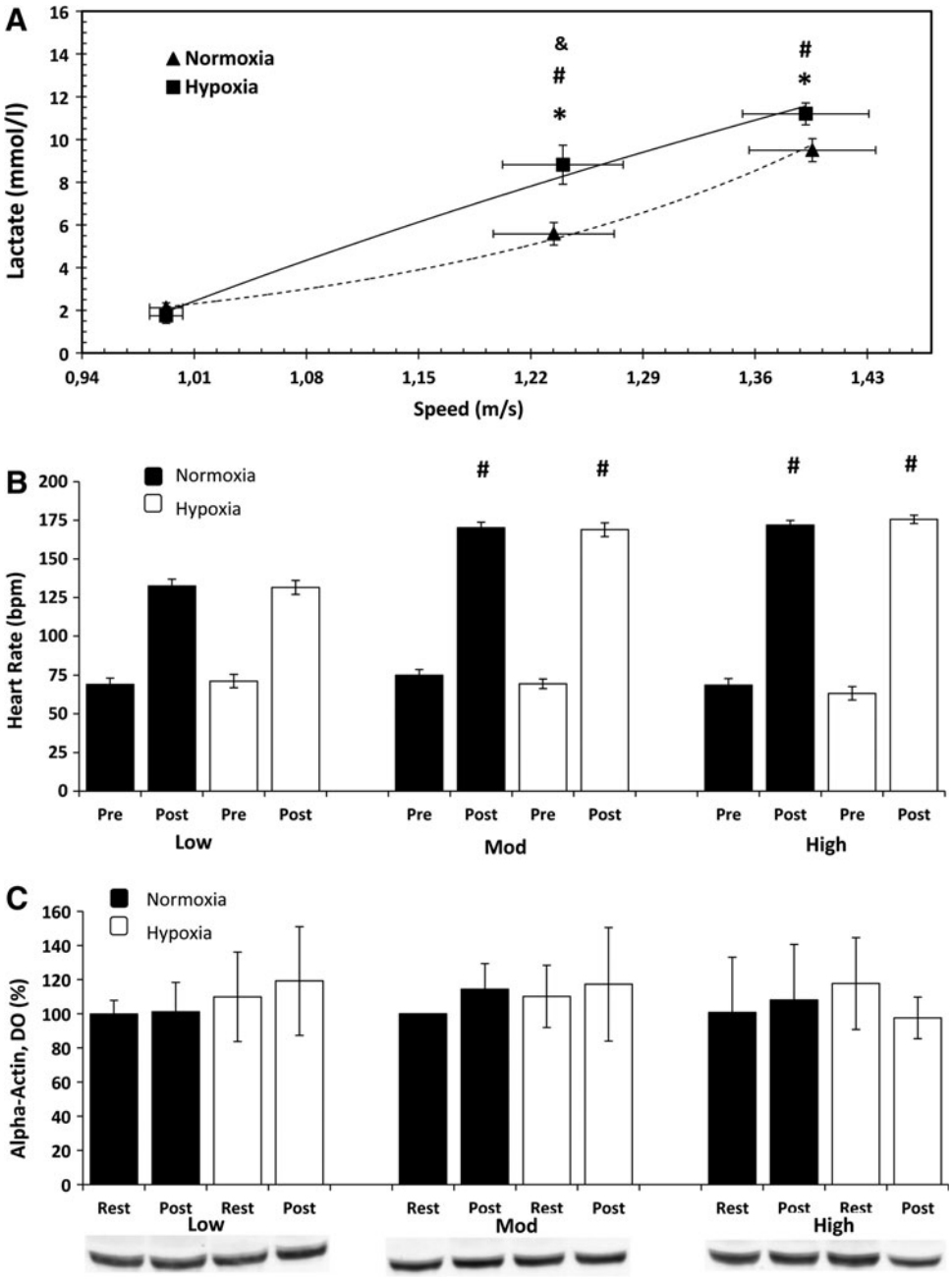

FIG. 1. BL, HR, and serum α-actin in response to swimming intensity and hypoxia. **(A)** Hypoxia induced a higher BL in response to MOD swimming training; this effect occurred despite the fact that the swimming intensity was almost identical between hypoxia and normoxia at all intensities. **(B)** HR was similar before the beginning of all protocols and was lower during LOW compared with MOD and HIGH, but no differences were found between MOD and HIGH. HR was not altered by hypoxia. **(C)** Serum α-actin remained unchanged throughout the time points examined. All the swimming intensities were statistically different between each other ($p < 0.05$). $^{*}p < 0.01$ for BL between normoxia and hypoxia and $^{\#}p < 0.001$ for BL and HR when LOW was compared to MOD and/or HIGH. $^{\&}p < 0.001$ when MOD was compared to LOW and HIGH in normoxia. BL, blood lactate; HR, heart rate; MOD, moderate.

Densitometric analysis was carried out by chemiluminescence (Prestained SDS-PAGE Standard, Low Range; Bio-Rad Laboratories). Light emission was detected with a digital imaging system (Fujifilm Image Analyzer LAS-4000, Tokyo, Japan) and analyzed with Multi Gauge software. The procedure was repeated for each study sample. The digital image obtained was formed of 40 wells: the resting of each subject was used as control. This resting sample was compared with the rest of the samples and expressed as relative change.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS 22.0 for Windows, Inc., Chicago, IL) was used for the statistical comparisons. The normal distribution of variables was tested with the Kolmogorov–Smirnov test. Two-way analysis of variance was carried out, and a Tukey's or Tamhane's T2 *post hoc* test was used to assess the statistically significant differences among the groups. The graphs were generated with Statistica version 7.1 (Stat Soft, Inc., Tulsa, OK). Results are considered statistically significant at $p < 0.05$ and presented as mean ± standard error of the mean. All variables were analyzed for a sex effect, but none of them showed any differences between men and women.

Results

Our protocol was able to discern between swimming intensities ($p < 0.05$) as the average swimming velocities were 1.00 ± 0.004 m/s for LOW, 1.24 ± 0.026 m/s for MOD, and 1.39 ± 0.031 m/s for HIGH. These intensities were almost identical between normoxia and hypoxia (Fig. 1A). Although there was a lower HR when LOW was compared with MOD and HIGH, no difference was found between MOD and HIGH; in addition, hypoxia did not modulate HR either at rest or while swimming (Fig. 1B). BL increased in MOD when compared with LOW and further increased during HIGH if compared with MOD (Fig. 1A).

During MOD hypoxic training we found a higher BL when compared with MOD normoxic swimming (Fig. 1A). This effect was not owed to an increased hemoconcentration as hypoxia did not significantly alter plasmatic volume change (Table 1). Moreover, no skeletal muscle damage was associated either with hypoxia or with swimming intensity as α-actin release to serum was similar among LOW, MOD, and HIGH at both normoxia and hypoxia (Fig. 1C).

Both TBARs and HPx were measured as markers of lipid peroxidation. We found that swimming intensity did not alter those parameters as they were similar during LOW, MOD, and HIGH (Fig. 2A, B). There were slight differences, however, between those parameters in response to hypoxic swimming. Regarding TBARs, we found a lower value Post Low in hypoxia compared with normoxia. Moreover, we found a lower basal value for TBARs before the HIGH hypoxic training (Fig. 2A). On the contrary, HPx were downregulated in hypoxia in response to LOW at Post and in response to MOD and HIGH at Post and Rec (Fig. 2B). It should be noted that HPx were downregulated by the hypoxic environment *per se* as we found lower HPx before the first hypoxic training. Regarding DNA damage to lymphocytes, we found no effect in percentage of DNA tails or heads in response to normoxia or hypoxia (Table 1).

In normoxia, SOD activity (Fig. 3A) decreased during HIGH at all the time points examined, thus suggesting that the antioxidant activity decreased as swimming intensity

Table 1. Hematological Parameters, DNA Oxidative Damage, and Serum Antioxidant Markers

	Low		*Moderate*		*High*	
	Normoxia	*Hypoxia*	*Normoxia*	*Hypoxia*	*Normoxia*	*Hypoxia*
HGB (g/100 mL) Pre	14.22 ± 0.54	14.72 ± 0.68	14.07 ± 0.49	14.89 ± 0.32	14.11 ± 0.52	15.06 ± 0.38
HGB (g/100 mL) Post	13.75 ± 0.49	14.91 ± 0.70	14.11 ± 0.38	14.70 ± 0.47	13.96 ± 0.44	14.83 ± 0.44
HGB (g/100 mL) Rec	13.59 ± 0.58	14.69 ± 0.63	13.94 ± 0.38	15.86 ± 0.60	14.15 ± 0.52	13.94 ± 0.37
HCT (%) Pre	41.50 ± 1.13	43.37 ± 1.57	45.17 ± 1.72	45.54 ± 1.36	43.60 ± 1.37	45.90 ± 1.61
HCT (%) Post	42.75 ± 1.47	45.75 ± 1.91	44.92 ± 1.65	46.54 ± 1.38	45.00 ± 1.32	47.20 ± 1.47
HCT (%) Rec	42.13 ± 1.60	45.25 ± 1.61	44.75 ± 1.63	45.18 ± 1.38	44.30 ± 1.55	44.90 ± 1.33
PVΔ (%) Post	1.4 ± 3.41	–6.1 ± 3.10	0.6 ± 3.33	0.32 ± 3.62	–1.2 ± 3.00	0.0 ± 4.08
PVΔ (%) Rec	3.8 ± 2.99	–2.8 ± 2.11	1.7 ± 1.85	–4.2 ± 3.98	–0.7 ± 4.47	11.3 ± 5.21
Comet (%) Tail Pre	13.97 ± 2.66	18.17 ± 1.63	23.19 ± 4.03	21.55 ± 3.01	20.55 ± 1.91	17.01 ± 0.82
Comet (%) Tail Post	16.43 ± 3.04	18.38 ± 1.50	24.68 ± 3.16	24.57 ± 1.59	20.70 ± 2.17	18.83 ± 1.42
Comet (%) Tail Rec	16.48 ± 2.72	18.37 ± 1.46	20.72 ± 1.66	22.80 ± 0.87	22.20 ± 1.93	17.39 ± 1.40
Comet (%) Head Pre	86.03 ± 302	81.52 ± 1.46	76.81 ± 4.74	78.45 ± 3.32	79.45 ± 1.58	82.99 ± 0.64
Comet (%) Head Post	84.43 ± 2.95	81.65 ± 1.49	75.23 ± 3.36	75.16 ± 2.78	79.30 ± 2.49	81.17 ± 1.87
Comet (%) Head Rec	83.23 ± 2.98	81.28 ± 1.58	79.29 ± 3.41	77.20 ± 2.01	77.80 ± 1.74	82.63 ± 1.41
CAT activity Pre	0.10 ± 0.02	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.01
CAT activity Post	0.09 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.07 ± 0.00	0.06 ± 0.01	0.07 ± 0.01
CAT activity Rec	0.10 ± 0.01	0.09 ± 0.05	0.10 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.06 ± 0.00
GRX (U/mg) Pre	10.65 ± 0.84	8.58 ± 0.95[a]	10.80 ± 0.42	8.50 ± 1.10[a]	8.60 ± 0.67	5.40 ± 0.30[a]
GRX (U/mg) Post	8.75 ± 0.99	8.66 ± 0.48	9.20 ± 0.85	8.50 ± 0.87	7.00 ± 0.60	5.40 ± 0.42
GRX (U/mg) Rec	9.72 ± 0.52	8.34 ± 0.71	9.70 ± 0.50	8.00 ± 0.80	8.20 ± 0.84	6.90 ± 0.18
RET (nmol/mL) Pre	4.16 ± 0.60	4.08 ± 0.33	4.17 ± 0.64	4.06 ± 0.33	3.37 ± 0.56	4.87 ± 0.88
RET (nmol/mL) Post	4.65 ± 0.51	3.38 ± 0.55	4.79 ± 0.62	3.53 ± 0.50	3.86 ± 0.37	5.30 ± 1.12
RET (nmol/mL) Rec	5.00 ± 0.55	4.78 ± 0.68	5.14 ± 0.59	4.60 ± 0.55	3.33 ± 0.48	5.39 ± 1.02

[a]$P < 0.05$, compared with normoxia at the same intensity.

CAT, catalase; GRX, glutation reductase; HCT, hematocrit; HGB, hemoglobin; PVΔ, plasmatic volume change; Rec, recovery; RET, retinol plasmatic concentration.

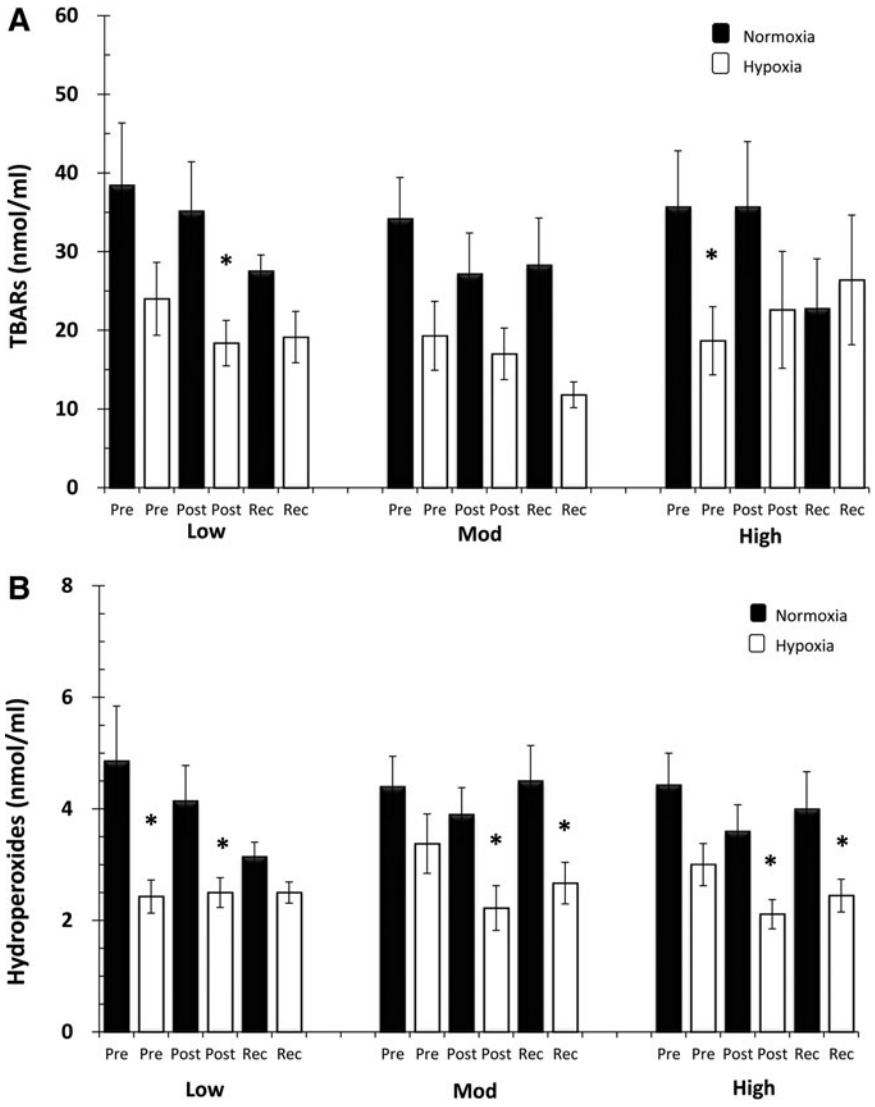

FIG. 2. Serum oxidative stress markers in response to swimming intensity and hypoxia. **(A)** Hypoxia induced lower TBARs in response to LOW, MOD, and HIGH; this effect was extended to the Rec period after MOD. **(B)** HPx were quantified as a direct marker of lipid peroxidation. Hypoxia induced lower HPx in response to LOW and MOD. It should be noted that HPx was reduced before the beginning of the LOW swimming training, but not before MOD and HIGH. $*p < 0.05$ between normoxia and hypoxia at the same condition and time point. HPx, hydroperoxides; Rec, recovery; TBAR, thiobarbituric acid.

increased. This is in accordance with the results shown for GPx activity (Fig. 3B) as it decreased Post High in normoxia. Moreover, the effects shown in response to HIGH were exacerbated in hypoxia as both SOD and GPx decreased their basal levels. Furthermore, GPx activity decreased in hypoxia, compared with normoxia, at Post and Rec during HIGH. Hypoxia decreased the basal level at all the intensities tested of GRx activity (Table 1).

In normoxia, we found lower levels of α-tocopherol during Rec after the HIGH swimming session (Fig. 4A). Moreover, under the normoxic condition, we found that coenzyme Q10 levels dropped in response to HIGH (Fig. 4B). Therefore, nonenzymatic antioxidants seem to be mobilized in a swimming intensity-dependent manner. This effect seems to be maximized in hypoxia, as α-tocopherol and coenzyme Q10 levels were lower during MOD swimming in hypoxia. Moreover, coenzyme Q10 levels dropped in response to HIGH, but no differences were found between normoxia and hypoxia.

Discussion

Our results provide new insights into the mechanisms that regulate oxidative stress during exercise in hypoxia. We found a systemic reduction of the oxidative stress when swimming in hypoxia, as denoted by a marked decrease in lipid peroxidation markers such as HPx and TBARs, and in addition, no DNA oxidative damage was observed. We also found that swimmers exhibit a high modulation of both enzymatic and nonenzymatic antioxidants in response to swimming intensity as demonstrated by coenzyme Q10 and GPx results.

BL is increased in response to hypoxia, which is statistically significant after MOD intensity, whereas skeletal muscle damage and HR are similar between hypoxia and normoxia. The regulation of HR during exercise in hypoxia is a complex process, which has not yet been fully understood. Some studies have reported an increased HR in athletes exercising in hypoxia (Lundby and Van Hall, 2002), which may

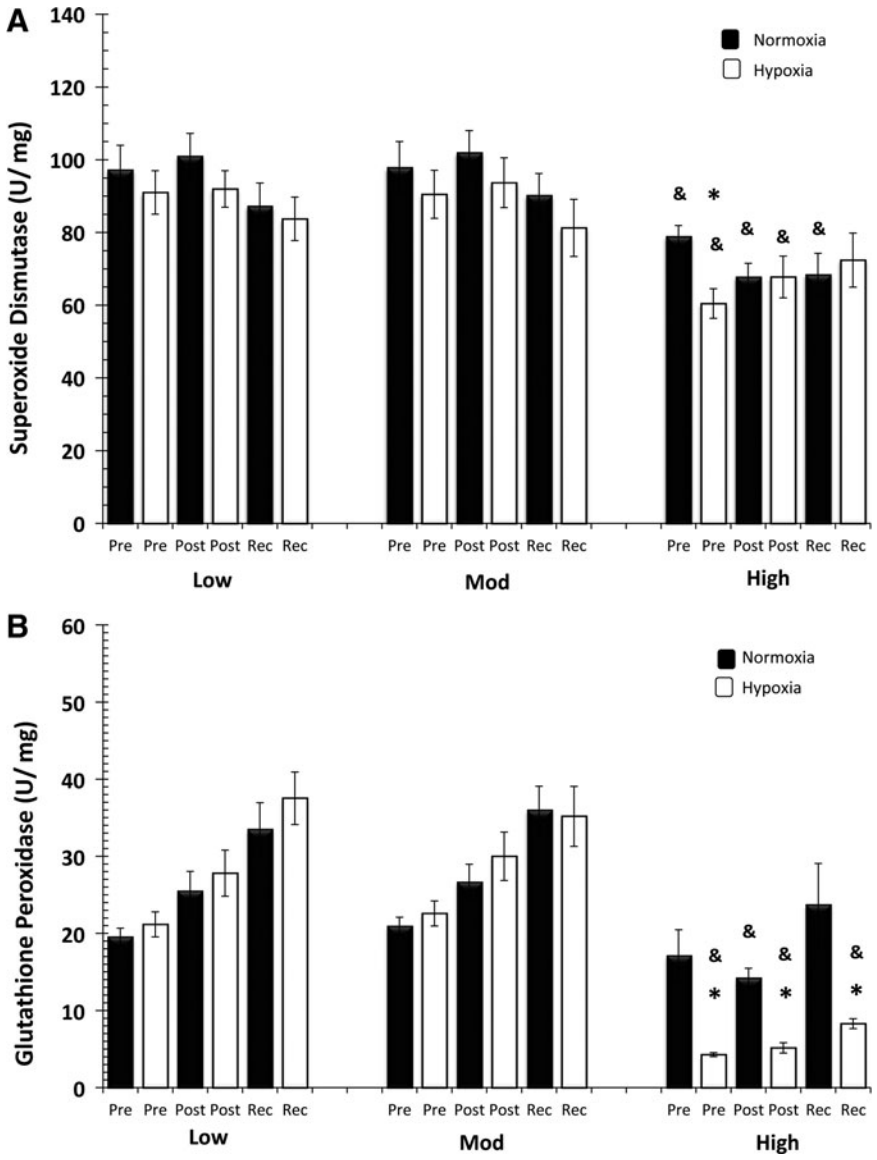

FIG. 3. Serum antioxidant enzymatic activity in response to swimming intensity and hypoxia. **(A)** SOD activity was decreased merely by the absence of a hypoxic environment as it decreased before the MOD and HIGH swimming sessions. On the contrary, swimming modulates SOD activity in a swimming intensity manner both in hypoxia and normoxia. **(B)** GPx showed a similar pattern as in response to the HIGH swimming there was a lower activity in both normoxia and hypoxia; however, the effect was greater for the hypoxic condition if compared with normoxia. $^{*}p < 0.05$ between normoxia and hypoxia at the same condition and time point. $^{\&}p < 0.05$ when compared to the same condition and time point of LOW and MOD. GPx, glutathione peroxidase; SOD, superoxide dismutase.

occur to enhance cardiac output (Talbot et al., 2005). Our results, however, are consistent with those showing no effect of hypoxia on HR (Calbet, 2003). It has been reported that swimming evokes a higher cardiac output than other exercise modes, mainly due to the horizontal position (Lazar et al., 2013), but also due to water immersion (Pendergast et al., 2015); moreover, upper limbs exercise induces a higher heart work than legs (Calbet et al., 2015). Then, moderate hypoxia may be insufficient to further increase cardiac output while swimming.

Moreover, the finding of an increased BL in hypoxia can be explained by an activation of the central nervous system. Indeed, at a given absolute intensity, muscle activation has been recently reported to be higher in hypoxia than in normoxia (Torres-Peralta et al., 2014). Therefore, the higher BL shown in hypoxia without changes in HR can be explained by an activation of the central nervous system, but also because of the nature of swimming.

Swimming mainly involves upper limbs, which have different metabolic responses to exercise than lower limbs. Indeed, mitochondrial responses to training differ between arms and legs (Boushel et al., 2014). Moreover, Larsen et al. (2015) showed that in response to high-intensity training, the triceps brachii shows a greater oxidative stress than the quadriceps, leading to a robust expression of mitochondrial antioxidant enzymes.

Our data on SOD and GPx activity show that the training of the upper limbs by swimming for years may lead to a significant enzymatic antioxidant efficiency. However, there are few studies aiming to describe oxidative stress in well-trained swimmers; it has been reported that adolescent swimmers show an oxidative damage to DNA and lipids in response to

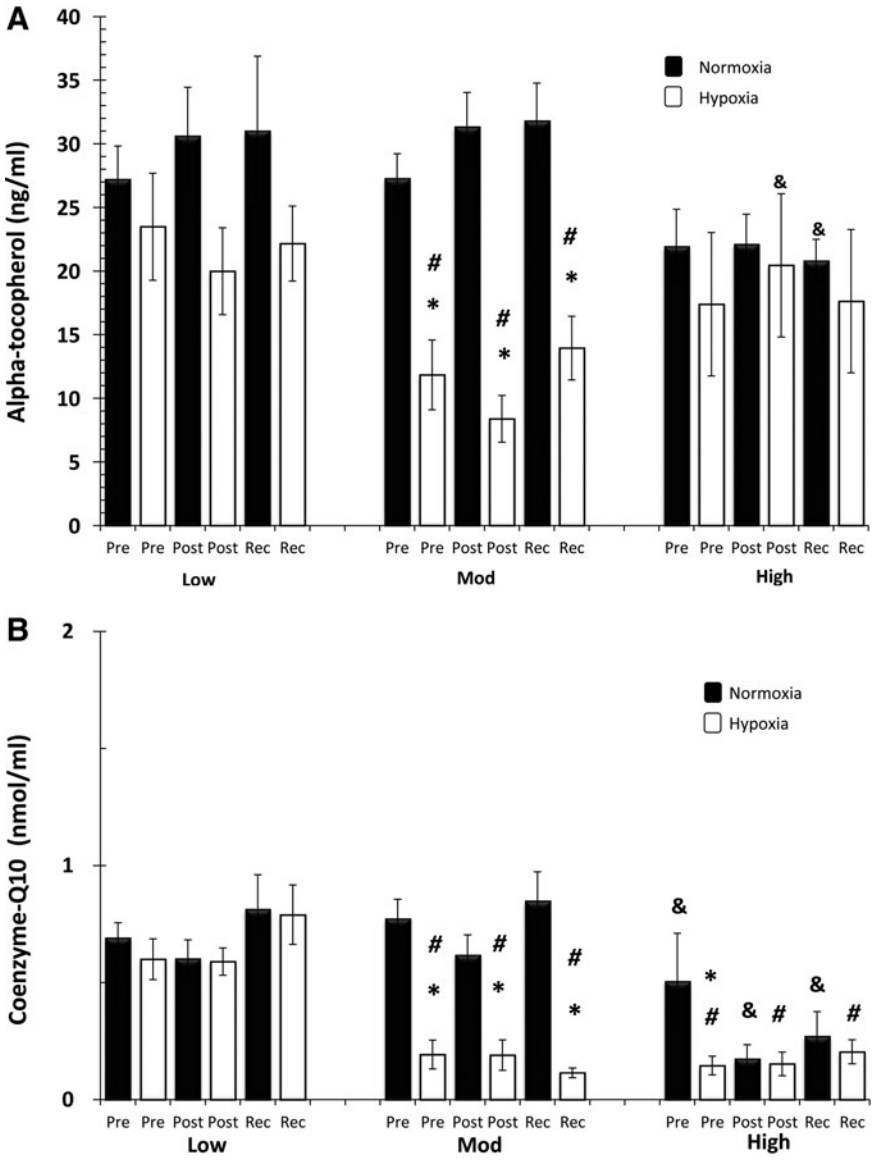

FIG. 4. Serum α-tocopherol and coenzyme Q10 in response to swimming intensity and hypoxia. **(A)** HIGH swimming induced a decreased α-tocopherol concentration in normoxia. Although α-tocopherol was not modulated in response to MOD if compared to LOW in normoxia, hypoxic MOD training showed a strong downregulation of the circulating levels of α-tocopherol when compared with LOW, but also if compared with MOD normoxic swimming. **(B)** Coenzyme Q10 was lower in response to HIGH swimming in normoxia, a modulatory effect which was maintained during hypoxic HIGH training. In response to MOD, there was a powerful effect on coenzyme Q10 toward a lower concentration induced by hypoxia. $^{*}p < 0.05$ between normoxia and hypoxia at the same condition and time point. $^{\#}p < 0.05$ when compared to LOW at the same condition and time point, and $^{\&}p < 0.05$ when compared to the same condition and time point of LOW and MOD.

an acute sprint swimming session (Kabasakalis et al., 2014), but also in response to moderate swimming intensity (Tauler et al., 2008). In contrast, adult swimmers subjected to high-intensity swimming show a rise in circulating TBARs rapidly counteracted by the antioxidant defenses (Deminice et al., 2010). Moreover, highly experienced swimmers are able to efficiently regulate a redox homeostasis during ultra-long duration swimming (Kabasakalis et al., 2011). These data suggest that as the swimming training status increases, there is a higher protection against oxidative stress induced by each swimming session.

Moreover, our results show a fall in the circulating levels of α-tocopherol and coenzyme Q10 in a swimming intensity manner. This result is consistent with the lower plasmatic α-tocopherol level ($\sim 2\%$) shown after an ''all out'' swimming session in highly trained swimmers (Deminice et al., 2010). Therefore, our results support the hypothesis that highly trained swimmers show an enhanced enzymatic and nonenzymatic antioxidant defense.

The high training status of the swimmers could also explain the unchanged DNA damage in response to hypoxic swimming. Indeed, training increases the resistance of lymphocytes to oxidant-induced DNA damage (Niess et al., 1996). Moreover, lipid peroxidation significantly decreases in response to all the swimming intensities in hypoxia and this effect is achieved despite a lower antioxidant activity. Before the first hypoxic swimming session, we found a nonsignificant $\sim 15\%$ α-tocopherol decrease but a significant

decrease of HPx if compared with the basal levels. This effect is even more marked after exercise, especially after hypoxic MOD intensity.

The mobilization of α-tocopherol from circulation to mitochondrial membranes has been previously reported to protect mitochondria from excessive ROS production (Mataix et al., 1998; Quiles et al., 1999). Previous studies conducted on elite swimmers and runners, who exercised in a hypoxic environment, also reported that swimmers mobilize more α-tocopherol in response to the same exercise intensity (Pialoux et al., 2009a, 2009b). The higher α-tocopherol mobilization, if compared with normoxic training, in response to the hypoxic LOW (∼30%) and MOD (∼65%) training intensity, may reflect an acquired mechanism to protect their biological membranes. This effect seems to efficiently work from low to moderate intensities. However, as the hypoxic swimming intensity increases, this mechanism may not be enough to counteract the further increase in ROS production (Quindry et al., 2016). At this point, as evidenced by MOD and HIGH hypoxic data, coenzyme Q10 is also mobilized to block excessive oxidative stress. Indeed, coenzyme Q10 has a double side effect, by blocking free radical production and by regenerating other antioxidants such as α-tocopherol under stressful conditions (Quiles et al., 1999).

The mechanism by which GPx and SOD activity is decreased in an intensity-dependent hypoxic training and the implications for swimming adaptations remain unclear. It has been reported that 4 weeks of high-intensity training attenuates mitochondrial function within upper limbs by a severe increase of oxidative stress, which, in turn, leads to a robust antioxidant enzyme expression (Larsen et al., 2015). Then, it could be possible that years of swimming exercise may improve the ability of nonenzymatic antioxidants to mobilize in response to oxidative stress, thus allowing proper cellular function and expression of antioxidant proteins. However, this hypothesis will require further investigations to be proved.

The present study has some limitations that should be mentioned. First, we found that hypoxia decreases basal levels of SOD, α-tocopherol, and coenzyme Q10 before MOD intensity. Hypoxia also decreases GPx, SOD, TBARs, and coenzyme Q10 before HIGH intensity; this effect could be because we did not randomize the swimming sessions. However, the fact that HPx and GRx also decrease before the LOW hypoxic session strongly suggests an effect induced by hypoxia *per se*. Another potential constraint is because the blood sample was not obtained immediately after exhaustion, since a 30-minute cooldown period was included. However, this can also be considered a strength because we tested the redox status under a real situation of swimming training.

Conclusions

Hypoxic swimming does not induce oxidative damage in well-trained swimmers, as evidenced by a decrease in the circulating levels of HPx and unchanged DNA damage. This effect is likely induced by a mechanism that stimulates the mobilization of nonenzymatic antioxidants, allowing an efficient antioxidant defense in an intensity-dependent manner.

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Author Disclosure Statement

No competing financial interests exist.

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III

CONTROL OF ANTIOXIDANT SUPPLEMENTATION THROUGH INTERVIEW IS NOT APPROPRIATE IN OXIDATIVE-STRESS SPORT STUDIES: ANALYTICAL CONFIRMATION SHOULD BE REQUIRED

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Control of antioxidant supplementation through interview is not appropriate in oxidative-stress sport studies: Analytical confirmation should be required

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ABSTRACT

Objective: Controlling antioxidant supplementation in athletes involved in studies related to oxidative stress and muscle damage is the key to ensure results. The aim of this study was to confirm through high-performance liquid chromatography (HPLC) analysis whether well-trained individuals lied during a personal interview when asked if they were taking supplements with antioxidants, and how this could affect oxidative stress, muscle damage, and antioxidant response.
Methods: A total of 94 men, well trained in endurance sports, volunteered in this study. They denied taking any antioxidant supplementation at initial interview. After a HPLC analysis, abnormal α-tocopherol concentrations were detected, probably due to a hidden antioxidant supplementation. Participants were classified into two groups: no evidence of antioxidant supplementation (NS group = α-tocopherol values <80 nmol/mL; n = 75) and evidence of antioxidant supplementation (S group = α-tocopherol values >80 nmol/mL; n = 19). Lipid peroxidation, muscle damage, antioxidant enzyme activity, and nonenzymatic antioxidant content were analyzed according to this classification. Statistical comparisons were performed using Student's *t* test.
Results: The α-tocopherol concentrations were significantly higher in the S group than in the NS group (MD = 725.01 ± 39.01 nmol/mL; $P = 0.001$). The S group showed a trend toward lower hydroperoxides than the NS group (MD = 1.19 ± 0.72 nmol/mL; $P = 0.071$). The S group showed significantly lower catalase activity than the NS group (MD = 0.10 ± 0.02-seg-1 mg-1; $P < 0.01$). Skeletal muscle damage markers did not differ between experimental groups.
Conclusions: Data from the present study reveal that 20% of participants lied in the exclusion criteria of antioxidant supplementation in a personal interview, as they showed high plasmatic α-tocopherol concentrations after HPLC verification. Catalase activity seems to be affected by high α-tocopherol plasma levels. Therefore, we strongly recommend the HPLC analysis as a necessary tool to verify the antioxidant intake and preserve results in studies linking oxidative stress and sport.

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Introduction

In studies measuring the oxidative stress induced by exercise and/or nutrition, researchers must take into account the intake of antioxidant supplements as exclusion criterion as this supplementation can alter results [1]. Despite several validated qualitative instruments to evaluate the supplementation [2,3], data collected using a self-report questionnaire might not be sufficient for obtaining reliable information. Although the personal interview seems to be reliable [4], it is susceptible to error [5]. Specifically, in sport studies, the majority of athletes and well-trained individuals do not feel comfortable discussing this topic with researchers. Thus, they might lie or they might not clearly express their opinions despite confidentiality guarantees [6]. Thus, studies in which is necessary to control the antioxidant intake may be subject to error if only indirect instruments such as interviews or questionnaires are used.

Moreover, the intake of antioxidant supplements should be controlled because there is a lack of scientific evidence regarding their long-term effects [7]. Although it is established that chronic endurance training generates beneficial effects by improving antioxidant defense and reducing lipid peroxidation [8,9], well-trained individuals commonly continue using vitamins and antioxidant supplements to aid recovery and performance and to improve health [10]. However, most of the studies about antioxidant supplementation in endurance exercise have concluded that there are controversial changes in oxidative response after acute exercise [11,12]. The physiological consequences of vitamin E supplementation in athletes regarding their health, prolonging their life, and improving sports performance is debatable [13,14]. Whereas some studies suggest beneficial effects [15], others showed damage in the cell function [16], no beneficial effects on muscle damage and oxidative stress [17], negative side effects [18], or still unknown long-term effects [19], probably due to the lack of standardized protocols and variety of doses [20].

For that reason, it is important to test the hypothesis that some athletes who respond to an interview in studies about oxidative stress and exercise might answer untruthfully to avoid being excluded because of antioxidant supplement intake. Additionally, this hidden antioxidant supplementation could affect antioxidant capacity, oxidative stress response, and skeletal muscle damage. Thus, a direct and quantifiable method should be used as there might be a significant difference among what athletes say, think, and do, and the results may be contaminated. Hence, the purpose of this study was to confirm through direct measure (high-performance liquid chromatography [HPLC]) whether individuals who are well trained in endurance sports lie during a personal interview when asked the following in the exclusion criterion: “Are you taking supplements with antioxidants?” and how antioxidant supplementation might interfere with oxidative stress, muscle damage, and antioxidant capacity.

Methods

Participants

Ninety-four healthy and well-trained men volunteered to participate in this study. The participants had competed in endurance modalities such as cycling or middle- or long-distance running at the amateur level in sports clubs in Granada (for $\geq$ 5 y). Study participants were in a competitive period at the time of the study. They performed endurance training between 7 and 10 h/wk including the weekend competition. Training loads were similar among the participants, which were ensured by the application of equation previously described [21]. In brief, 2 wk before the study we controlled time taken within each training session and multiplied by the Borg Rating Perceived Exertion scale.

Exclusion criteria included a history of cardiovascular diseases, diabetes mellitus, dyslipidemia, and intake of antioxidant supplements. All participants were informed of the methods of the study, and each provided written informed consent. The study was conducted in accordance with the Helsinki declaration and it was approved by the Ethics Committee of the University of Granada (case No. 417).

Study design

All participants were evaluated in fasting condition on the same day in the Laboratory of Exercise Physiology in the Faculty of Sciences in Physical Activity and Sport (University of Granada). First, they underwent a personal interview in which they had to state whether they were currently consuming any type of antioxidant supplement. Additionally, an initial health report (Par-Q) [22] was completed to check exclusion criteria. Then, an anthropometric evaluation was performed, and a blood sample was obtained to determine further plasma concentration of vitamin E and coenzyme Q10 through HPLC analysis, which verified whether participants had lied during the interview. All participants were evaluated $\geq$72 h after the last training or exercise day.

After HPLC analysis, some abnormal plasmatic α-tocopherol concentrations were detected probably due to an omitted supplementation. In fact, the plasmatic α-tocopherol concentrations showed a multimodal distribution, where the divisor mixing parameter at 80 nmol/mL was established (Fig. 1A). This led us to establish two study groups: a group of participants who showed no evidence of antioxidant supplementation (NS group: α-tocopherol values <80 nmol/mL; n = 75) and a group of participants who showed evidence of antioxidant supplementation (S group: α-tocopherol values >80 nmol/mL; n = 19). This distribution allowed us to study the effect of high plasma concentrations of α-tocopherol on oxidative stress, muscle damage, and antioxidant response (Table 1).

Experimental procedures

Anthropometry

After the initial interview, all participants underwent an anthropometric assessment by skinfold thickness. Body mass index (BMI) was calculated as weight (kg)/height (m)^2. Body fat and muscle mass were calculated using the procedures described in the ISAK method [23]. The body fat mass was calculated by the Faulkner equation [24]. From this former equation, the other body compartments (muscle, bone and residual masses) were obtained using the methodology of four compartments described by De Rose and Guimaraes derived from Matiegka equation [25]. All final measures were expressed in relative terms of percentages.

Blood sampling

Qualified nursing staff drew 3-mL blood samples from the antecubital vein using vacutainers. The samples were deposited in two different containers, one for whole-blood analysis (plasma and erythrocytes) and the other for serum. All samples were centrifuged and frozen (−80°C) waiting further analysis. Specifically plasma nonenzymatic antioxidants were analysed 3 mo after freezing by HPLC analysis.

Biochemical blood analysis

Plasma biochemical parameters (glycemia, cholesterol, high-density lipoprotein, and low-density lipoprotein) were measured spectrophotometrically in the analyzer Biochemist BS-200 (Chemistry Analyzer, Shenzhen Mindray Biomedical Electronics Co., Ltd) using commercial kits home Spinreact (Sant Esteve de Bas, Girona, Spain).

Antioxidant quantification

α-Tocopherol and coenzyme Q10 content in plasma were quantified as previously described [26]. First, an extraction with ethanol-petroleum ether (60:40) by HPLC equipped with a diode array detector (Beckman Instruments, Inc., Fullerton, CA, USA) and an HPLC column with a reverse-phase C18 Spherisorb ODS1 of 25 × 0.46-cm was performed. For chromatographic separation, a 120-μL sample (150 μL of pure ether plus the extracted antioxidants sample) was injected. The mobile phase used was a mixture of ethanol and doubly distilled water for HPLC in proportion 97:3 (air was removed with a vacuum pump). The flow rate was 1 mL/min and the total duration of the method was 25 min. TM automatic Waters 717 plus Autosampler (Gen Tech Scientific, Arcade, NY, USA) also was used. The images were analyzed by chromatograms that were quantified later.

Blood markers of oxidative stress

Hydroperoxides. Plasma hydroperoxides (Hpx), as direct marker of lipid peroxidation, were determined using the Sigma PD1 kit (Saint Louis, MO, USA). Peroxide Detect Kit for the Determination of Aqueous and Lipid Hydroperoxides. Absorbance changes at 560 nm were monitored spectrophotometrically.

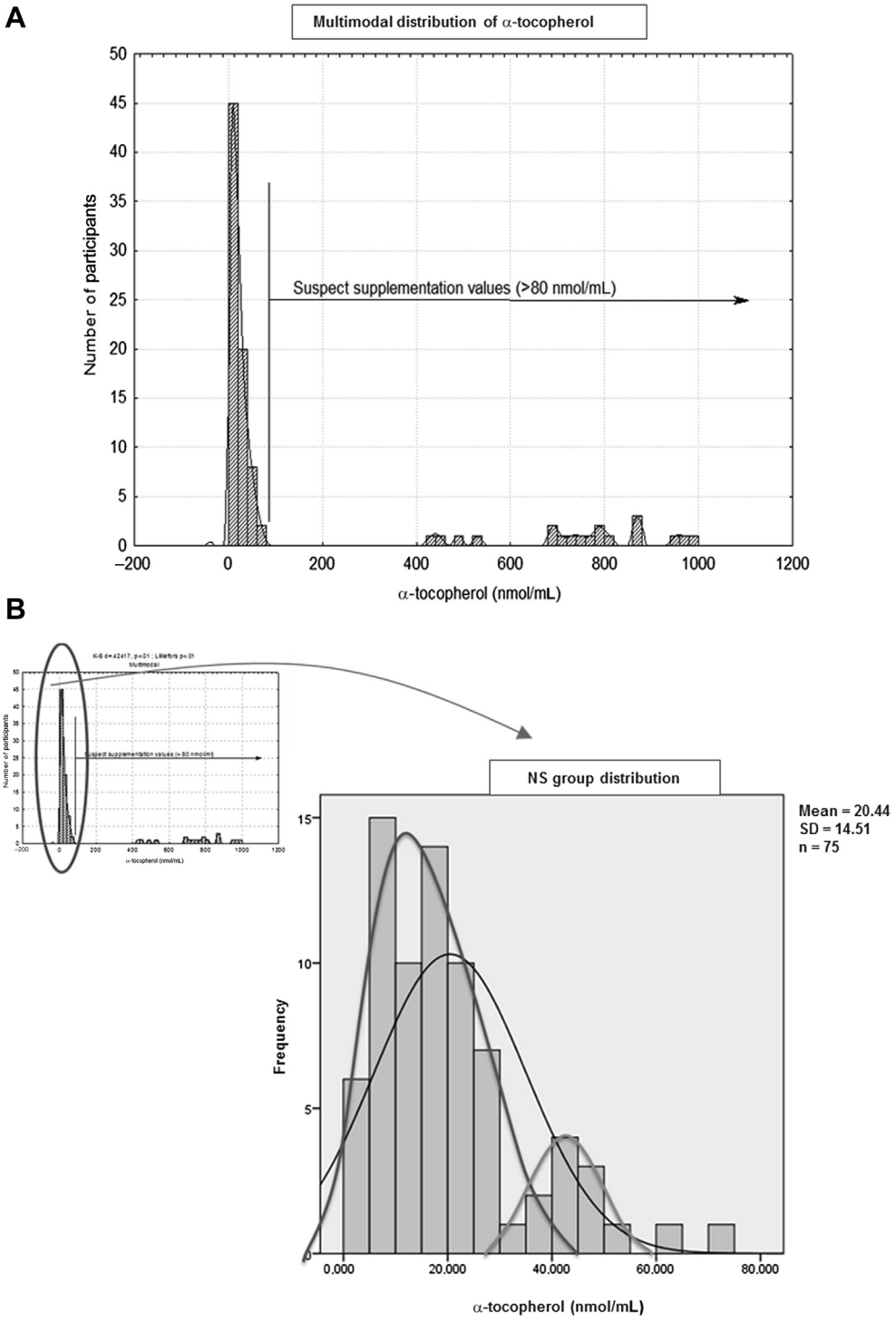

Fig. 1. (A) Multimodal distribution of α-tocopherol concentrations after analysis with high-performance liquid chromatography. (B) α-Tocopherol distribution for the NS group.

Thiobarbituric acid-reactive substances assay. Plasma thiobarbituric acid-reactive substances (TBARSs) assay is an indirect method to estimate the malondialdehyde (MDA) concentration as described by Orrenius in 1997 [27], through the spectrophotometric measurement of substances that react with TBA. The reaction of MDA-TBA led to orange pigmentation, which was then read spectrophotometrically on a U-bottomed microplate at 532 nm.

Skeletal muscle damage

Muscle damage was determined by analysis of several serum biomarkers: lactate dehydrogenase (LDH) and creatine kinase (CK-MB) activities, and α-actin concentrations.

LDH and CK-MB analysis. Samples were centrifuged at 3000*g* for 15 min (at 4°C) to obtain serum. LDH and CK-MB serum activities were measured spectrophotometrically using a commercial test kit (Spinreact, S.A. Girona, Spain, ref: TK41254 for CK-MB and ref: 41220 for LDH) according to the manufacturer's recommendations. CK-MB and LDH values were expressed by U/L.

Alfa-actin quantification was measured through Western blot and densitometric analysis as described previously [28]. In brief, a sample of serum (5 μL) from each study participant was dissolved in Laemmli's sample buffer (62.76 mm Tris-HCl pH 6.8, 1% 2-mercaptoethanol, 1% sodium dodecyl sulphate, 10% glycerol, and 0.01% bromophenol blue) at a 1:5 ratio, boiled for 5 min, microfuged for 1 min, and analyzed by sodium dodecyl sulphate-polyacrylamide

Table 1
Characteristics of the participants who were divided into two groups after analysis of the α-tocopherol quantification by high-performance liquid chromatography analysis

Parameters	NS group* n = 75	S group* n = 19	*P* value
Age, y	42.71 ± 2.57†	31.30 ± 4.68	0.040
Training, h/wk	7.07 ± 0.90	7.50 ± 1.70	0.824
BMI, kg/m^2	25.30 ± 0.51	24.01 ± 0.66	0.206
Fat mass, %	19.28 ± 0.32	18.27 ± 0.99	0.250
Muscle mass, %	46.36 ± 0.32	46.80 ± 0.88	0.563
α-tocopherol, nmol/mL	20.45 ± 1.68	745.45 ± 38.96†	<0.001
Glycemia, mg/dL	87.06 ± 2.56	82.76 ± 3.46	0.470
Cholesterol, mg/dL	182.32 ± 5.97	176.76 ± 14.38	0.699
HDL, mg/dL	41.87 ± 1.29†	34.69 ± 1.64	0.018
LDL, mg/dL	110.40 ± 10.48	94.72 ± 12.67	0.102

BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein
Data presented as mean ± SEM
* NS group: Participants (n = 75) who showed no evidence of antioxidant supplementation (α-tocopherol values <80 nmol/mL). S group: Participants (n = 19) who showed evidence of antioxidant supplementation (α-tocopherol values >80 nmol/mL).
† Statistical differences between the two experimental groups. Student's *t* test was set at $P < 0.05$.

gel electrophoresis in a Mini Protean II cell (Bio-Rad, Hercules, CA, USA) at 60 mA for 1 h at room temperature. Gels with samples of serum were run in duplicate in all cases. Gels for immunoblot analyses were separated electrophoretically and transferred to a nitrocellulose membrane by applying a current of 20 V at room temperature for 30 min. Blots were treated with blocking solution (20 mM Tris, 0.9 NaCl, 10% non-fat milk) for 3 h at room temperature and then reacted with a 1:2000 dilution of antisarcomeric actin monoclonal antibody (Alpha-Sr-1 Clones, Dako, Glostrup, Denmark). Primary antibodies were incubated overnight at 4°C. Membranes were washed (15 min in 5% Tris-buffered saline [TBS] with Tween) and incubated with horseradish peroxidase conjugated goat antimouse immunoglobulin M (whole molecule) conjugated with type VI horseradish peroxidase (1:1000, Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at room temperature, followed by additional washes (15 min in 5% TBS with Tween). Proteins were visualized by enhanced chemiluminescence (Bonus, Amersham, Little Chalfont, UK).

Densitometric analysis. Densitometric analysis was performed by scanning radiographic images of membranes. Image resolution was 100 points per inch. Image treatment software (Adobe Photoshop 5.0, Adobe Systems) was used to treat images, which were stored in TIF format to allow them to be accessed by the software for quantification. This method yielded digital images of 10 wells: one for positive control (pure actin), another for molecular weight (Prestained SDS-PAGE Standard, Low Range, Bio-Rad Laboratories), and the remaining eight for the study samples. Quantity One 1-D Analysis Software for densitometric image analysis (Bio-Rad) was used to compare the different wells with the positive control. This method was used to calculate absolute α-actin values in all study samples.

Enzymatic activity

Superoxide dismutase

For determining the superoxide dismutase (SOD) 1 activity enzyme in erythrocytes, a previously described, slightly modified technique was used [29]. A unit of SOD was defined as the amount of SOD that decreases the rate of reduction of cytochrome C (CC) by 50%, a xanthine-xanthine oxidase (XO) controlled system. First, the amount of XO that reduces CC 100% (amount of XO was an increase of between 0.025 and 0.05 absorbance) was calculated. Samples of 30 μL xanthine, 30 μL CC, and relationship-sample buffer with 30 μL of XO were used. Then, it was proceeded to the fast spectrophotometric reading, at 550 nm, 25°C for 3 min with intervals of 15 s.

Glutathione peroxidase

The enzymatic antioxidant activity of glutathione peroxidase (GPx) was measured in erythrocytes by spectrophotometry (BioTek Synergy HT, Winooski, Vermout, USA) [30]. Phosphate-buffered saline potassium phosphate buffer (KH2 PO4 0.1 pH 7.0) (56 μL) containing 0.1 mM EDTA, 19 μL glutathione 99% in bidistilled H_2O, 19 μL glutathione reductase, and 18 μL sample erythrocytes were incubated at 37°C for 10 min. GEN 5 program was used in the spectrophotometer. A 19-μL NADPH sample (1.5 mM in 0.1% $NaHCO_3$) was added for monitoring the independent chemical reaction of hydroperoxide for 3 min at 340 nm; immediately, 19 μL of 30% H_2O_2 sample was added for full monitoring of the second enzymatic reaction at 340 nm for 5 min. The decrease in concentration of NADPH (Δ[NADPH] min) was calculated from the decrease in absorbance at 340 nm, using the Lambert-Beer theory and molar extinction coefficient.

Catalase activity

Catalase (CAT) enzymatic activity was measured in erythrocytes by spectrophotometry (BioTek Synergy HT, Winooski, Vermout, USA). Previously, hemoglobin (Hb) concentration was measured by the Drabkin method. CAT activity was analyzed as previously described [30]. A sample of erythrocytes (10 μL) was used. The enzymatic activity of CAT was determined by kinetic reaction spectrophotometrically analyzed. H_2O_2 decomposition was conducted by absorbance decrease at 240 nM ($\varepsilon 240 = 0.00394 \pm 0.0002$ L mmol-1). The absorbency difference by unit time is the mean of CAT.

Statistical analysis

Data are expressed as mean difference (MD) ± standard error of the difference (SED) in the results test, whereas mean ± SEM were used in tables and figures. Normality of distribution was assessed by the Kolmogorov–Smirnov test. Homogeneity of variance was analyzed through the Levene test. According to this, a nonparied Student's *t* test was used to compare the mean values of Hpx, TBARS, α-actin, LDH, CK-MB, coenzyme Q10, SOD, CAT, and GPx between the NS and S groups. The level of significance was set at $P < 0.05$. All statistical procedures were carried out using SPSS/PC V. 22 (SPSS Inc., Chicago, IL, USA).

Results

α-tocopherol quantification

Eighty percent of the sample (n = 75) showed α-tocopherol values <80 nmol/mL, which are considered physiologically normal levels from a balanced diet, whereas the remaining 20% (n = 19) presented significantly high levels (>80 nmol/mL), suggesting a vitamin E supplementation (Table 1).

Blood markers of oxidative stress and muscle damage

No statistical differences in plasma Hpx and TBARS were found when comparing the NS group with the S group. Nevertheless, there were trends toward a lower plasma Hpx in the S group. Additionally, LDH and CK activities, and α-actin release were similar in both experimental groups. The mean and SEM of blood markers of oxidative stress and muscle damage are shown in Table 2.

Endogenous antioxidant response

Coenzyme Q10 plasma concentrations, and SOD, GPx, and CAT activities are represented in Table 2. All these endogenous antioxidants analyzed showed lower levels in the S group than in the NS group. Statistical differences were reported only for CAT.

Discussion

In the present study about oxidative status in well-trained endurance athletes, a possible antioxidant supplementation with vitamin E was revealed by an HPLC analysis in some athletes, despite their claim in the initial interview that they were not taking antioxidant supplements. The group with high plasma α-tocopherol concentrations showed trends toward to lower Hpx levels, and significantly lower enzymatic activity of CAT than the group with normal plasma α-tocopherol values.

A combination of two measurements for detecting antioxidant supplementation was used in this study: a personal interview (indirect and qualitative measure) and an HPLC analysis [31] (direct and quantitative measure). In this sense, a previous study recommended the combination of indirect and

Table 2
Oxidative stress, skeletal muscle damage, and endogenous antioxidant status related to α-tocopherol plasma concentration in well-trained endurance athletes

	NS group* n = 75	S group* n = 19	*P* value
Oxidative stress blood markers			
Hydroperoxides (nmol/mL)	3.51 ± 0.34	2.32 ± 0.37	*0.071*
TBARS (nmol/mL)	8.16 ± 0.63	6.72 ± 0.77	0.289
Skeletal muscle damage blood marker			
Lactate dehydrogenase (U/L)	394.24 ± 36.26	386.55 ± 44.71	0.661
Creatine kinase-MB (U/L)	22.82 ± 2.29	25.23 ± 4.19	0.928
α-actin (μg/mL)	5.74 ± 0.49	6.04 ± 1.30	0.793
Endogenous antioxidants			
SOD (U/mg)	0.11 ± 0.01	0.09 ± 0.01	0.619
GPx (U/mg)	5.17 ± 0.68	4.49 ± 1.06	0.644
Catalase (seg^{-1} mg^{-1})	0.22 ± 0.02	0.11 ± 0.02	*0.020*†
Coenzyme Q1 O (nmol/mL)	1.10 ± 0.28	0.73 ± 0.30	0.510

GPx, glutathione peroxidise; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances
Data are presented as mean ± SEM. The most relevant results are indicated in italics.
* NS group: Participants (n = 75) who showed no evidence of antioxidant supplementation (α-tocopherol values <80 nmol/mL). S group: Participants (n = 19) who showed evidence of antioxidant supplementation (α-tocopherol values >80 nmol/mL).
† Statistical differences between the two experimental groups. Student's *t* test was set at $P < 0.05$.

direct measurement to control doping or supplementation in athletes [31]; however, this study does not recommend a specific technique to detect antioxidant supplementation.

The distribution of plasma α-tocopherol given for HPLC analysis in this study led us to establish two study groups: a group of participants who showed normal α-tocopherol levels of a balanced diet, who were characterized as the NS (nonsupplemented) group; and another group that showed α-tocopherol concentrations above normal range, who were characterized as the S (supplemented) group. To establish this classification, we relied on plasma average values shown in individuals with a balanced diet as described in scientific literature. At present, there appear to be no internationally normal values accepted for plasma α-tocopherol in humans, as the plasma levels of tocopherol depend on many factors: nutritional (e.g., tocopherol intake, degree of unsaturation of the fat, gene polymorphisms associated with lipid metabolism) and physiological and pathophysiological conditions (e.g., pregnancy, aging, oxidative stress, sports practice) [32]. For the evaluation of the state of vitamin E, there is no index that accurately reflects consumption or their deposits in the body [33,34]. However, in the present study, the concentrations showed by the NS group (α-tocopherol mean value = 20.44 nmol/mL) are within the range of normal plasma concentrations according to several studies [35–38], wherein the normal values of α-tocopherol in plasma with a balanced diet could be 9 to 46 nmol/mL, showing similar concentrations as in this study.

Additionally, a detailed analysis of the plasmatic α-tocopherol concentrations distribution in the NS group showed two peaks (Fig. 1B). The second peak corresponded to only five participants with values between 46 and 80 nmol/mL, surpassing the average of the group without antioxidant supplementation evidence. Results from a previous study demonstrated median values and the 5th, 25th, 75th, and 95th percentiles between 18.34 and 45.97 nmol/mL for a male Spanish population [39]. Additionally, the mentioned peaks can be due to various factors. Previous studies have reported that blood α-tocopherol levels decrease after a stress such as exercise due to a mitochondrial mobilization [40]. It is possible that these five participants had a lack of exercise for >72 h, thus affecting the α-tocopherol mobilization to mitochondria holding it in the bloodstream. Another possible explanation is that these participants included a high tocopherol intake in their daily diet. Although most studies about vitamin E in athletes are made with exogenous antioxidant supplements [41], one study reported that female endurance athletes, who maintained a diet rich in vitamin E, had higher values of α-tocopherol in plasma (~35 nmol/mL) than those participants who maintained normal intake of vitamin E 48 h after the last training session [42]. However, a study in ultra-endurance athletes with low dietary vitamin E intake showed a mean value of 30 nmol/mL of plasma α-tocopherol concentrations [43]. Another study, in which participants followed a diet of food rich in tocopherol (consumption of 10.66–22.51 mg/d), reported α-tocopherol plasma levels ranging between 34.99 and 53.19 nmol/mL [44]. Therefore, it has been considered that these irregular peaks of α-tocopherol plasma concentrations can be because of diets rich in vitamin E or a pause in exercise of more than 48 h, but not as a result of an exogenous antioxidant supplementation. Thus, we have considered these individuals as participants without evidence for antioxidant supplementation.

Moreover, concentrations >80 nmol/mL might be due to the ingestion of different doses of supplements that contain high concentrations of vitamin E (the higher vitamin supplement intake, the higher values of α-tocopherol in plasma). Several studies conducted from 1991 [45] to 2014 [46] detected plasmatic α-tocopherol concentrations ≤90 nmol/mL after a supplementation of 235 to 800 mg/d of vitamin E from 1 wk, 21 d, or even 3 mo of vitamin supplementation. Therefore, we assume that the high plasmatic values of α-tocopherol in some participants identified in the S group might be explained by a high vitamin supplementation.

Given the discovered omitted antioxidant supplementation, oxidative stress, skeletal muscle damage, and endogenous antioxidant capacity were compared in the two experimental groups established by α-tocopherol concentrations. Regarding lipid peroxidation and skeletal muscle damage blood markers, data gathered in this study revealed that they are not statistically different between the experimental groups. However, only in the case of Hpx, the S group showed a trend toward lower concentrations. This might be due to a transient protection to the overproduction of reactive oxygen species attributable to the intake of supplements with high doses of vitamin E [47]. Regarding to exercise-induced muscle damage, it seems that the omitted antioxidant supplementation did not affect the skeletal muscle damage biomarkers of LDH, CK-MB, and α-actin. A previous study concluded that vitamin supplementation only prevents increases in lipid peroxidation but had no apparent effect on inflammatory and muscle damage responses in endurance athletes [48]. Other authors concluded that there is insufficient scientific evidence to verify that vitamin E supplementation decreases muscle damage [17], and that it might even increase the probability of mortality [49]. Additionally, the S group, with high α-tocopherol concentrations, showed lower levels of endogenous antioxidants, with statistical differences in the case of CAT enzyme. Our results are in accordance with recent studies that reported that, after exercise, there might be a punctual decrease in reactive oxygen species production by the action of antioxidant supplementation, which, however, might inhibit the activation of antioxidant enzymes whether antioxidant intake is chronically used [50,51]. This seems to be explained by exercise-induced oxidative stress that might cause an adaptive response that improves endogenous antioxidant defense

capacity by promoting the transcriptional regulation of SOD1, SOD2, GPx1, and CAT, and which could be completely abrogated by a chronic vitamin E or C supplementation [52]. Therefore, antioxidant supplementation appears to suppress endogenous antioxidant mechanisms [53], which might explain the decrease of antioxidant activities in the present study, specifically in the case of CAT where statistical differences are observed. Likewise, another endogenous antioxidant measure as coenzyme Q10 showed slightly lower concentrations in the S group than in the NS group without significant differences, probably due to an endogenous antioxidant synthesis inhibition, which could compromise the efficiency of the energy production, or even block the antioxidant defense mechanism actions as coenzyme Q10 is a key component of the electron transport chain [54]. Conversely, several studies defended a protective effect of this antioxidant supplementation, which was reflected in an increase in antioxidant defense and a reduction in lipid peroxidation and muscle damage [55,56]. More research is needed to elucidate the long-term interrelationship among various antioxidants.

This study had some limitations. By the nature of the study, we do not know what kind of supplementation (type, amount, frequency) or how long it had been ingested; however, it was not part of the scope of this study as the main objective was to determine whether athletes can lie about antioxidant intake during a personal interview. Moreover, this study presented some strengths; for instance, we used a large sample size (~100 participants). Additionally, several blood biomarkers of skeletal muscle damage contrasted with several blood markers of oxidative stress were included. Finally, we used a combination of qualitative-subjective assessment (personal interview) and a direct measurement (HPLC) to check antioxidant supplementation as exclusion criterion. The present study is, therefore, an opportunistic study, given that it pushes us to reflect on the accuracy of the published data and their conclusions in which exclusion criteria were not directly verified.

Conclusions

The high plasma α-tocopherol concentrations derived from a possible antioxidant supplementation seem to affect CAT activity in well-trained endurance athletes. For that reason, it is relevant to take into account that participants can lie about antioxidant intake in a personal interview during the exclusion process. Thereby, we strongly suggest including a specific technique for quantification of plasma antioxidants such as the HPLC analysis to detect the possible hidden antioxidant supplementation and, thus, preserve the veracity of the final results and conclusions in future studies of oxidative stress and sport.

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IV

A NOVEL APPROACH TO ESTIMATE ELECTROMYOGRAPHY FATIGUE THRESHOLD IN MAXIMUM INCREMENTAL STRENGTH TESTS

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Control Motor.

AN APPROACH TO ESTIMATE ELECTROMYOGRAPHIC FATIGUE THRESHOLD FROM MAXIMAL INCREMENTAL STRENGTH TESTS

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AN APPROACH TO ESTIMATE ELECTROMYOGRAPHIC FATIGUE THRESHOLD FROM MAXIMUM INCREMENTAL STRENGTH TESTS

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Running Head: Fatigue threshold identified by Electromyography

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Abstract

The aim of the present study was to provide an approach to estimate electromyographic fatigue threshold from maximal incremental strength tests. Ten men and 10 women performed a half-squat strength test consisting of five incremental intensities of the one repetition maximum. Surface electromyographic activities of the right side *vastus lateralis*, *vastus medialis*, and *rectus femoris* muscles were recorded, finding a break point corresponding to the electromyographic fatigue threshold occurring in men at 70.74%, 71.48% and 72.52%, respectively. The break point corresponded in women to 76.66% and 72.10 % for *vastus medialis* and *rectus femoris*, respectively. In summary surface electromyography is a useful and non-invasive tool to determine the fatigue threshold of independent muscle groups during a maximal half-squat strength test.

Keywords: Area under the RMS, non-invasive, fatigue, break point, half-squat.

Introduction

Performance evaluation is important for athletes and coaches in planning and monitoring daily training, (Candotti et al., 2008) as well as during muscle injury (Naik & Nguyen, 2015). In this line, the main physiological parameters used during training are lactate threshold, (Faude, Kindermann, & Meyer, 2009) ventilatory threshold, (Plato, McNulty, Crunk, & Tug Ergun, 2008) and heart rate (HR), (Borresen & Lambert, 2007) which are good predictors of physical fitness. However, these variables are used to assess physical activities involving large muscle masses and they are not applicable for the assessment of independent muscle groups during strength tests.

Thus, it has been previously reported that, during incremental effort exercise test that involve large muscle masses, an electromyographic break point appears, called surface electromyographic fatigue threshold (EMG_{FT}) (Lucía, Sánchez, Carvajal, & Chicharro, 1999). This point is a result of the recruitment of fast-twitch motor units and/or higher neural firing rate to counterbalance the muscle deficit contraction caused by the fatigued motor units activated during the effort (Mäestu et al., 2006). In addition, several authors have confirmed that the EMG_{FT} correlated with the threshold of transition from aerobic to anaerobic metabolism, measured by classic methods such as the kinetics of VO^2 and VCO^2 and/or ventilatory equivalents, (Moritani, Takaishi, & Matsumoto, 1993) heart rate (Bunc, Hofmann, Leitner, & Gaisl, 1996) and lactate threshold (Faude et al., 2009). Surface electromyography (EMG) has also been used to determine EMG_{FT} during isometric, (Beck et al., 2007) isokinetic (Beck et al., 2007) and dynamic (Travis, Arthmire, Baig, Goldberg, & Malek, 2011) muscle actions. However, some sport activities including strength components and fitness training involve specific muscle groups with short-term explosive

movements. In these cases, classic methods such as maximal oxygen consumption (VO_2 max), heart rate or lactate concentration do not suffer large variations and, accordingly, they are not very helpful in detecting the EMG_{FT}. In these situations, EMG monitoring could be an adequate method to evaluate the intensity at which fast-twitch fibers can be recruited in short-time maximum incremental strength test (MIST).

We propose a method to detect the EMG_{FT} in different muscles involved in both men and women when undertaking a half-squat maximum strength test, at different one repetition maximum (1RM) rates.

Methods

Subjects

A total of 20 healthy college-age volunteers (10 men and 10 women) were enrolled in the study. Table 1 shows the baseline features of the subjects in this study. All subjects were familiar with strength training and half-squat exercise. All subjects had recreational experience (1 to 5 years) of strength training including half-squat exercise. Subjects trained their lower body musculature 2–3 times per week by lifting loads of 60–90% of one repetition maximum (1RM). No scheduled training programme was followed for volume or intensity. No specific resistance programme for any sport was followed either. Smoking, high intake of antioxidant-rich foods, ergogenic supplementation or drugs were considered exclusion criteria. All subjects were thoroughly informed of the purpose, nature, practical details and potential risks associated with the study before signing an informed consent.

The study was approved by the Ethics Committee of the University of Granada (Spain) and was in accordance with the Declaration of Helsinki.

All procedures were performed out at the exercise physiology laboratory of the Faculty of Sport Sciences of the University of Granada (Spain). All subjects were assessed in the morning, in particular, two hours after breakfast, at a physiology laboratory with controlled humidity and temperature according to the guidelines established by the National Strength and Conditioning Association (NSCA -National Strength & Conditioning Association, 2008).

Design

All the procedures were performed during spring. Half-squat exercise was the chosen procedure because it is a common method used for the assessment of maximum strength in athletes (McMaster, Gill, Cronin, & McGuigan, 2014). Pre-test sessions were conducted 2 weeks prior to the experiment onset. During the first week, subjects became familiar with the experimental testing procedures. During the second week, subjects participated in a control test to identify their 1RM (1RM: the heaviest load that can be correctly pressed only once using the correct technique) (Sanchez-Medina, Perez, & Gonzalez-Badillo, 2010). Four days of testing were required to evaluate the subjects (Tuesdays and Thursdays over two consecutive weeks).

Anthropometry

Before performing the MIST, all subjects underwent an anthropometric study by skinfold thickness assessment. Body mass index (BMI) was calculated as weight (kg)/height (m^2). Body fat and muscle mass were calculated using the procedures described by the International Society for the Advancement of Kinanthropometry (ISAK), and their values were expressed in relative terms (%) and kg.

Initial 1RM Strength Test

One week before the experiment onset, all the subjects underwent a maximal progressive exercise test in order to determine their 1RM. We used the 1RM test described by Sanchez-Medina et al., (2010). Immediately before the test, the subjects performed a warm-up consisting of 5 minutes on a cycloergometer, dynamic stretching and two sets of five repetitions with 20 kg and 30 kg each. In brief, the test was carried out on a Smith Machine where the subjects performed 1RM test. The load was increased by 10 kg when the subjects succeeded, whereas the load was reduced by 5 kg when the subjects failed the task. The test finished when the subjects failed to perform two consecutive attempts. All the repetitions were interspersed by a 3-minutes rest and the 1RM was achieved between the third and sixth attempt.

Maximum Incremental Strength Test

The subjects were asked to refrain from exercise for a minimum of 72 hours prior to the experiment. The MIST was performed between 8.30 a.m. and 03.00 p.m. The subjects performed the warm-up routine consisting of 5 minutes of jogging and leg stretching, as

well as a familiarization set of 15 repetitions with a load at 40% of 1RM in the half-squat exercise (Smilios, Häkkinen, & Tokmakidis, 2010). The MIST was performed in a half-squat apparatus (Smith Machine) in which the barbell was attached at both ends with linear bearings on two vertical bars allowing only vertical movements. Shoulders were in contact with the bar, and the starting angle of the knee was 180º. Eccentric and concentric phases were continuously performed, and knee flexion angle was kept at 90º. To guide the displacement path, the position that the individual needed to adopt to initiate the half-squat was determined using a manual goniometer and marked using a rod on a tripod. The subject was then indicated in each repetition to half-squat until touching the rod with the glutei. The accuracy error of the procedure was, 1 cm (Flanagan et al., 2014). Repetitions in which the complete distance was not covered were not considered. Foot spacing on the half-squat was set at approximately the same width as, or slightly wider than, shoulder width with toes pointed slightly outward (Cotterman, Darby, & Skelly, 2005). The torso was kept as straight as possible and a security belt was used by all subjects (Izquierdo et al., 2003).

Five incremental intensities derived from the individual 1RM tests were used: 20%, 40%, 60%, 80% and 100% during the strength test. For the first three intensities, 5 repetitions of 5 seconds each at 20%, 40% and 60% intensities were performed. Two repetitions were performed at 80% intensity without a time limit. Finally, only 1RM (100% intensity) was completed by each participant without any help for the last load. A resting period of 3 minutes long was allowed after each of the first four intensities (20%, 40%, 60% and 80%) and a 5 minute resting period was allowed between 80% and 100% intensities.

Electromyography

Electrical muscle activation was monitored using surface electromyography (EMG). EMG signals obtained during extension and flexion actions of the knee were recorded from the *vastus lateralis* (VL), *vastus medialis* (VM) and *rectus femoris* (RF). The muscle contractions were performed in dynamic conditions. Before the application of the EMG electrodes, the skin surface was carefully shaved and wiped with alcohol to reduce skin impedance. Bipolar single differential electrodes were placed longitudinally on the muscles following the SENIAM recommendations (Hermens, Freriks, Disselhorst-Klug, & Rau, 2000) and taped to the skin to minimize movement artefacts. The position of the electrodes was marked on the skin with indelible ink, and these references were used for precise electrode placement on repeated experiments according to Torres-Peralta *et al*. (2016).

The EMG signals were acquired using a 4-channel recording system (Myomonitor IV, Delsys Inc., Boston, MA) at a sampling rate of 1000 Hz using rectangular shaped (19.8 mm wide and 35 mm long) bipolar surface electrodes with 1 × 10 mm 99.9% Ag conductors, and with an inter-conductor distance of 10 mm (DE-2.3 Delsys Inc.). A 12-bit analog-to-digital data acquisition card BNC-2090 (National Instruments Corporation, Austin, TX, USA) was used to sample the signal at 4000 Hz. The EMG signal corresponding to each muscle contraction was acquired and analyzed using a specific program (EMGworks 4.1.7 Analysis; Delsys, Inc. Boston). After visual inspection, EMG recordings were filtered using a Butterworth filter class with a high pass filter of 100 Hz and low pass of 450 Hz (IIR Filter, Delsys, Inc. Boston). Then the root mean square (RMS)

was calculated for each contraction. In this study we determined the area under the RMS (aRMS) for each contraction, as shown in Figure 1.

The aRMS average of the second, third and fourth repetition of the intensities from 20% to 60% was calculated in order to ensure that the analyses were performed with repetitions involving correct rhythm and techniques. At 20%, 40% and 60% intensities, the first and last repetitions were always excluded from the calculation to avoid registering movements derived from the bar removal at the Smith machine.

The EMG_{FT} were determined by examining the exponential relation between the aRMS and its corresponding intensities (20%, 40%, 60%, 80% and 100%) during the strength test. The break point (EMG_{FT}) of each exponential curve was calculated according to Muggeo, (2008) based on a segmented linear regression model (two segments separated by a break-point).

Blood Lactate Concentration and Heart Rate Assessment

During the MIST, blood samples were collected using microcapillary tubes on micropunctures at the fingertips, during the first 30 seconds of rest, at each intensity (20%, 40%, 60%, 80% and 100%). Blood lactate concentrations were analyzed using a Lactate Pro analyzer (Lactate Pro, Carlton, Australia). In addition, HR was recorded continuously (Polar TEAM 2 PRO, Kempele, Finland) along the test.

Statistical Analysis

The data obtained were analyzed using PASW V.22 (SPSS for Windows Chicago, IL, USA) and presented as mean ± standard error of the mean (SEM). Normality of distribution was confirmed using the Shapiro–Wilk test. Characteristics of the subjects (age, weight, height, BMI, fat mass and muscle mass percentages, thigh and leg circumferences, total leg muscle areas) and EMG_{FT} values were compared by sex through Student's *t* test or the Mann–Whitney U test for nonparametric samples.

The mean of the aRMS response for the concentric phase of the VL, VM and RF muscles was analysed by using a one-way ANOVA followed by a Bonferroni *post-hoc* test. The break point (EMG_{FT}) of each exponential curve from VL, VM and RF muscles was calculated by Segmented, an R package developed by Muggeo (Muggeo, 2008) (R statistical Package "Segmented". Version 1.4. University of Palermo, Italy). Significance was set at $p < 0.05$ for all statistical analyses.

Results

Features of Subjects

Table 1 shows the body composition and other relevant features of the subjects. Despite the age of subjects was homogeneous, other features (weight, height, BMI, fat mass, muscle mass and leg muscle area) showed significant differences between men and women.

Lactate and Heart Rate

Neither HR nor blood lactate concentrations showed significant differences at the different intensities tested (Figure 2). Furthermore, no statistically significant differences between men and women regarding HR and blood lactate concentration were found.

Evaluation of the Electromyographic Fatigue of VM, VL and RF during an Incremental Strength Test

In men, the exponential relationships between the aRMS and its corresponding intensities (20, 40, 60, 80 and 100%) during the endurance test were: $y = 2.256^{e0.019x}$ ($R^2 = 0.962$) for VM; $y = 2.494^{e0.017x}$ ($R^2 = 0.989$) for VL; and $y = 1.785^{e0.020x}$ ($R^2 = 0.978$) for RF. VM, VL and RF electromyographic break-points occurred at 70.74%, 71.48% and 72.52% of 1RM, respectively, values differing significantly from 100% of 1RM ($p < 0.009$, $p < 0.004$ and $p < 0.023$, respectively) (Figure 3).

In the case of women, the VM and RF electromyographic break points occurred at 76.66% and 72.1% of 1RM, respectively, values differing significantly from 100% of 1RM ($p < 0.010$ and $p < 0.008$, respectively) (Figure 4).

Discussion

In the present study we provide an innovative method to estimate the EMG_{FT} of independent muscle groups in MIST of short duration, where classic ergospyrometry is not applicable. We tested this methodological modification by using half-squat exercise as a

model and the average area of single RMS for every load, instead of the average RMS for short-time windows. Similarly to the classic methods, our methodological alternative allows correlating the aRMS and the incremental loads by verifying that the correlation fits an exponential curve. The segmented linear regression model gives the EMG_{FT}. In men, the VM, VL and RF EMG_{FT} occurred at 70.74%, 71.48% and 72.52% of 1RM, respectively. In women, the VM, VL and RF EMG_{FT} occurred at 76.66%, 76.27% and 72.1% of RM, respectively. The intensity of each EMG_{FT} was found similar between the three muscles studied, both in men and women. Our results are consistent with the findings of Andersen et al., (1985) and Escamilla et al., (1998) who described that these three muscles are the most relevant in half-squat.

Our method does not allow to confirm that the EMG threshold of such a rapid sport gesture is due to the metabolic threshold or the EMG_{FT} described by other authors for classical effort tests (Galen & Malek, 2014). In classical tests, it is easy to confirm that the EMG break point coincides with that of lactate (4 mmol/l), the kinetics of VO_2 and VCO_2 and ventilatory equivalents. Under our experimental conditions the gesture lasts few seconds, thus the physiological response is not sufficient to increase circulating lactate concentration, nor to modify partial pressures of O_2 and CO_2 and the gesture occurs without the corresponding ventilatory readjustment. In fact, lactate concentrations at different loads are very similar to those at rest, below 3 mmol/l in men and 2.5 mmol/l in women. Similarly, the HR remains steady at all loads (below 150 ppm for men and 140 ppm for women). Our results do not imply the absence of an important mechanical and metabolic activity in the muscular groups involved at submaximum and maximum loads. Likely, the metabolic readjustments are so rapid that no systemic response is found (Maté-Muñoz et

al., 2015). With regard to the concentrations of lactate, rapid fibres recruited massively after overcoming the threshold are widely known to generate a great volume of lactate that is exported to the extracellular medium through monocarboxylate transporters MCT4 (Iizuka, Machida, & Hirafuji, 2014). Since the gesture is so fast, it is probably absorbed by the adjacent slow fibres through monocarboxylate transporters MCT1 (Kobayashi, 2004). This homeostatic readjustment would lead to circulating concentrations of lactate very similar to concentrations of lactate found at rest which would explain our results. Invasive techniques (serial biopsies and/or continuous microperfusion of the interstitial fluid of the muscles involved) might be used to confirm this metabolic readjustment.

With the regard to the absence of significant increases of the HR at incremental loads, our results are consistent with those described by Avelar et al., (2011) who did not find any change in this parameter during squat even though VO_2 increased from 3.33 ± 0.13 to 5.69 ± 0.23 $ml/kg^{-1}.min^{-1}$. Our lactate and HR values were consistent with those reported by Garnacho-Castaño et al., (2015) who compared half-squat exercise and cycloergometer at an anaerobic threshold intensity.

This break point estimated with our method would be the consequence of a massive recruitment of rapid fibres which always induces an exponential increase in the electromyographic signal, responsible for the break point of the curve (Mäestu et al., 2006). Altogether, we believe that the method based on short-duration strength tests described in the present study is useful to elucidate the metabolic threshold of independent muscle groups.

Conclusion

In the present study we propose a method which is able to discriminate EMG_{FT} within particular skeletal muscle portions while performing strength tests. Athletes would benefit from this approach by ensuring adaptations of a particular muscle. Moreover, it would also be used by clinicians in order to assess proper recovery from an injury within a specific muscle portion. Therefore, the results presented here suggest that EMG performed by aRMS may be a useful tool to determine EMG_{FT} in single muscles during an explosive and general strength exercise such as half-squat. The reported method is rapid and non-invasive, ideal for sports with very short duration and explosive movements for which classic tests are not applicable.

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Table 1. Characteristics of participants

Parameter	Units	Men			Women		
Age	Years	22.30	±	0.91	22.80	±	0.39
Weight	kg	75.87	±	1.82	60.01 *	±	2.23
Height	cm	178.50	±	2.23	167.6 *	±	2.15
BMI	kg/m^2	23.81	±	0.48	21.30 *	±	0.42
Fat mass	%	8.77	±	0.58	18.80 *	±	1.11
Muscle mass	kg	38.36	±	0.95	26.52 *	±	0.92
Thigh circumference	cm^2	53.05	±	0.90	49.04	±	1.08
Leg - circumference	cm^2	37.53	±	0.54	35.57	±	0.52
Leg - muscle area	cm^2	195.01	±	6.80	132.35 *	±	6.67

BMI: Body mass index. Results are expressed as mean ± SEM (n = 10 for men and n= 10 for women group). * /p<0.05/ Men vs women

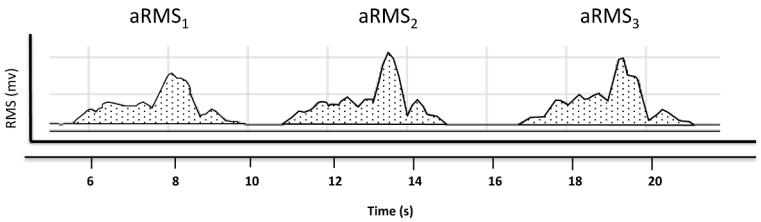

Figure 1

An example of the data obtained from the values of the area under the RMS curve (aRMS) of three consecutive muscle concentrations at 20% of the 1RM (aRMS1, aRMS2 and aRMS3).
Figure 1
254x190mm (300 x 300 DPI)

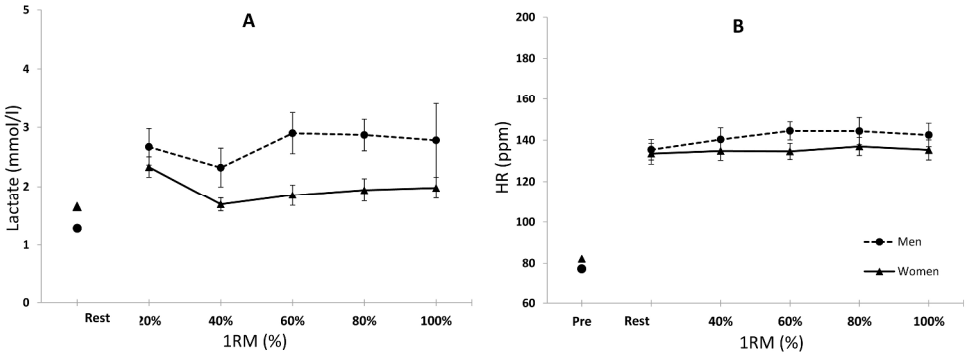

Figure 2

Lactate (A) and heart rate (B) obtained at rest and during the maximum incremental strength test (one repetition maximum). The Student's t test did not present significant differences at the various intensities of test performed.

Figure 2
302x148mm (300 x 300 DPI)

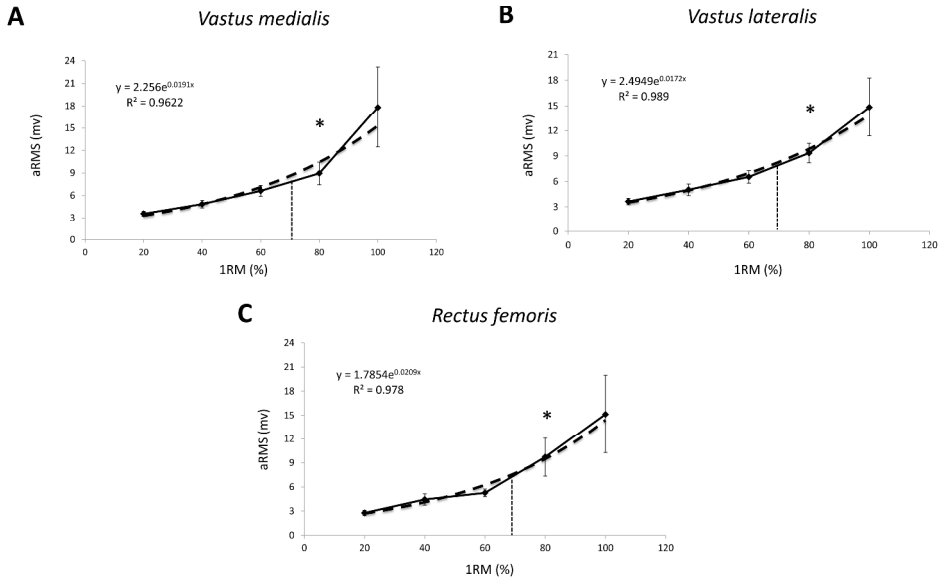

Figure 3

aRMS vs intensities in vastus medial (A), vastus lateralis (B) and rectus femoris (C) in male subjects. * Indicate significant different between 80 % of the1RM with respect other intensities ($p < 0.009$ in A, $p < 0.004$ in B and $p < 0.023$ in C).

Figure 3
399x299mm (300 x 300 DPI)

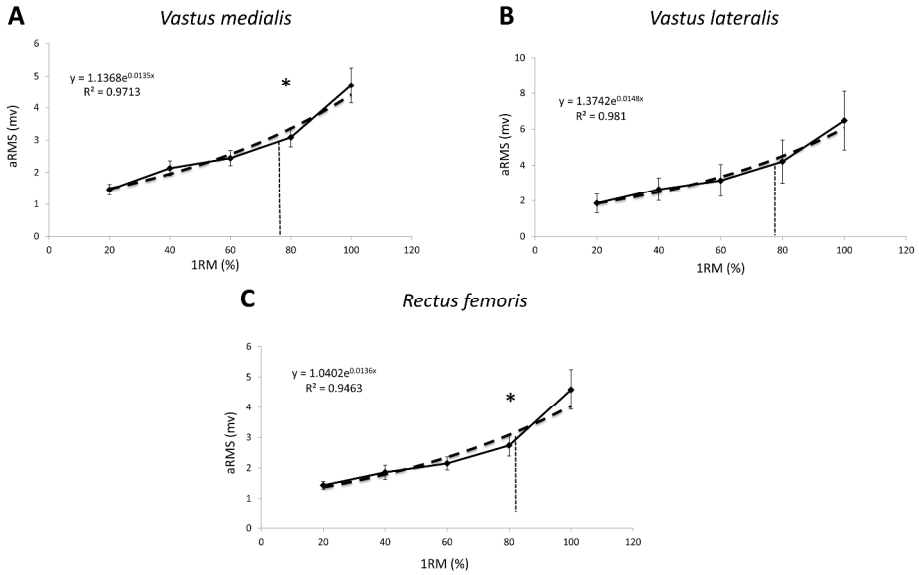

Figure 4

aRMS vs intensities in vastus medial (A) and rectus femoris (C) in female subjects. * Indicate significantly different between 80 % of the 1RM with respect other intensities ($p < 0.010$ in A, and $p < 0.008$ in C).
Figure 4
399x299mm (300 x 300 DPI)

V

CARDIAC MUSCLE DAMAGE IN BENCH PRESS VERSUS HALF-SQUAT EXERCISES: ARE THEY HEART-HEALTHY SPORTING ACTIVITIES

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CARDIAC MUSCLE DAMAGE IN BENCH PRESS VERSUS HALF-SQUAT EXERCISES: ARE THEY HEART-HEALTHY SPORTING ACTIVITIES?

--Manuscript Draft--

Manuscript Number:	
Full Title:	CARDIAC MUSCLE DAMAGE IN BENCH PRESS VERSUS HALF-SQUAT EXERCISES: ARE THEY HEART-HEALTHY SPORTING ACTIVITIES?
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Abstract:	Squat and bench press are two types of exercise commonly used to increase and assess muscle strength; however, each of these exercises involve different amount of muscle mass. Despite their wide use in sport centres by all kind of athletes, the mechanism by which equivalent loads of squat or bench press affect muscle damage and oxidative stress is still unknown. The aim of this study was to identify whether the muscle mass involved affects skeletal and cardiac muscle damage and oxidative stress in these two strength exercises. Ten well-trained men were subjected to two maximal incremental strength tests (half-squat and bench press) with 20, 40, 60, 80 and 100% 1RM. Blood samples were collected at rest, 15-minutes post-test and 24 hours post-test. During the test, an electromyographic record was performed. Serum α-actin was determined by western blot as a marker of skeletal muscle damage. In addition, serum lactate dehydrogenase (LDH) and creatine kinase isoenzyme MB (CK-MB) activities and plasma hydroperoxide concentration were measured by spectrophotometry. Statistical differences were determined using a one-way ANOVA. The highest values of muscle damage markers were obtained in bench press vs. half-squat at 15 min after exercise (LDH: 326.26 ±24.22 U/L vs. 306.27±18.48 U/L, p=0.04. CK-MB: 26.43±1.94 U/L vs. 19.07±2.04 U/L, p=0.01). At equivalent workloads, the bench press exercise induced greater muscle damage, at both skeletal and cardiac levels, compared with half-squat exercise, likely because in half-squat the load is supported by many muscle groups, including the lower body muscles.
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CARDIAC MUSCLE DAMAGE IN BENCH PRESS VERSUS HALF-SQUAT EXERCISES: ARE THEY HEART-HEALTHY SPORTING ACTIVITIES?

Brief running head: Are bench press and half-squat exercises heart-healthy?

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ABSTRACT

Squat and bench press are two types of exercise commonly used to increase and assess muscle strength; however, each of these exercises involve different amount of muscle mass. Despite their wide use in sport centres by all kind of athletes, the mechanism by which equivalent loads of squat or bench press affect muscle damage and oxidative stress is still unknown. The aim of this study was to identify whether the muscle mass involved affects skeletal and cardiac muscle damage and oxidative stress in these two strength exercises. Ten well-trained men were subjected to two maximal incremental strength tests (half-squat and bench press) with 20, 40, 60, 80 and 100% 1RM. Blood samples were collected at rest, 15-minutes post-test and 24 hours post-test. During the test, an electromyographic record was performed. Serum α-actin was determined by western blot as a marker of skeletal muscle damage. In addition, serum lactate dehydrogenase (LDH) and creatine kinase isoenzyme MB (CK-MB) activities and plasma hydroperoxide concentration were measured by spectrophotometry. Statistical differences were determined using a one-way ANOVA. The highest values of muscle damage markers were obtained in bench press vs. half-squat at 15 min after exercise (LDH: 326.26 ±24.22 U/L vs. 306.27±18.48 U/L, p=0.04. CK-MB: 26.43±1.94 U/L vs. 19.07±2.04 U/L, p=0.01). At equivalent workloads, the bench press exercise induced greater muscle damage, at both skeletal and cardiac levels, compared with half-squat exercise, likely because in half-squat the load is supported by many muscle groups, including the lower body muscles.

Key words: Oxidative stress, α-actin, muscle mass, creatine kinase-MB and lactate dehydrogenase.

INTRODUCTION

Bench press and half-squat are two classic push exercises for strengthening the body (29), frequently used to assess maximal muscular strength (15). In both exercises, the force is produced through a combination of eccentric, concentric, and joint-stabilizing isometric muscle actions. Together, these muscle actions form the basic components of the movements performed in daily living and strength exercise (11). However, the amount of muscle mass involved during the performance of bench press and half-squat differs largely (2), being half-squat the exercise that involves larger number of muscle groups.

Exercise-induced muscle damage, especially caused by eccentric exercises, is characterized by changes in calcium (Ca^{2+}) homeostasis, reactive oxygen species (ROS) production, muscle fatigue and inflammatory processes, among others. Bench press and half-squat exercises involve ischemia-reperfusion injury, during which a critical increase of superoxide by the xanthine oxidase pathway could occur (1). However, the consumption of the mitochondrial oxygen would also be involved in the ROS production to a lesser extent (25). In any case, an excessive ROS production can lead to an increase in cardiac and skeletal muscle damage and a decrease in the athlete performance.

The level of ROS production largely depends on the maximal oxygen consumption occurring during exercise. The study described by Ratamess et al., (27), conducted using several protocols of acute strength exercises, both bench press and squat, concluded that VO_2 mean values during squat were higher than values obtained during bench press. The authors speculated that this difference could be because squat

exercise involves a higher muscle mass that leads to higher VO_2 values than exercises involving lower muscle mass (23,28). Since lower muscle mass is involved in bench press, we hypothesized that serum markers of skeletal and heart damage, induced by ROS production and/or the mechanical stress of exercise, would be increased during half-squat exercise. We analysed the creatine kinase MB isoenzyme (CK-MB) and lactate dehydrogenase (LDH) activities to test our hypothesis, since both have been used extensively as markers for cardiac and systemic cell damages, respectively (10). We also determined α-actin by Western blot to detect sarcomeric muscle damage (22).

To date there are no available studies that identify muscle damage based on the amount of muscle mass involved in these two classic strength exercises, bench press and half-squat, which are the most commonly used exercises in the sport training field. For this reason, the aim of the present study was to ascertain whether the involvement of muscle mass induces a higher production of lipid peroxidation and associated muscle damage at both skeletal and heart level, through a maximal incremental strength test.

METHODS

Subjects

Baseline features of the participants in this study are shown in table 1. A total of 10 well-trained male participants familiarised with half-squat exercise and bench press enrolled voluntarily in the study. All participants had recreational experience (1 to 5 years) with strengh training involving these exercises. Participants trained their body musculature 2-3 times per week by lifting loads of 60-90% of one repetition maximum (1RM: the heaviest load that could be correctly pressed only once using the correct technique) (14). Smoking, high intake of antioxidant-rich foods, ergogenic supplementation or drug consumption were considered exclusion criteria. Subjects were

thoroughly informed of the purpose, nature, practical details and possible risks associated with the study before they provided their written informed consent to participate. The study was conducted in accordance with The Declaration of Helsinki (2000) and it was approved by the Ethics Committee of the University of Granada (Spain).

Design

In this study, the differences in lipid peroxidation and muscle damage between bench press and half-squat strength exercises were evaluated. All the procedures were performed during the spring, so the environmental temperature was similar for the running protocol. All methods were performed at the Exercise Physiology Laboratory of the Faculty of Sport Sciences, at the University of Granada (Spain). Subjects were assessed in the morning, in particular two hours after breakfast, at a laboratory with controlled humidity and temperature according to the guidelines established by the National Strength and Conditioning Association (NSCA-National Strength & Conditioning Association 2008).

All subjects refrained from vigorous physical activity during the 48 hours prior to each test. Pre-test sessions were conducted 2 weeks before the experiment. During the first week, subjects underwent an anthropometric assessment and were familiarised with the experimental testing procedures. The second week, the subjects participated in a control testing day to know their 1RM in both strength exercises, on different days allowing 5 days of rest between tests. The same technique was used for all tests and familiarisation procedures.

Finally, participants performed maximal incremental strength tests for both bench press and half-squat exercises, allowing a one-week rest between tests. Four days

of experiment were required to evaluate the subjects. During each test, the electromyographic (EMG) signal was recorded to evaluate the neuromuscular activation and blood samples were collected at rest, 15-min post-test and 24 hours post-test.

Anthropometry

All participants underwent an anthropometric assessment. Anthropometric measurements were obtained following the protocol developed by the International Society for Advanced of Kinanthropometry (ISAK) (20). Anthropometric variables included body mass, height, 7 skinfolds (biceps, triceps, subscapular, supraspinal, abdominal, front thigh, and medial calf), 3 circumferences (flexed upper arm, thigh, and medial calf) and 3 breadths (humeral and femoral epicondyles and wrist). Body fat was assessed by applying the following formula for sport males (5): Body Fat (%) = (∑6skinfolds*0.1051)+2.58, where the 6 skinfolds were triceps, subscapular, supraspinal, abdomen, thigh, and medial calf expressed in millimetres. Body muscle mass was calculated as described by Wurch (33). All variables are expressed in kg.

One repetition maximum (1RM) strength testing sessions

All the participants underwent a maximal progressive test in order to determine their 1RM. Before the test, the subjects performed a warm-up which included 5 minutes on a cycle ergometer, dynamic stretching and 2 sets of 5 repetitions with 20 and 30 kg, respectively. We used the 1RM test described by Fernández-Gonzalo (10), for both half-squat and bench press exercises. In brief, the test was carried out in a Smith Machine (Life Fitness, Brunswick Corporation, USA) where the subjects performed one repetition maximum test. The load was increased by 10 kg when the subjects succeeded, whereas the load was reduced by 5 kg when the subjects failed the task. The test

finished when the subjects failed to perform two consecutive attempts. All the repetitions were interspersed by a 3-minutes rest and the 1RM was achieved between the third and sixth attempt.

Maximal incremental strength test

Each subject completed 2 testing visits, one visit each week. The subjects were asked to refrain from exercise for a minimum of 72 hours prior to the experiment. The maximal incremental strength test was performed between 8.30 a.m. and 03.00 p.m. In both visits, the maximal incremental strength test was performed in a Smith Machine (Life Fitness, Brunswick Corporation, USA). The subjects performed a warm-up which included 5 minutes on a cycle ergometer, dynamic stretching and 2 sets of 5 repetitions with 20 and 30 kg, respectively.

In half-squat, the barbell was attached at both ends with linear bearings on 2 vertical bars allowing only vertical movements. Shoulders were in contact with the bar, and the starting angle knee was 180º. Eccentric and concentric phases were continuously performed, and knee flexion angle was kept at 90º. To guide the displacement path, the position that the individual needed to initiate the half-squat was determined using a manual goniometer and marked using a rod on a tripod. The subject was then indicated in each repetition to half-squat until touching the rod with the glutei. The accuracy error of the procedure was 1 cm (9). Repetitions in which the complete distance was not covered were discarded. Foot spacing on the half-squat was set at approximately the same width as, or slightly wider than, shoulder width with toes pointing slightly outward (8). The torso was kept as straight as possible and a security belt was used by all subjects (17).

During the bench press test protocol, the head, shoulders and hips were supported by the bench with 90º flexion into knees, as suggested by Cotterman L. et al., (8). Then, the barbell was lowered in a continuous motion until the bar position was 1-2 cm above their intermammary line, and they were required to maintain this position for 1 s (velocity = 0 m/s). From that position, every participant was instructed to perform a purely concentric action (as quickly as possible) to return to the initial position (12). This technique allows an optimal use of the *pectoralis major* whereas still allowing the triceps to add to initial explosiveness (6). Press hand spacing was set at 165–200% of bisacromial width, which has been shown to provide the highest strength values of all grip widths for the supine bench press (19). Hand and foot spacing were recorded for replication in subsequent 1RM tests (8).

In both tests, five intensities derived from the individual 1RM test were incrementally used during the strengh test: 20, 40, 60, 80 and 100% 1RM. For the first three intensities, the participants performed 5 repetitions of 5 seconds each. At the 80% 1RM incremental intensity, the participants performed two repetitions without a time limit. A positive feedback was provided by attendees cheering to the test subjects during the eccentric and concentric phases, taking into account that the concentric phase was always performed explosively at the maximum possible speed (12). Finally, only 1RM (100%) was completed by each participant without any help for the last load. At the same time, several researchers were located behind the athlete during the test to help in raising the bar on a failed attempt and to help the participant place the bar back on the rack. A 3-minute resting period was allowed after each of the first four intensities (20%, 40%, 60% and 80%), and a 5-minute resting period was allowed between 80% and 100% intensities.

Electromyography

Electrical muscle activation was monitored using surface electromyography (EMG). The EMG signals obtained during extension and flexion actions of the right leg muscles were recorded from the *vastus lateralis* (VL), *vastus medialis* (VM), *rectus femoris* (RF) and *biceps femoris* (BF) in half-squat. The arm muscle values were recorded from the right *pectoralis major* (PM) (approximately 4 cm medial to the axillary fold (30), *anterior deltoid* (AD) (1.5 cm distal and anterior to the acromion), *latissimus dorsi* (LD) and *triceps brachii* (TB) (long head, approximately 3 cm medial and on 50% on the line between acromion and olecranon) (32), in bench press. The muscle contractions were performed in dynamic conditions. The skin surface was carefully shaved and wiped with alcohol before the application of the EMG electrodes to reduce skin impedance. Bipolar single differential electrodes were placed longitudinally on the muscles following the Surface ElectroMyoGraphy for the Non-Invasive Assessment of Muscles (SENIAM) recommendations (16) and fixed to the skin to minimize movement artefacts. The reference electrode was placed on the skin over the acromion. The position of the electrodes was marked on the skin with indelible ink, and the electrodes were maintained fixed throughout the test.

The EMG signals were acquired using a 4-channel recording system (Myomonitor IV, Delsys Inc., Boston, MA) at a sampling rate of 1000 Hz using rectangular shaped (19.8 mm wide and 35 mm long) bipolar surface electrodes with 1 × 10 mm 99.9% Ag conductors, and with an inter-conductor distance of 10 mm (DE-2.3 Delsys Inc.). A 12-bit analog-to-digital data acquisition card BNC-2090 (National Instruments Corporation, Austin, TX, USA) was used to sample the signal at 4000 Hz. The EMG signal corresponding to each muscle contraction was acquired and analysed using a specific program (EMGworks 4.1.7 Analysis; Delsys, Inc. Boston). After visual

inspection, EMG recordings were filtered using a Butterworth filter class with a high pass filter of 100 Hz and low pass of 450 Hz (IIR Filter, Delsys, Inc. Boston). Then the root mean square (RMS) was calculated for each contraction. In this study we determined the area under the RMS (aRMS) for each contraction.

The aRMS average of the second, third and fourth repetition of the intensities from 20% to 60% was calculated in order to ensure that the analyses were performed with repetitions involving correct rhythm and techniques. At 20%, 40% and 60% intensities, the first and last repetitions were always excluded from the calculation to avoid registering movements derived from the bar removal at the Smith machine.

Blood sampling

Blood samples were collected from the antecubital vein when the subjects arrived to the experimental site after 10-15 minutes of resting passively. Then new blood samples were collected again from the antecubital vein at 15 minutes post-test and at 24 hours post-test. Qualified nursing staff was responsible of conducting this procedure. The samples were stored in a container for serum analysis. Samples were transported at -4 ºC to the Biomedical Research Centre of the University of Granada. Samples were centrifuged at 3,000 rpm for 15 min at 4 °C, the resulting supernatants (serum) were transferred to clean tubes and frozen (-80 ºC) until analysis of oxidative stress and muscle damage.

Lipid Peroxidation

Hydroperoxides were measured as a specific and direct biomarker of lipid peroxidation process (34). Plasma hydroperoxides were determined using the Sigma

PD1 kit (Saint Louis, MO, USA). Absorbance changes at 560 nm were monitored spectrophotometrically.

Malondialdehyde (MDA) was measured by the thiobarbituric acid reactive substances (TBARS) assay. TBARS is one of the various low-molecular-weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products and is considered a sub-product of cellular oxidative stress. Plasma MDA was measured as described by Orrenius (24) through the spectrophotometric measurement of substances which react with TBARS. The reaction of MDA-thiobarbituric acid (TBA) leads to an orange pigmentation, which was then read spectrophotometrically on a U-bottomed microplate at 532 nm.

Serum CK-MB and LDH activities

CK-MB and LDH activities were measured by spectrophotometry according to the manufacturer's recommendations, by a manual procedure using commercial kits (Spinreact, S.A. Girona, Spain, for LDH, and Spinreact, S.A. Girona, Spain, for CK-MB). Results are expressed as U/L.

α-Actin quantitation

Sarcomeric α-actin was determined by Western blot. A sample of serum (5 µl) from each subject was dissolved in sample buffer (62.76 mm Tris-HCl pH 6.8, 1% 2-mercaptoethanol, 1% SDS, 10% glycerol, and 0.01% bromophenol blue) at 1:3 ratio, boiled for 10 minutes, microfuged for 10 seconds and analyzed by SDS-PAGE (sodium dodecylsulphate polyacrylamide gel electrophoresis) in a Mini Protean transfer system (Bio-Rad, Hercules, California, USA) at 60 mA for 90 minutes at room temperature. Proteins were separated electrophoretically and transferred onto a 0.2 µm nitrocellulose

membrane (Trans-Blot transfer pack, Bio-Rad) by applying a current of 25 v at room temperature for 7 minutes using a Trans-Blot Turbo apparatus (Bio-Rad). The blots were treated with blocking solution (5% non-fat milk in TBST buffer: 20mM Tris, 0.9 % NaCl) for 1 h at room temperature. Later on, the blots were incubated with the primary antibody (Actin (C-11): sc-1615, 1:1000 dilution, Santa Cruz Biotechnology, Dallas), overnight at 4^{o} C. Membranes were subjected to three 15-minute washes in 5% TBST, incubated with the secondary antibody (bovine anti-goat IgG-HRP: sc-2350, 1:5000 dilution, Santa Cruz Biotechnology, Dallas) for 1 h at room temperature, and again washed 3 times in 5% TBST.

Immunoreactive signals were detected via enhanced chemiluminescence (SuperSignal West Dura Chemiluminescent Substrate, 34075, Thermo Scientific, Europe), and the membranes were digitally imaged and analyzed using ImageJ software for densitometric analysis. Results are expressed as the fold-change in expression relative to the control. α-Actin was quantitated by comparing the bands with a standard curve obtained by immunoblotting known amounts of pure α-actin.

Statistical analysis

Data are expressed as mean ± standard error of the mean. The normality of distribution was assessed by the Shapiro-Wilk test. The homogeneity of variance was analysed through the Levene test. One-way ANOVA test was used to compare the mean values of oxidative stress and muscle damage between squat vs bench press exercise, followed by Bonferroni post-hoc test. The level of significance was set at $p<0.05$. All statistical procedures were performed using SPSS/PC V. 22 (SPSS Inc., Chicago, IL).

RESULTS

The goal of this work was to determine whether the muscle mass involved in bench press and squat exercises affects skeletal and cardiac muscle damage and oxidative stress. A total of 10 well-trained athletes were recruited for this purpose. Features of these athletes are described in table 1. The 1RM values of half-squat and bench press were 131.00 ± 1.24 kg and 86.1 ± 1.61 kg (mean ± SEM), respectively. The measurements of hematocrit and haemoglobin concentrations of athletes were used as haematological parameters. The TBARS and HPX concentrations were markers as markers of lipid peroxidation (table 2). All four parameters remained unchanged 15 min and 24 h after bench press exercise. However, HPX concentration significantly increased 24 h after squat exercise compared with its value at 15 min.

The evolution of the electromyographic activity from 20% RM to 1RM is shown in table 3. Values followed an upward trend regardless of the muscle involved, and were maximal for 1RM. The main finding of electromyography was that records were much greater at 1RM for muscles participating in bench press exercise compared with squat.

We then determined CK-MB activity, LDH activity and α-actin as markers of cardiac, systemic and skeletal muscle damage, respectively. We found differences in CK-MB and LDH between the two types of exercise and also throughout time (figures 1A and 1B). Both CK-MB and LDH activities decreased after 24 h of squat compared with values at rest and at 15 min post-exercise. In addition serum CK-MB values were significantly higher in bench press athletes compared with squat athletes. In contrast, α-actin values remained constant regardless of the time and type of exercise assessed (figure 1C).

DISCUSSION

This study aimed to determine whether muscle damage and oxidative stress are higher in sporting movements that involve large muscle mass, such as half-squat and bench press. Results obtained show that no increase in lipid peroxidation took place at 15 minutes post-test after a maximal incremental strength test with either bench press or half-squat. However, two muscle damage parameters, CK-MB and LDH, were higher after the bench press exercise compared to the half-squat exercise.

No differences were found in the blood markers of lipid peroxidation, despite the performed effort, at 15 minutes post-test. This absence of differences might be explained by the low number of muscle groups involved in each exercise and the short duration of the explosive movements. Likely this short duration is insufficient to allow the production of ROS to trigger the lipid peroxidation cascade (31). Most researchers suggest that xanthine oxidase-dependent ROS production, activated during repeated periods of ischemia-reperfusion, can be produced in this kind of sports (25). Nevertheless, the xanthine oxidase-dependent ROS production was not found at our experimental conditions, likely because as we stated above, the ischemia-reperfusion phase was too short in our study.

Muscle damage markers showed a tendency to release larger amounts of CK-MB, LDH and α-actin in bench press compared with half-squat. This finding, together with the absence changes in lipid peroxidation, indicates a non-metabolic origin of the muscle damage. Previous studies have shown that muscle damage could be induced by mechanical stress, especially eccentric muscle contraction (26). The mechanism is still unknown and probably requires a transient increase in intracellular calcium. In fact, an overload of intracellular calcium activates calpain (a calcium dependent protease), which destroys muscle protein (13). Therefore these findings lead us to think that this

muscle damage could be mostly due to a mechanical stress rather than a metabolic stress caused by eccentric exercise.

Paradoxically, greater CK-MB and LDH activities were found in bench press compared with half-squat in our study, that is, greater activities in the exercise involving lower muscle mass. Likely, the maximum load in half-squat is distributed throughout the body, thus decreasing the potential damage. This interpretation is supported by the results described by Athanasios Z. et al., who compared the eccentric action between arms and legs at the same relative intensity, finding a larger increase in muscle damage markers (CK, LDH and myoglobin) during the exercise of the arms (18). Another explanation for this difference could be the systoles produced during load (overload at press) when physical activity is performed at high loads sustained over time, likely producing a higher heart damage. Our results are consistent with data of the study conducted by Calbet et al., who showed greater blood pressures during small mass exercise compared to large muscle mass exercise (4). In addition, another study determining whether the estimated work of the heart close to exhaustion is greater during arm cranking compared to two-legged pedalling, concluded that the estimated work performed by the heart at maximal exercise intensity during arm cranking in the upright position is even higher than that measured during leg pedalling (3). Also, the lower CK-MB and LDH values found in half-squat in comparison with bench press could be caused by a great efficiency of the post-test clearance mechanisms and related with the conservation of energy and essential micronutrients. In fact, these proteins have a high content of essential amino acids, which would be used for the synthesis of enzymes and even for the energy production during the recovery. However, no significant decrease in these markers over time was found in our study in bench press.

Furthermore, the muscle damage was not exclusive of heart origin but also systemic, since both CK-MB and LDH activities increased in serum. Since the increase was much lower for CK-MB, these data suggest a predominance of systemic damage.

The electromyography performed during our study showed higher average values of aRMS at 1RM (that is, at intensities that involve a great effort) in bench press than in half-squat. Therefore, exercises involving lower muscle mass leading to higher electomyographic values under equivalent workloads would suggest that an increased muscle damage can be produced. Thus, our electromyographic results are consistent with those described by Farup and Sørensen (9) who examined the force development characteristics in the upper body after a maximal bench press intervention, which could explain this paradigm. These authors found that a higher muscle fatigue is induced in bench press compared to squat at equivalent workloads, explaining these results by the induction of higher levels of muscle activation in the elbow extensors compared to the hip extensors (9). This increase in muscle fatigue suggest the presence of a larger muscle damage induction (7).

In contrast with the enzyme results, no significant differences were found in α-actin results, likely because CK-MB and LDH are cytoplasmic enzymes whereas the α-actin is a sarcomeric protein and the muscle damage would affect more the cytoplasmic membrane than the myofibrils. This is consistent with the results of Amat et al., (2005). These authors analysed the muscle damage induced in subjects that had undergone a severe accident and found that the cytosolic markers of muscle damage reached values 10 times above the control group. However, the α-actin in their study only reached 3-times the value of the resting conditions (21). Therefore, our α-actin values would require a severe muscle damage at sarcomeric level to be able to increase α-actin release in blood in an equivalent proportion than those of CK-MB and LDH.

We are aware that our study had some limitations, the main being that a specific marker of skeletal muscle damage does not exist and, accordingly, could not be used to perfectly identify the origin of the muscle damage in both exercises.

In summary, our study evidences that the muscle damage would be caused by mechanical more than by metabolic stress at maximum efforts of short duration repeated over time. In addition, the redistribution of a maximum load between several muscle groups, as occurs in half-squat compared to bench press, attenuates the skeletal and heart muscle damage.

PRACTICAL APPLICATIONS

When coaches set up a training program to their athletes based on strength exercises according to their percentage of 1RM, they should carefully choose the exercise depending on the amount of muscle mass used. Therefore, subjects starting a training program in sport centres should begin with exercises involving large muscle groups, as they are less aggressive than the exercises with lower muscle mass involvement. Moreover it would be desirable to add a period of initiation where the user acquires physical adaptations necessary to avoid any injury at heart level.

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FIGURE LEGENDS

Figure 1. Values of muscle damage in bench press (black bars) and squat (white bars) exercises in response to maximum incremental strength tests at different time-points. (A) Creatine kinase-MB (CK-MB). (B) Lactate dehydrogenase (LDH). (C) α-Actin. *$p<0.05$, bench press vs squat. #$p<0.05$ as indicated in the figure.

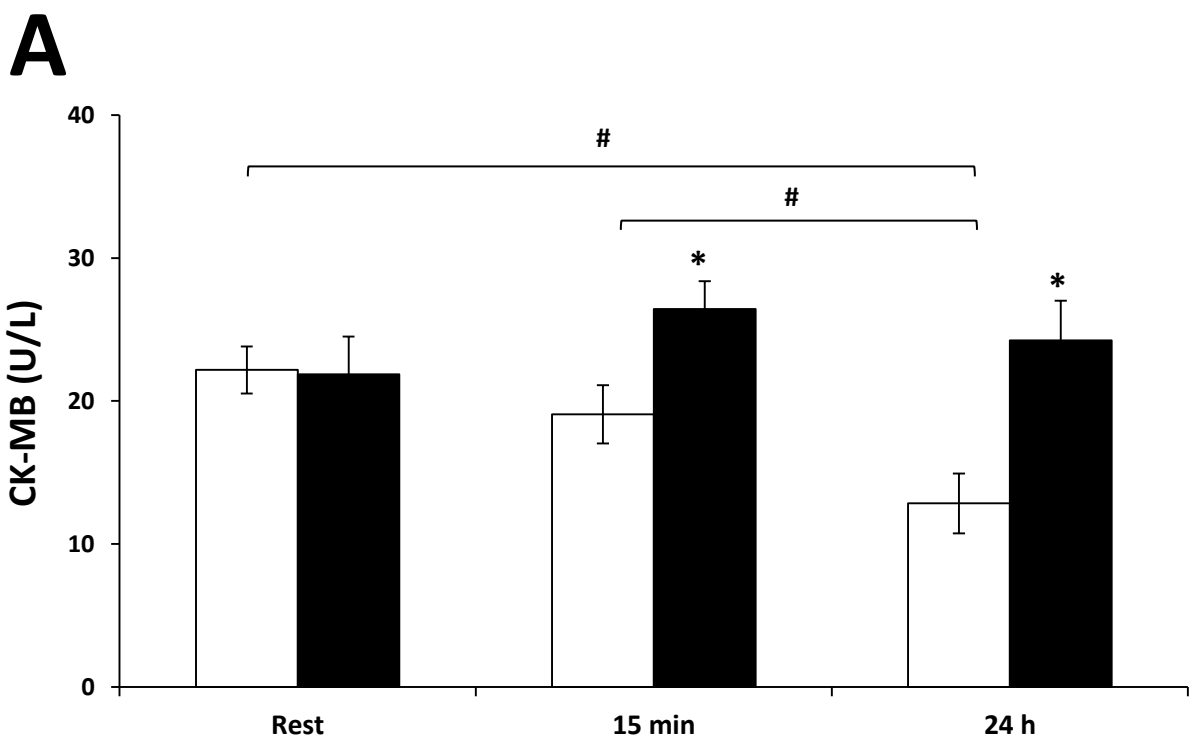

Figure 1A

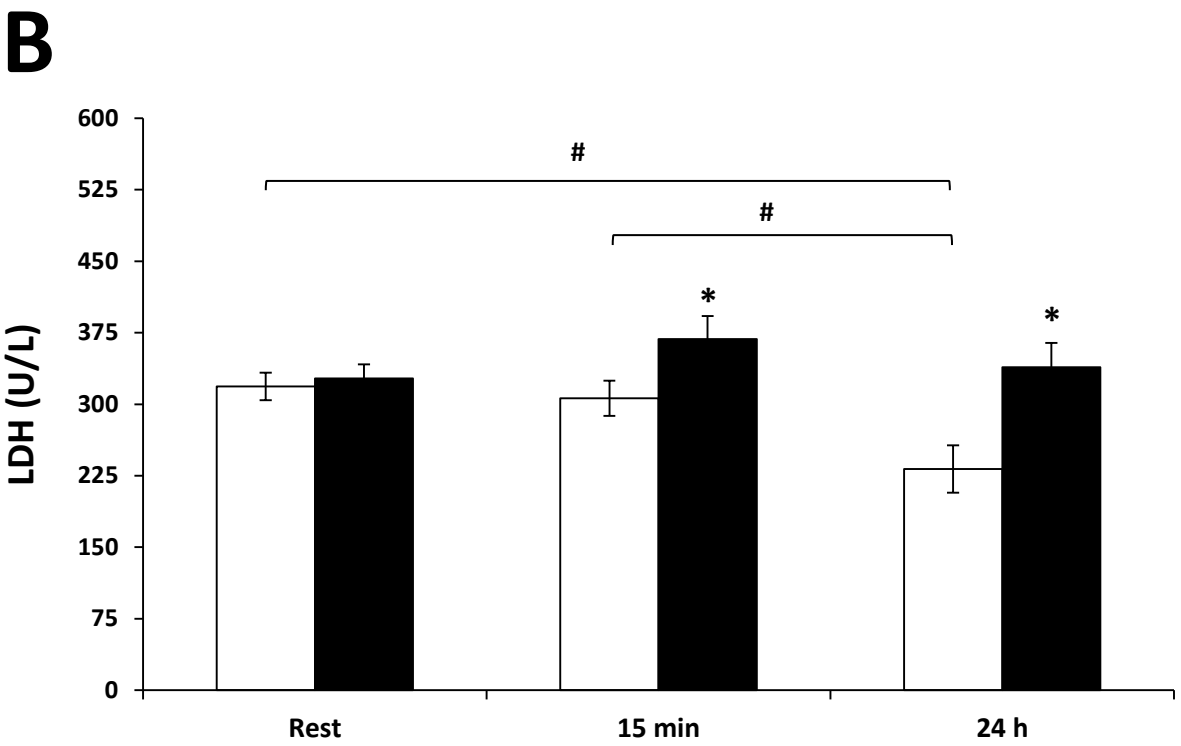

Figure 1B

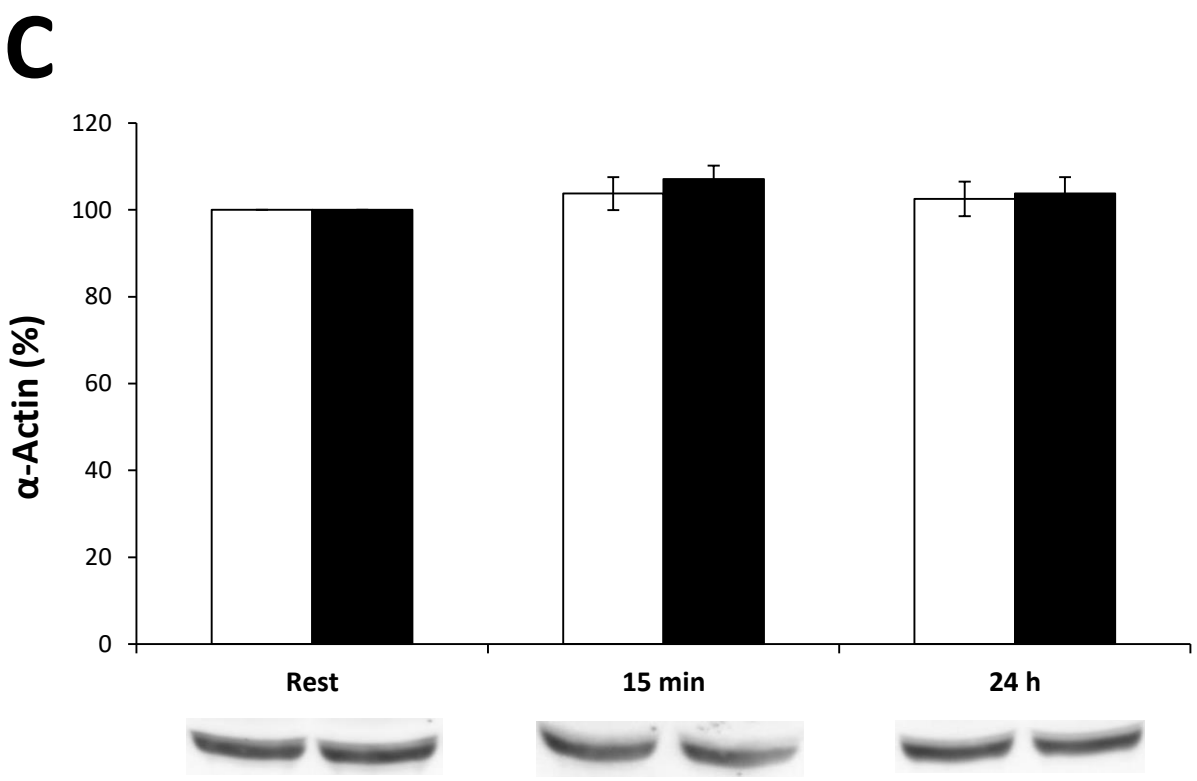

Figure 1C

Table

Table 1. Characteristics of participants

Parameter	**Units**	*Mean*		*SEM*
Age	Years	22,76	±	0,77
Weight	kg	76,97	±	1,11
Height	cm	180,00	±	1,44
BMI	kg/m^2	23,81	±	0,48
Fat mass	kg	6,78	±	0,48
Muscle Mass	kg	38,86	±	0,60
Perimeter Thigh	cm	53,30	±	0,79
Leg - Perimeter	cm	37,64	±	0,48
Leg - Muscle Area	cm^2	195,01	±	6,80
1RM of Squat	kg	131,00	±	1,24
1RM of Press Bench	kg	86,10	±	1,61

1 RM: One repetition maximum; BMI: Body mass index. Results are expressed as mean ± standard error of the mean (SEM). n = 10 athletes.

Table 2. Haematological parameters and serum lipid peroxidation markers

		Squat			Bench Press		
Parameter	Unit	Rest	15' Post	24 H. Post	Rest	15' Post	24 H. Post
Tbars	nmol/ml	6,97 ± 0,67	7,72 ± 0,70	6,61 ± 0,86	6,53 ± 0,73	7,30 ± 0,80	7,22 ± 0,41
HPX	nmol/ml	4,00 ± 0,32	3,72 ± 0,50	4,73 * ± 0,36	4,12 ± 0,34	4,10 ± 0,30	4,33 ± 0,31
Hemoglobin	g/100ml	12,21 ± 0,26	12,63 ± 0,19	±	13,53 ± 0,29	13,34 ± 0,24	±
Hematocrit	%	46,10 ± 0,50	46,30 ± 0,55	±	47,20 ± 0,61	47,30 ± 0,73	±

TBARS, Thiobarbituric acid reactive substances; HPX, Lipid peroxidation. Results are expressed as mean ± standard error of the mean (SEM). * $p < 0.05$ 15 min vs 24 h. n = 10 athletes

Table 3. The electromyographic recording

	Bench Press				
Muscle	**20%**	**40%**	**60%**	**80%**	**100%**
Anterior Deltoid	2,89 ± 0,49	5,24 ± 1,33	11,05 ± 2,22	24,80 ± 7,85	94,87 ± 21,85
Pectoralis Major	0,83 ± 0,18	1,25 ± 0,19	2,72 ± 0,54	6,60 ± 1,04	44,45 ± 13,28
Latissimus Dorsi	0,56 ± 0,14	0,71 ± 0,22	1,94 ± 1,16	3,37 ± 1,43	18,38 ± 5,10
Triceps Brachii	1,18 ± 0,17	2,51 ± 0,77	4,42 ± 1,12	10,31 ± 1,94	46,20 ± 10,39
	Squat				
Vastus Medialis	3,24 ± 0,46	4,42 ± 0,66	6,02 ± 0,92	8,05 ± 1,72	15,84 ± 5,43
Vastus Lateralis	3,31 ± 0,50	4,57 ± 0,81	5,95 ± 0,95	8,42 ± 1,47	13,18 ± 3,67
Rectus Femoris	2,52 ± 0,40	4,00 ± 0,76	4,74 ± 0,67	8,65 ± 2,35	13,24 ± 4,61
Biceps Femoris	0,85 ± 0,15	1,03 ± 0,16	1,20 ± 0,23	1,46 ± 0,32	2,96 ± 0,93

Results are expressed in millivolts as mean ± SEM. n = 10 athletes.

VI

SEX DIFFERENCES IN MUSCLE AND OXIDATIVE DAMAGE AFTER A MAXIMAL STRENGTH EXERCISE

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SEX DIFFERENCES IN MUSCLE AND OXIDATIVE DAMAGE AFTER A MAXIMAL STRENGTH EXERCISE

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SEX DIFFERENCES IN MUSCLE AND OXIDATIVE DAMAGE AFTER A MAXIMAL STRENGTH EXERCISE

Running title: Sex differences in muscle and oxidative damage.

ABSTRACT

Purpose: High intensity exercise induces muscle damage and lipid peroxidation. Whether there are differences between sexes in these two variables or not is a matter that has been scarcely studied. The aim of the present work was to investigate lipid peroxidation and muscle damage at both cardiac and skeletal levels in male and female athletes subjected to a maximal incremental strength test. Method: Twenty college athletes (10 males and 10 females) performed a half squat exercise consisting of five incremental intensities: 20%, 40%, 60%, 80% and 100% of the one repetition maximum. Neuromuscular involvement during the test was measured by recording the surface electromyographic activities of the right side of *vastus medialis* and *rectus femoris*. Blood samples were collected at rest, 15-min and 24-hr post-test. Lactate dehydrogenase and creatine kinase MB isoform activities and lipid peroxidation were measured in serum. Serum α-actin was measured as a marker of sarcomeric damage. Results: Electromyographic values were significantly higher in males compared with females at all intensities. No differences were found in muscle damage or lipid peroxidation. Conclusion: No sex differences in muscle damage or lipid peroxidation were found after the performance of a high-intensity and short-duration exercise under our experimental conditions. However, statistically significant differences for α-actin were found between males and females when this variable was normalised by their theoretical volemia.

Key words: EMG, strength, Gender, Exercise.

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INTRODUCTION

Lipid peroxidation and muscle damage in response to exercise have been extensively studied during the past decade due to their association with processes such as cell signalling and muscle adaptation to exercise (Gomez-Cabrera, Viña, & Ji, 2016). Muscle damage has been assessed in anaerobic exercises, especially weight lifting and endurance exercises (Polotow et al., 2016). These two types of exercise induce adaptive microtraumas, during both concentric and eccentric phases, although it is not elucidated if these microtraumas are caused by the mechanical action of sarcomeres or by the metabolic oxidative stress. Despite an excess of reactive oxygen species (ROS) is related to a performance decrease, a suitable production of ROS is essential for the muscle hypertrophy (Finaud, Lac, & Filaire, 2006).

Potential differences between males and females regarding the degree of oxidative stress and/or the sarcomeric damage have been poorly studied for these sport activities. In addition, most studies take into account neither the neuromuscular function nor the muscle mass involved. There are physiological differences between men and women that led us to hypothesise a distinct muscular damage and lipid peroxidation based on the sex, such as differences in: a) endocrine profile (Liu et al., 2010), b) antioxidant defence mechanisms (Barranco-Ruiz et al., 2016), and c) muscle mass and sarcomeric protein profile (Casuso et al., 2016). In fact, women have a lower percentage of slow-twitch oxidative fibres (type I) compared with men (Wiecek, Maciejczyk, Szymura, & Szygula, 2015). Moreover, men develop more strength and power than women because men have more muscle mass. In contrast, men could be more susceptible to oxidative stress (Hunter, 2014) and to muscle damage (Wolf et al., 2012), since their metabolism and oxygen consumption is greater (Monteiro et al., 2008).

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In the present study we hypothesised that men generate more lipid peroxidation and muscle damage than women because the former have a larger muscle mass and are able to lift heavier loads. Therefore, our aim **was to investigate whether there are sex differences in lipid peroxidation and muscle damage at both cardiac and skeletal levels after a maximal incremental strength test (MIST). The finding of such sex differences would allow athletes to adjust their resting periods so as to improve their performance during training and competition.**

METHODS

Subjects

A total of 20 healthy college-aged volunteers (10 males and 10 females) were enrolled in this study. Table 1 shows the baseline features of these volunteers. All subjects were familiar with strength training and half-squat exercise. All subjects had recreational experience (1 to 3 years) in strength training including half-squat exercise. Subjects trained their lower body musculature 2–3 times per week by lifting loads of 60–90% of one repetition maximum (1RM). Smoking, high intakes of antioxidant-rich foods, ergogenic supplementation or drugs were considered exclusion criteria. All subjects were thoroughly informed of the purpose, nature, practical details and potential risks associated with the study before signing an informed consent. The study was approved by the Ethics Committee of the University of Granada (Spain) and it was in accordance with the Declaration of Helsinki.

Design

All the procedures were performed at the exercise physiology laboratory of the Faculty of Sport Sciences of the University of Granada (Spain). Half-squat exercise was the chosen procedure because it is a common method used for the assessment of maximum strength in athletes (McMaster, Gill, Cronin, & McGuigan, 2014). All subjects were assessed in the morning, in particular two hours after breakfast, at a laboratory with controlled humidity and temperature according to the guidelines established by the National Strength and Conditioning Association (NSCA -National Strength & Conditioning Association, 2008). Pre-test sessions were conducted 2 weeks prior to the experiment onset. During the first week, subjects became familiar with the experimental testing procedures. During the second week, subjects participated in a control test to identify their 1RM (Sanchez-Medina, Perez, & Gonzalez-Badillo, 2010). Four days of testing over two consecutive weeks were required to evaluate the subjects.

Anthropometry

All participants underwent an anthropometric assessment. Anthropometric data were obtained following the protocol developed by the International Society for Advanced of Kinanthropometry (ISAK) (Marfell-Jones, M, Olds, T, Stewart, A, and Carter, 2006). Anthropometric variables included body mass, height, 7 skinfolds (biceps, triceps, subscapular, supraspinal, abdominal, front thigh, and medial calf), 3 circumferences (flexed upper arm, thigh, and medial calf) and 3 breadths (humeral and femoral epicondyles and wrist). Body fat was assessed by applying the following formula (Carter, 1982):

$$\text{Body Fat (\%)} = (\sum 6 \text{ skinfolds} \times 0.1051) + 2.58$$

where the 6 skinfolds were taken from triceps, subscapular, supraspinal, abdomen, thigh, and medial calf expressed in millimetres. Body muscle mass was calculated as described by Matiegka (Brozek & Prokopec, 2001).

One repetition maximum strength testing sessions

All the participants underwent a maximal progressive test in order to determine their 1RM. Before the test, the subjects performed a warm-up which included 5 minutes on a cycle ergometer, dynamic stretching and 2 sets of 5 repetitions with 20 kg and 30 kg each. We used the 1RM test described by Fernandez-Gonzalo et al. (Fernandez-Gonzalo, Lundberg, Alvarez-Alvarez, & de Paz, 2014) for half-squat exercise. The test was carried out in a Smith machine (Life Fitness, Brunswick Corporation, USA). The load was increased by 10 kg when the subjects succeeded, whereas the load was reduced by 5 kg when the subjects failed the task. The test finished when the subjects failed to perform two consecutive attempts. All the repetitions were interspersed by a 3-minute rest and the 1RM was achieved between the third and sixth attempt.

Maximal Incremental Strength Test

The subjects were asked to refrain from exercise for at least 72 hours prior to the experiment. The MIST was performed between 08:30 and 15:00 hr. The subjects performed a warm-up routine consisting of 5 minutes of jogging and leg stretching, as well as a familiarisation set of 15 repetitions with a load at 40% of 1RM in the half-squat exercise (Smilios, Häkkinen, & Tokmakidis, 2010). The MIST was performed in a half-squat apparatus (Smith Machine) in which the barbell was attached at both ends with linear bearings on two vertical bars allowing only vertical movements. Shoulders were in contact with the bar, and the starting angle of the knee was 180º. Eccentric and concentric phases were performed continuously. Knee flexion angle was kept at 90º. To

guide the displacement path, the position that the individual needed to adopt to initiate the half-squat was determined using a manual goniometer and marked using a rod on a tripod. The subject was then indicated in each repetition to do a half-squat until touching the rod with the glutei. The accuracy error of the procedure was 1 cm (Flanagan et al., 2014). Repetitions that did not cover the whole distance were not considered. Foot spacing on the half-squat was set at approximately the same width as, or slightly wider than, shoulder width while keeping toes pointed slightly outward (Cotterman, Darby, & Skelly, 2005). The torso was kept as straight as possible and a security belt was used by all subjects (Izquierdo et al., 2003).

Five incremental intensities derived from the individual 1RM tests were used: 20%, 40%, 60%, 80% and 100% during the strength test. For the first three intensities, 5 repetitions of 5 seconds each at 20%, 40% and 60% intensities were performed. Two repetitions were performed at 80% intensity without a time limit. Finally, only 1RM (100% intensity) was completed by each participant without any help for the last load. A resting period of 3 minutes was allowed after each of the first four intensities (20%, 40%, 60% and 80%), and a 5-minute resting period was allowed between 80% and 100% intensities.

Electromyography (EMG)

Electrical muscle activation was monitored using surface EMG. The EMG signals obtained during the extension and flexion actions of the knee were recorded from the *vastus medialis* (VM) and the *rectus femoris* (RF). The muscle contractions were performed in dynamic conditions. Before the application of the EMG electrodes, the skin surface was carefully shaved and wiped with alcohol to reduce skin impedance. Bipolar single differential electrodes were placed longitudinally on the muscles following the SENIAM recommendations (Hermens, Freriks, Disselhorst-Klug, & Rau,

2000) and taped to the skin to minimize movement artifacts. The position of the electrodes was marked on the skin with indelible ink, and these references were used for precise electrode placement on repeated experiments according to Torres-Peralta et al. (2016).

The EMG signals were acquired with the help of a 4-channel recording system (Myomonitor IV, Delsys Inc., Boston, MA) at a sampling rate of 1000 Hz using rectangular shaped (19.8 mm wide and 35 mm long) bipolar surface electrodes with 1 × 10 mm 99.9% Ag conductors, and with an inter-conductor distance of 10 mm (DE-2.3 Delsys Inc.). A 12-bit analog-to-digital data acquisition card BNC-2090 (National Instruments Corporation, Austin, TX, USA) was used to sample the signal at 4000 Hz. The EMG signals corresponding to each muscle contraction were acquired and analysed using a specific program (EMGworks 4.1.7 Analysis; Delsys, Inc. Boston). After a visual inspection, the EMG recordings were filtered using a Butterworth filter with a high pass filter of 100 Hz and low pass of 450 Hz (IIR Filter, Delsys, Inc. Boston). Then the root mean square (RMS) was calculated for each contraction. In this study we determined the area under the RMS (aRMS) for each contraction.

The aRMS average of the second, third and fourth repetition of the intensities from 20% to 60% was calculated in order to ensure that the analyses were performed with repetitions involving correct rhythm and techniques. At 20%, 40% and 60% intensities, the first and last repetitions were always excluded from the calculation to avoid registering movements derived from the bar removal at the Smith machine.

Blood sampling

Blood samples were collected from the subjects' antecubital vein at time 0 as well as 15 minutes and 24 hours post-test. Samples were centrifuged at 3,000 rpm and 4 °C for 15 min to separate serum from cells.

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Lipid peroxidation

Hydroperoxides (HPX) were measured in serum as a specific and direct biomarker of lipid peroxidation (Yoshida, Umeno, & Shichiri, 2013) with the PD1 kit from Sigma (St. Louis, MO, USA) following the manufacturer's instructions. Serum Malondialdehyde (MDA) was measured by the thiobarbituric acid reactive substances (TBARS) assay as described by Orrenius (Orrenius, Moldeus, Thor, & Högberg, 1976).

Serum creatine kinase MB isoform (CK-MB) and lactate dehydrogenase (LDH) activities

CK-MB and LDH activities were measured using commercial kits purchased from Spinreact, S.A. (Gerona, Spain) following the manufacturer's instructions.

α-Actin quantification

Sarcomeric α-actin was determined by Western blot as described by Casuso et al. (Casuso et al., 2016). A sample of serum (5 µl) from each subject was placed in sample buffer (62.76 mM Tris-HCl pH 6.8, 1% 2-mercaptoethanol, 1% SDS, 10% glycerol, and 0.01% bromophenol blue) at 1:3 ratio, boiled for 10 minutes, microfuged for 10 seconds and analyzed by SDS-PAGE (sodium dodecylsulphate polyacrylamide gel electrophoresis) in a Mini Protean transfer system (Bio-Rad, Hercules, California, USA) at 60 mA and room temperature for 90 minutes. Proteins were separated electrophoretically and transferred onto a 0.2 µm nitrocellulose membrane (Trans-Blot transfer pack, Bio-Rad) by applying a current of 25 V at room temperature for 7 minutes using a Trans-Blot Turbo Transfer system (Bio-Rad). The blots were treated with blocking solution (5% non-fat milk in TBST buffer: 20mM Tris, 0.9 % NaCl) for 1 hr at room temperature. Then, the blots were incubated with the primary antibody (Actin (C-11): sc-1615, 1:1000 dilution, Santa Cruz Biotechnology, Dallas), overnight at 4^{o} C.

Membranes were subjected to three 15-minute washes in 5% TBST, incubated with the secondary antibody (bovine anti-goat IgG-HRP: sc-2350, 1:5000 dilution, Santa Cruz Biotechnology, Dallas) for 1 hr at room temperature, and again washed 3 times in TBST. Proteins were visualized by enhanced chemiluminescense (SuperSignal West Dura Chemiluminescent Substrate, 34075, Thermo Scientific).

Densitometric analysis was performed by scanning radiographic images of membranes. Image resolution was 100 points per inch. Image treatment software (Adobe Photoshop 5.0, Adobe Systems) was used to treat images, which were saved in TIF format to allow them to be accessed by the software for quantification (Quantity One 1-D, Bio-Rad). α-Actin in samples was quantified by comparison with a standard curve done with increasing concentrations of the pure actin (Sigma-Aldrich Química, S. L., Madrid, Spain) and run in parallel (Casuso et al., 2016).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). The normality of distribution was assessed by the Shapiro-Wilk test. The homogeneity of variance was analysed through the Levene test. A two-way mixed analysis of variance was used with gender as the between-subjects factor (men vs women) and time as the within-subjects factor (at rest, 15-min post-test and 24-hr post-test). Multiple comparisons were performed through the Bonferroni post-hoc test. The level of significance was set at $p<0.05$. All statistical procedures were carried out using SPSS/PC V. 22 (SPSS Inc., Chicago, IL, USA).

RESULTS

Anthropometry

The features of the participants are described in Table 1. Male and female participants were of the same age. However, participants significantly differed in weight, height, body mass index (BMI), 1RM, fat and muscle mass, leg muscle area, and perimeters of thigh and leg.

Lipid peroxidation and muscle damage

TBARS and HPX were used as biomarkers of oxidative stress (Figure 1). No statistically significant differences between sexes were found in TBARS and HPX concentrations (Figure 1A and 1C).

CK-MB and LDH activities along with α-actin concentration were used as markers of muscle damage (Figure 2). There were no differences in CK-MB, LDH and α-actin between sexes (Figure 2A, 2C and 2E). In males, LDH was significantly lower at 24 hours post-test than at rest and 15 minutes post-test (Figure 2C).

Values of lipid peroxidation normalised by blood volume are shown in Figure 1B and 1D. HPX values were different in males at 24 hours post-test compared with females (Figure 1D). TBARS values showed no differences between sexes (Figure 1B). Values of muscle damage normalised by blood volume are shown in Figure 2. At rest, CK-MB activity was significantly higher in males than in females (Figure 2B). The LDH activity was significantly higher in males compared with females at rest and 15 minutes post-test (Figure 2D). α-Actin concentration was significantly higher in males than in females at rest, 15 minutes and 24 hours post-test (Figure 2F).

Electromyographic record

Vastus medialis and *rectus femoris* were analyzed electromyographically throughout the MIST in male and female athletes. EMG signals obtained from both muscles were significantly higher in males compared with females at all intensities (Figure 3A and 3B). In males, the EMG signals obtained with 1RM from both muscles were significantly greater compared with the rest of intensities (Figures 3A and 3B). Statistical differences in EMG signals between males and females remained when the values were normalised by muscle mass (Figures 3C and 3D). There was a statistically significant correlation between aRMS and the load lifted in the *vastus medialis* (Figure 4A) and *rectus femoris* (Figure 4B), although there were no significant differences between sexes.

DISCUSSION

The aim of this work was to investigate lipid peroxidation and muscle damage at both cardiac and skeletal levels in young male and female athletes subjected to a maximal incremental strength test. Physical activity did not affect the markers of damage in skeletal and cardiac muscles in males nor in females. TBARS and HPX also remained unchanged. These results could be due to the sport movement involved in the test, which is explosive and of short duration and therefore could not stimulate the xanthine oxidase system, activated during the repetitive periods of ischemia/reperfusion typical in strengthening exercises (Powers, Nelson, & Hudson, 2011). Therefore, due to the limited participation of the aerobic metabolism, the main way of obtaining energy would be through the reserve phosphagen system, decreasing the production of reactive oxygen species. Consistently with our results, although using

267 a different experimental design, Child et al. (1999) could not find evidence that chronic
268 muscle inflammation compromises antioxidant status or increases lipid peroxidation
269 after 70 maximal eccentric muscle contractions. Other authors, using a strength protocol
270 (7 x 10 eccentric repetitions of the knee extensors at 150% 1RM) did not show
271 differences in oxidative stress at 24, 48 and 72 hours post-exercise (Kerksick, Taylor,
272 Harvey, & Willoughby, 2008).

273 In our study, we only found a significant decrease of LDH in men, induced by
274 the physical activity at 24 hours post-test. This decrease in LDH could be due to the
275 larger muscle mass of men, which may lead to a greater ability to clear blood enzymes
276 compared to women. However, other mechanisms may not be ruled out.

277 When comparing by sex, we did not find statistically significant differences in
278 the markers of lipid peroxidation or muscle damage at each of the time points studied.
279 Stupka et al. (2000) found similar results in CK after a protocol of eccentric exercises in
280 both males and females. Similarly, although using indirect methods such as delayed
281 onset muscle soreness and muscle thickness, Radaelli et al. (2014) showed that both
282 males and females experienced similar muscle damage after a traditional strength
283 training. These results are striking because males involve a larger muscle mass and lift
284 larger loads than women and accordingly lipid peroxidation and the muscular damage
285 would be expected to be greater in men.

286 The electromyographyc records showed values of aRMS significantly higher in
287 males for both VM and RF, even when data were normalised to muscle mass (Figure 3).
288 Similarly to our results, Ebben et al. (2009), when normalising electromyographic data
289 by muscle mass obtained significantly higher values in men than in women. Moreover,
290 when aRMS values were correlated with their respective lifted loads, we found that they

adjusted to a statistically significant exponential regression curve ($p<0.05$) (Figure 4). For equal loads lifted by males and females, EMG signals were similar. The only difference was that males lifted heavier loads than females, and in fact, when females reached their 1RM, males were only mobilising 60% of their 1RM.

The different volemia of males and females may account for the finding that even though males i) have a larger muscle mass, ii) lift heavier loads and iii) show higher electromyographic signals, they do not generate more muscular damage at both skeletal and cardiac levels than females (Green et al., 1999). CK-MB, LDH and α-actin would be more diluted in the blood of males. Therefore, we calculated the theoretical blood volume of our athletes using the formula proposed by Nadler et al. (1962) obtaining an average volemia of 5.22±0.07 L for males and 3.82±0.13 L for females. This difference of 1.4 L might mask larger muscle damage and lipid peroxidation markers in males, as shown in Figures 1 and 2. However, further studies would be needed to confirm these results because, to date, there are no experimental designs that normalise the blood markers analysed by the actual values of blood volume. After the normalisation by blood volume, males exhibited increases in markers of both lipid peroxidation and muscle damage, since the α-actin values were higher in males than in females for the three time points analysed. These results are consistent with our initial hypothesis.

Distinct expression patterns in genes encoding sarcomeric proteins, mainly the proteins responsible for the binding of α-actin to desmin, might also account for the differences obtained between males and females. In fact, α-actinin-3 is known to be expressed mainly in type IIx fibres, prevailing in males, and that would be related with a stronger actin-desmin binding (Lek & North, 2010).

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WHAT DOES THIS ARTICLE ADD?

No sex differences in muscle damage or lipid peroxidation were found after the performance of a high-intensity and short-duration exercise under our experimental conditions. However, statistically significant differences were found between males and females for α-actin, at rest and after exercise, when the variable was normalised to the theoretical blood volume. The larger concentrations of α-actin found in males could be related to their larger muscle mass and heavier loads lifted.

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438

439 **FIGURE LEGENDS**

440

441 **Figure 1:** Lipid peroxidation of males and females at rest and 15 min and 24 hr post-
442 test. A, Hydroperoxide (HPX) concentration. B, HPX concentration normalised by
443 blood volume. C, Thiobarbituric acid reactive substances (TBARS) concentration. C,
444 TBARS concentration normalised by blood volume. * $p<0.05$ males *vs*. females.

445

446 **Figure 2:** Markers of muscle damage of males and females at rest and 15 min and 24 hr
447 post-test normalised by blood volume. A, Creatine kinase MB isoform (CK-MB)
448 activity. B, CK-MB activity normalised by blood volume. C, Lactate dehydrogenase
449 (LDH) activity. D, LDH activity normalised by blood volume. E, α-Actin concentration.
450 F, α-Actin concentration normalised to blood volume. *$p<0.05$ males *vs*. females. #
451 $p<0.05$ 24 hr post-test *vs*. rest. @ $p<0.05$ 24 hr post-test *vs*. 15 min.

452

453 **Figure 3:** Electromyographic signals of *vastus medialis* and *rectus femoris* in males and
454 females. Panels A and C show results for *vastus medialis*. Panels B and D show results
455 for *rectus femoris*. Panels C and D show results normalised to muscle mass. *$p<0.05$
456 males *vs*. females. # $p<0.05$ 1 RM (100% intensity) *vs*. other intensities.

457

458 **Figure 4:** Correlation between aRMS and 1RM load. A, *vastus medialis*. B, *rectus*
459 *femoris*.

460

Table 1. Characteristics of participants

		Males		Females	
Parameter	**Units**	**Mean**	**SEM**	**Mean**	**SEM**
Age	Years	22.76 ±	0.77	22.70 ±	0.42
Weight	kg	76.97 ±	1.11	60.01 ±	2.23*
Height	cm	180.00 ±	1.44	167.60 ±	2.15*
BMI	kg/m^2	23.79 ±	0.15	21.30 ±	0.13*
1 RM	kg	131.00 ±	1.24	73.45 ±	3.31*
Fat mass	kg	6.78 ±	0.48	11.35 ±	0.29*
Muscle mass	kg	38.86 ±	0.60	26.52 ±	0.92*
Leg - Muscular area	cm^2	195.01 ±	6.80	132.35 ±	6.67*
Thigh Perimeter	cm^2	53.30 ±	0.79	49.03 ±	0.79*
Leg Perimeter	cm^2	37.64 ±	0.48	35.56 ±	0.52*

1 RM: One repetition maximum. BMI: Body mass index. Results are expressed as mean ± SEM. n = 10 each males and females. * $p<0.05$ males vs females.

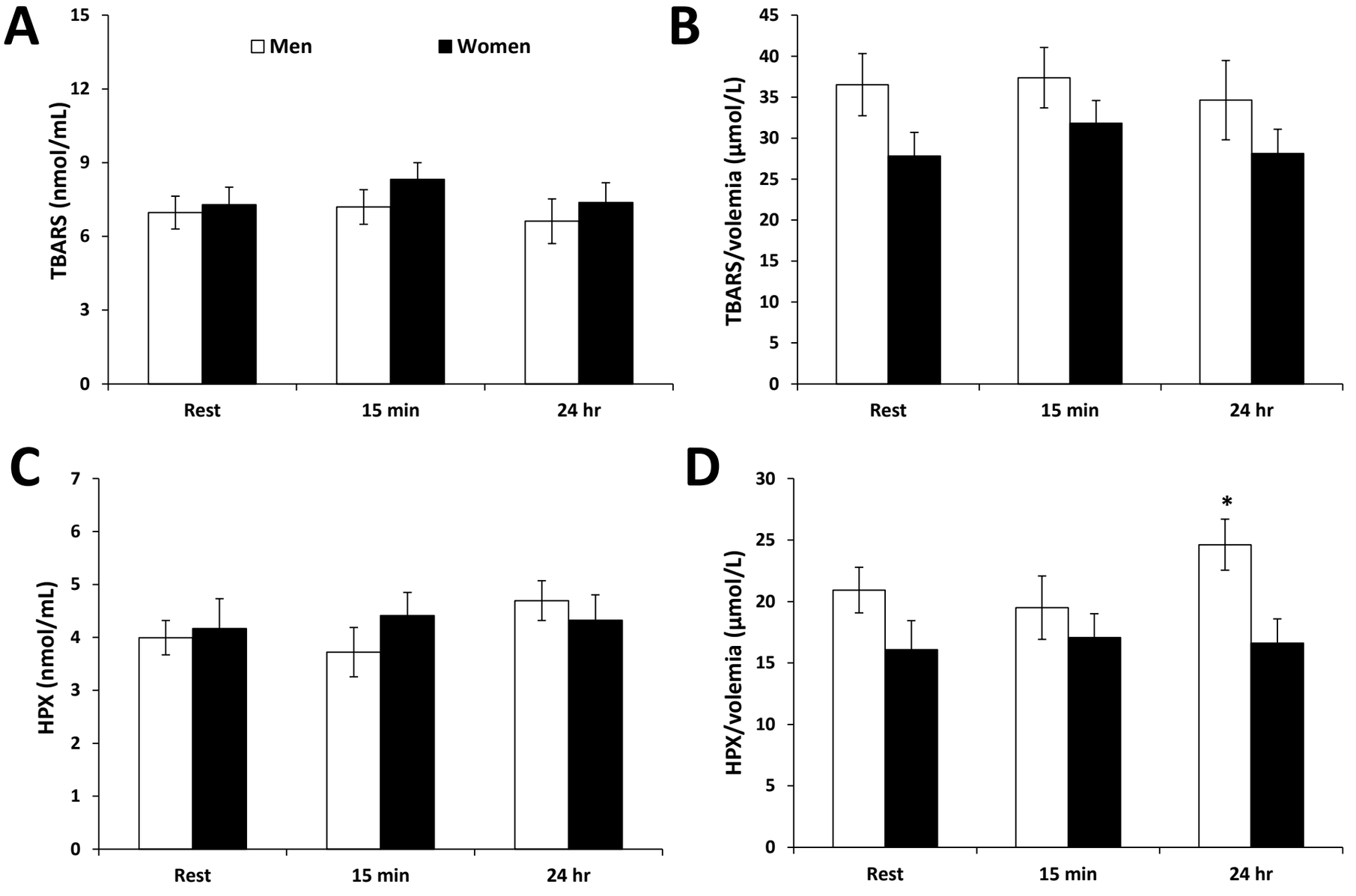
A
Men
Women
TBARS (nmol/mL)
0
3
6
9
12
15
Rest
15 min
24 hr
B
TBARS/volemia (µmol/L)
0
5
10
15
20
25
30
35
40
45
Rest
15 min
24 hr
C
HPX (nmol/mL)
0
1
2
3
4
5
6
7
Rest
15 min
24 hr
D
HPX/volemia (µmol/L)
0
5
10
15
20
25
30
*
Rest
15 min
24 hr

Figure 1

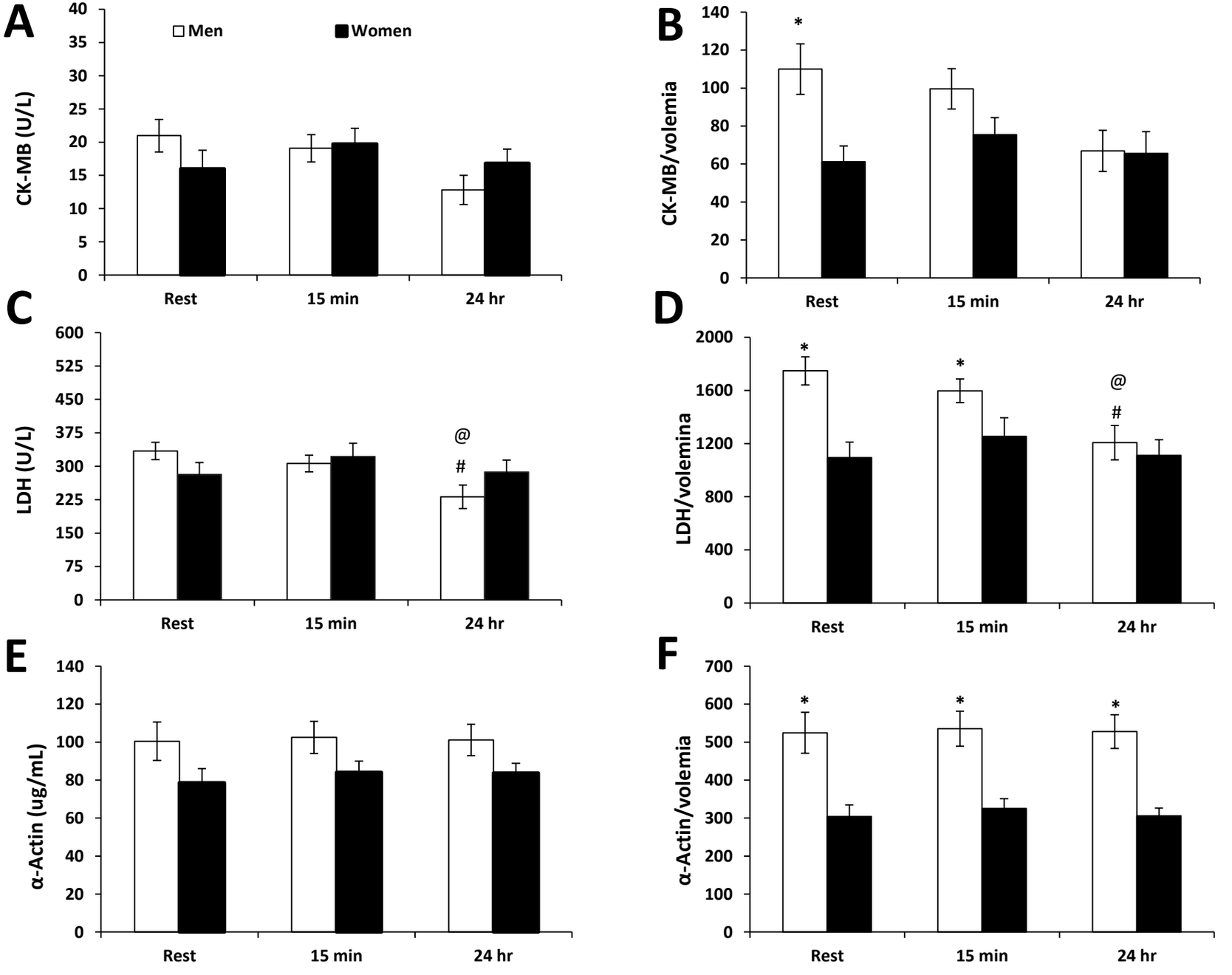
A
Men
Women
CK-MB (U/L)
0
5
10
15
20
25
30
35
40
Rest
15 min
24 hr
B
CK-MB/volemia
0
20
40
60
80
100
120
140
*
Rest
15 min
24 hr
C
LDH (U/L)
0
75
150
225
300
375
450
525
600
@
#
Rest
15 min
24 hr
D
LDH/volemina
0
400
800
1200
1600
2000
*
*
@
#
Rest
15 min
24 hr
E
α-Actin (ug/mL)
0
20
40
60
80
100
120
140
Rest
15 min
24 hr
F
α-Actin/volemia
0
100
200
300
400
500
600
700
*
*
*
Rest
15 min
24 hr

Figure 2

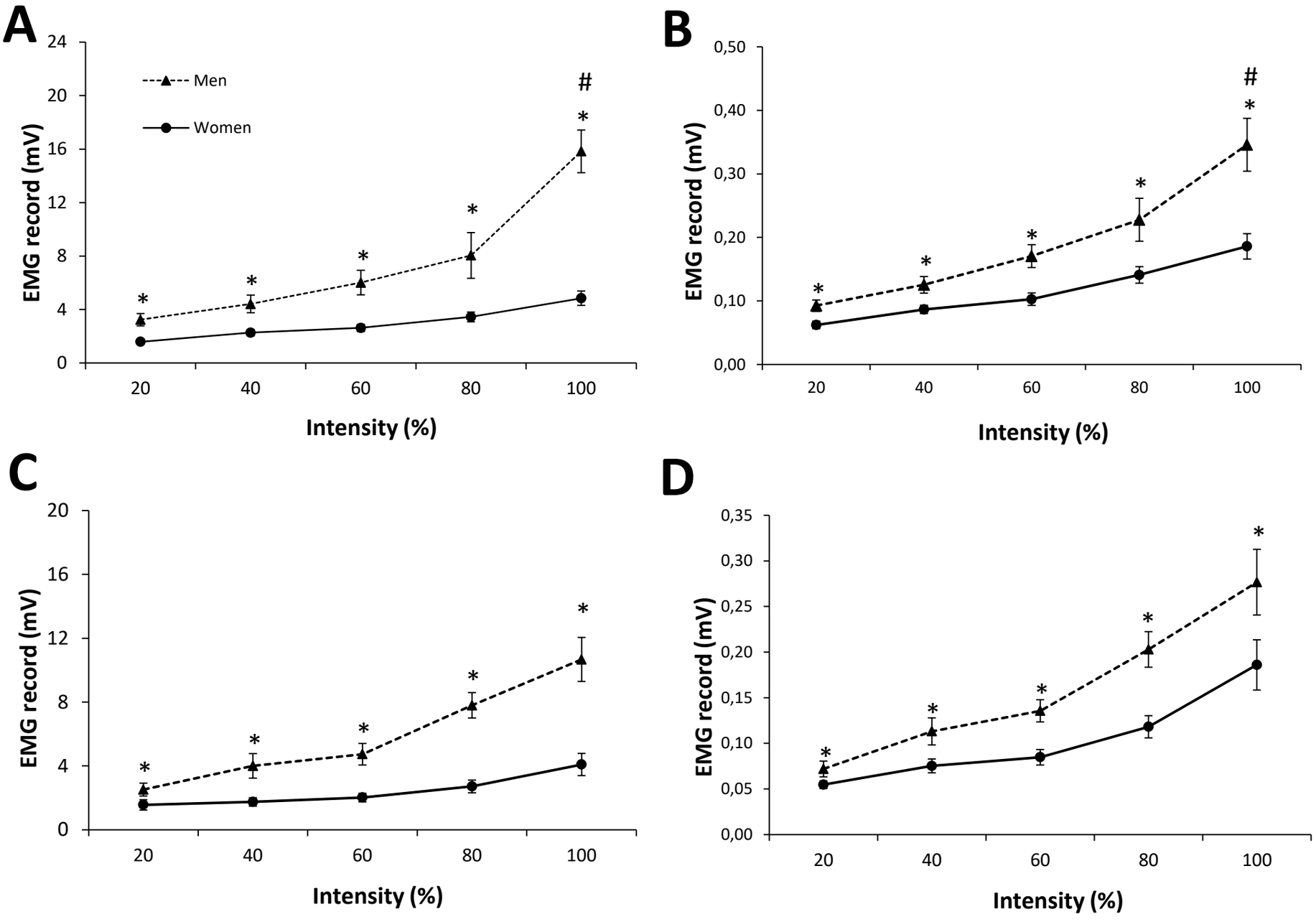

Figure 3

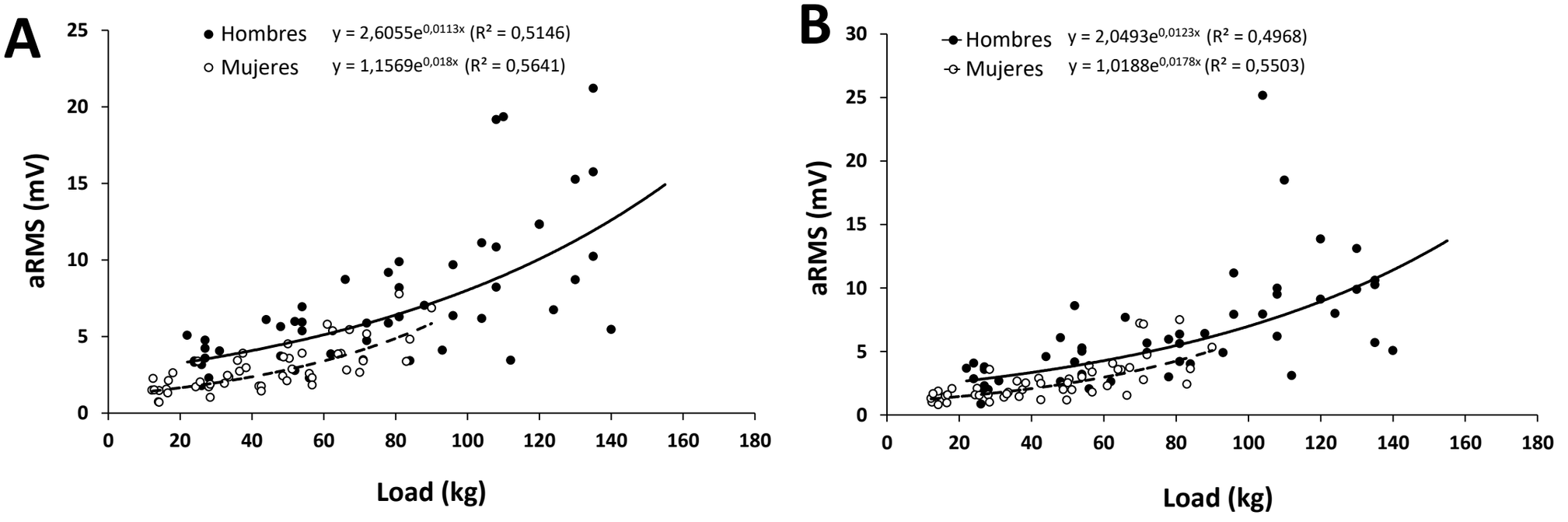
A
Hombres y = 2,6055e0,0113x (R² = 0,5146)
Mujeres y = 1,1569e0,018x (R² = 0,5641)
aRMS (mV)
Load (kg)
B
Hombres y = 2,0493e0,0123x (R² = 0,4968)
Mujeres y = 1,0188e0,0178x (R² = 0,5503)
aRMS (mV)
Load (kg)

Figure 4

CONSENTIMIENTO INFORMADO

TÍTULO DEL PROYECTO: Marcadores de daño sarcomérico y metabólicos y registros EMG con cargas crecientes en levantadores de pesas.

INVESTIGADORES: Luis Javier Chirosa Ríos, Ignacio Chirosa Ríos y Jesús Francisco Rodríguez Huertas

Yo ....., mayor de edad, y con N° de DNI: ..... por el presente consiento que se me tomen 3 muestras de sangre de 2 ml (reposo, tras el test y a los 15 minutos de recuperación), se me realice una antropometría y se me someta a un cuestionario médico y nutricional.

Asimismo consiento se haga una analítica de mis muestras biológicas, reservándome el derecho de su destrucción y la posibilidad de solicitar cualquier información que se desprenda de ellas.

Afirmo que se me ha explicado la naturaleza, el diseño experimental y el objetivo de lo que se me propone, incluyendo riesgos significativos. Estoy satisfecho con esas explicaciones y las he comprendido.

Granada..... de ..... de 2013

Firma y DNI ...

_____

Por la presente certificamos que hemos explicado la naturaleza, propósito, beneficios, riesgos y alternativas del procedimiento propuesto, nos hemos ofrecido a contestar cualquier pregunta y hemos contestado completamente todas las preguntas hechas. Creemos han comprendido completamente lo que hemos explicado y contestado.

Firma y DNI del responsable de la investigación: