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Efectos de la hipoxia hipobárica intermitente en la recuperación del daño muscular inducido por ejercicio excéntrico en ratas entrenadas

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Índice

Abreviaturas

ANT	Translocador del adenosín-nucleótido (*Adenosine Nucleotide Transporter*)
CCA	Número de capilares por cada 1.000 μm^2 de área de fibra (C*apillary Counts per Area*)
CD	Densidad capilar (*Capillary Density*)
C/F	Cociente entre el número de Capilares y el número de Fibras (C*apillary-to-Fibre ratio*)
CK-MM	Creatina quinasa MM (*creatine kinase MM*)
CS	Citrato Sintasa
DOMS	Dolor muscular de aparición tardía (*Delayed Onset Muscle Soreness*)
Drp-1	Proteína relacionada con la dinamina-1 (*Dynamin-related protein-1*)
eATPasa	Adenosín-trifosfatasa endotelial
EEE	Ejercicio Excéntrico Extenuante
EEIMD	Daño muscular inducido por ejercicio excéntrico (*Eccentric Exercise-Induced Muscle Damage*)
FCSA	Área de la sección transversal de la fibra *(Fibre Cross-Sectional Area)*
FD	Densidad de fibras (*Fibre Density*)
FOG	Fibras rápidas oxidativas glicolíticas (*Fast Oxidative Glycolytic*)
GAS	Gastrocnemio
HHI	Hipoxia Hipobárica Intermitente
HIF	Factor inducible por hipoxia (*Hypoxia Inducible Factor*)

ICC	Coeficiente de correlación intraclase (*Intraclass Correlation Coefficient)*
mATPasa	Miosina adenosín-trifosfatasa
Mfn2	Mitofusina 2
NCF	Número de Capilares por Fibra
OPA-1	Proteína atrófica óptica-1 (*Optic atrophy protein-1*)
OSA	Obstructive Sleep Apnea
PGC-1α	Co-activador 1α del receptor gamma activado del proliferador de peroxisomas (*Peroxisome proliferator-activated receptor Gamma Coactivator 1α*)
SDH	Succinato Deshidrogenasa
Sirt3	Sirtuina 3
SO	Fibras lentas oxidativas (*Slow Oxidative*)
SOL	Músculo soleus
TA	Músculo tibial anterior
Tfam	Factor de transcripción A mitocondrial (*mitocondrial Transcription Factor A*)
TOM20	Translocasa de la membrana externa 20 (*Translocase of the Outer Membrane* 20)
VEGF	Factor de crecimiento vascular endotelial (*Vascular Endothelial Growth Factor*)

1.

Introducción general

1.1 Presentación de las publicaciones

La presente tesis consiste en una serie artículos científicos fruto del trabajo realizado entre 2012 y 2016 en el Laboratorio de Fisiología del Ejercicio y la Hipoxia (Departamento de Biología Celular, Fisiología e Inmunología) de la Facultad de Biología de la Universidad de Barcelona. La investigación llevada a cabo durante estos años se enmarca principalmente en el proyecto *Efecto de la hipoxia hipobárica intermitente en la recuperación del daño muscular inducido en ratas de laboratorio*, contando también con el soporte económico del proyecto *Efecto sinérgico de frío e hipoxia en la reparación del daño muscular inducido en ratas de laboratorio*, ambos financiados por el Ministerio de Economía y Competitividad del Gobierno de España (DEP2010-22205-C02-01 y DEP2013-48334-C2-1-P, respectivamente).

Los trabajos que conforman esta tesis aparecen listados a continuación en números romanos (se utilizará esta enumeración para su referencia a lo largo de la tesis):

I. Rizo-Roca D, Ríos-Kristjánsson JG, Núñez-Espinosa C, Ascensão AA, Magalhães J, Torrella JR, Pagès T, Viscor G. (2015). A semiquantitative scoring tool to evaluate eccentric exercise-induced muscle damage in trained rats. *European Journal of Histochemistry* 59(44):1-7.

II. Rizo-Roca D, Ríos-Kristjánsson JG, Núñez-Espinosa C, Santos-Alves E, Gonçalves IO, Magalhães J, Ascensão AA, Pagès T, Viscor G, Torrella JR. (Artículo en prensa). Intermittent hypobaric hypoxia combined with aerobic exercise

improves muscle morphofunctional recovery after eccentric exercise to exhaustion in trained rats. *Journal of Applied Physiology*.

III. Rizo-Roca D, Ríos-Kristjánsson JG, Núñez-Espinosa C, Santos-Alves E, Magalhães J, Ascensão AA, Pagès T, Viscor G, Torrella JR. (Artículo en segunda revisión). Modulation of mitochondrial biogenesis, dynamics, bioenergetics and oxidative stress markers by intermittent hypobaric hypoxia and aerobic exercise after strenuous eccentric exercise in trained rats. *Applied Physiology Nutrition and Metabolism*.

El artículo **I** recoge la creación de una herramienta para semicuantificar histológicamente el daño muscular inducido por ejercicio excéntrico extenuante en ratas entrenadas. Se trata de un trabajo claramente metodológico, que nace de la necesidad de evaluar de manera rápida y precisa muestras con un tipo de daño muy característico. A diferencia del daño producido por modelos traumáticos, el ejercicio excéntrico, en individuos entrenados, induce un tipo de daño mucho más moderado, en el que la mayoría de fibras musculares permanecen sanas y viables. Así pues, a partir de cortes histológicos teñidos mediante hematoxilina-eosina, se elaboró un sistema de puntaje semicuantitativo que evaluaba diferentes aspectos histopatológicos de la muestra, tales como la morfología y estado de las miofibras, el grado de inflamación y los espacios extracelulares.

El artículo **II** constituye el trabajo central de la tesis. En él se analizan a nivel histológico los efectos del ejercicio excéntrico extenuante en la morfofuncionalidad y capilarización de las fibras del músculo sóleo, así

como las alteraciones inducidas por diferentes protocolos de recuperación (recuperación pasiva, recuperación con hipoxia hipobárica intermitente (HHI) y recuperación con HHI seguida de ejercicio aeróbico ligero). Se evalúa el estado histopatológico del músculo (mediante una versión modificada de la herramienta presentada en el artículo **I**) y su capacidad oxidativa haciendo especial énfasis en los parámetros relacionados con la capilarización del tejido y un posible fenómeno de neovascularización.

Finalmente, el artículo **III** es fruto de la colaboración, a través de una estancia doctoral, con en el *Laboratório de Metabolismo e Exercício* de la Faculdade de Desporto de la Universidade de Porto, especializado en el estudio de la funcionalidad mitocondrial. Este manuscrito analiza una notable cantidad de marcadores mitocondriales relacionados con la biogénesis, dinámica y bioenergética mitocondrial, complementando los resultados obtenidos a nivel histológico en el artículo **II**.

El lector encontrará en las próximas páginas una introducción en la que se presenta el contexto de la tesis y los principales conceptos teóricos, seguida de la presentación de los objetivos de esta tesis doctoral, artículos publicados y una discusión general de los resultados obtenidos.

1.2 Contexto

Las lesiones del sistema muscoloesquelético tienen un gran impacto socioeconómico y sobre el bienestar de la población. Según el *Informe sobre el estado de la seguridad y salud laboral en España* (Instituto Nacional de Seguridad e Higiene en el Trabajo 2013), el 79% de las enfermedades no traumáticas relacionadas con el trabajo diagnosticadas en 2011

correspondió a patologías del aparato locomotor. Además, las lesiones musculares son prácticamente inherentes a la práctica de la actividad física, sea recreativa o profesional. De hecho, la práctica de cualquier tipo de ejercicio, si se da con suficiente intensidad o frecuencia, puede producir dolor y daño muscular.

La cotidianidad e impacto de este fenómeno, especialmente en deportistas, suscitó el interés del grupo de trabajo de *Fisiología del Ejercicio y la Hipoxia*, dirigido por el Dr. Ginés Viscor Carrasco. Desde la última década del siglo pasado, este grupo de investigación ha venido utilizando la exposición intermitente a hipoxia hipobárica (HHI) como herramienta para la mejora del rendimiento deportivo en humanos, y ha estudiado sus efectos a nivel bioquímico e histológico en modelos humanos y animales (Rodríguez et al. 1999; Casas et al. 2000; Panisello et al. 2008; Esteva et al. 2010), reportando mejoras en la capilarización y metabolismo muscular. En este contexto, el próximo paso parecía evidente: si la HHI resulta eficaz para mejorar y modular tanto el rendimiento deportivo como el tejido muscular esquelético, ¿podría inducir alteraciones beneficiosas durante la recuperación del daño muscular? Así, en 2010 fue concedida por el Ministerio de Economía y Competitividad la financiación para ejecutar el proyecto *Efecto de la hipoxia hipobárica intermitente en la recuperación del daño muscular inducido en ratas de laboratorio*, en el cual se encuadra la presente tesis.

1.3 Daño muscular inducido por ejercicio excéntrico

La inducción experimental del daño muscular en modelos animales data de finales de los años 70 (Vihko et al. 1978). Desde entonces, la

diversidad de métodos para la inducción del daño muscular se ha incrementado notablemente. Pueden encontrarse modelos *in vivo*, tanto en animales conscientes como anestesiados, y modelos *in vitro*, utilizando desde músculos enteros hasta fibras aisladas (Warren y Palubinskas 2007). De entre todos los modelos animales disponibles, el más habitual ha sido siempre el modelo roedor, especialmente ratas. La inducción del daño puede clasificarse en dos grandes modalidades: 1) mediante contracciones (extenuantes y/o excéntricas) y 2) mediante traumatismo (como la aplicación de una toxina, laceración o fuertes impactos). Tradicionalmente, se ha considerado que las lesiones producidas por contracción son más frecuentes que las traumáticas (Warren et al. 1999), en tanto que el modelo por traumatismo se suele utilizar más cuando el objeto de interés es la regeneración y no la lesión *per se*.

Debido a ello, en este trabajo se ha optado por la utilización de un modelo de daño inducido por ejercicio excéntrico (EEIMD), descrito en el clásico artículo de Schwane y Armstrong (1983). Es bien sabido que la realización inusual de ejercicio excéntrico puede derivar en una pérdida temporal de la fuerza muscular, aumentar la tensión pasiva del tejido y producir hinchazón y agujetas. Todo ello puede dificultar la posterior realización del trabajo rutinario o el seguimiento de un protocolo de entrenamiento.

1.3.1 Etiología del EEIMD

La etiología del EEIMD ha sido ampliamente estudiada, especialmente en la década de los 80. Se consideran eventos clave en el desarrollo de la lesión la disrupción de los sarcómeros de las fibras musculares y la alteración del sistema de acoplamiento excitación-contracción (E-C).

Estos eventos conducen a una pérdida de fuerza y a la aparición del DOMS (*Delayed Onset Muscle Soreness*), también conocido vulgarmente como *agujetas*, típicamente producto de un ejercicio extenuante o un esfuerzo no habitual. Sin embargo, aún a día de hoy no hay consenso sobre cuál es el mecanismo inicial desencadenante y responsable de la pérdida de fuerza. Algunos autores, entre los que destaca Uwe Proske, del Departamento de Fisiología de la Universidad de Monash (Australia) defienden un origen mecánico del daño: la disrupción física de los sarcómeros, debido a un sobreestiramiento heterogéneo de los mismos durante la contracción, acabaría por dañar la membrana y retículo sarcoplasmático, lo cual produciría un fallo en el sistema de acoplamiento E-C y una liberación masiva de Ca^{2+}, lo que llevaría a contracturas a nivel local, un aumento en la tensión pasiva y, potencialmente, a la muerte de la fibra muscular (Proske y Allen 2005). La Figura 1 muestra una esquematización de estos eventos.

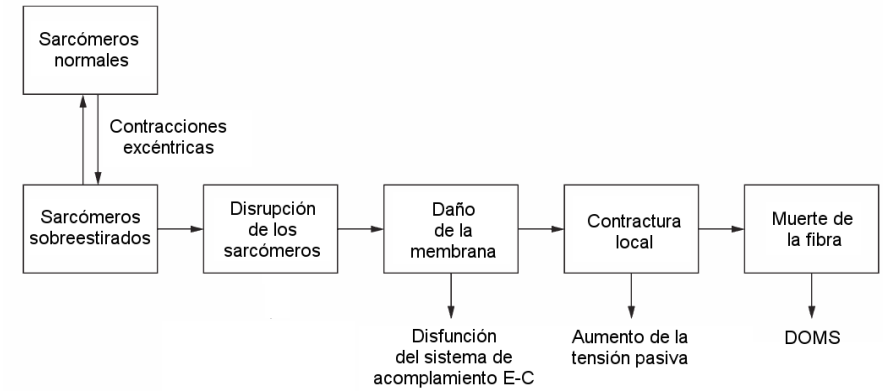


Figura 1. Cadena de eventos desencadenantes del daño muscular inducido por contracciones excéntricas propuesta por Proske y Allen. *Diagrama adaptado de Proske y Allen (2005).*

Sin embargo, otros autores, como Gordon L. Warren, del Departamento de Terapia Física de la Universidad de Georgia (Estados

Unidos) sugieren que el evento principal responsable de la disminución de la fuerza es el fallo del sistema de acoplamiento E-C (Warren et al. 2001), mientras que la disrupción física sería una causa secundaria. La Figura 2 muestra una estimación de la evolución temporal y la contribución de cada factor a la pérdida de fuerza, según Warren et al. (2001).

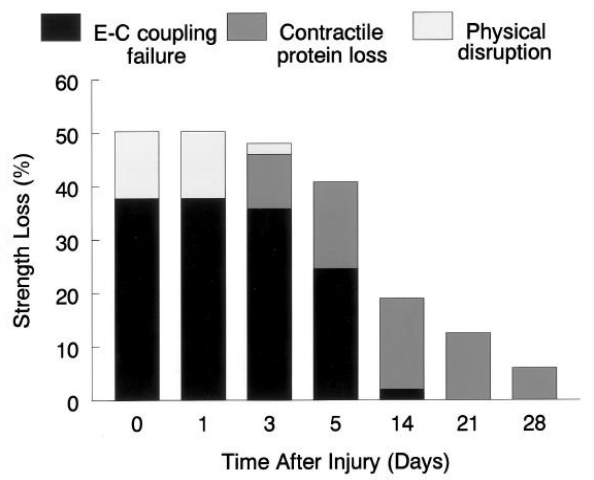


Figura 2. Contribución de los diferentes factores implicados en la pérdida de la fuerza muscular después de contracciones excéntricas según las estimaciones de Warren. *Figura extraída de Warren et al. (2001).*

En cualquier caso, parece claro que la disrupción de los sarcómeros y la pérdida de la homeostasis del Ca^{2+} derivada de la alteración del sistema de acoplamiento E-C son los fenómenos desencadenantes del daño muscular.

1.3.2 Manifestaciones histológicas y bioquímicas del EEIMD

A pesar de que está generalmente aceptado que las pruebas funcionales son las más fiables para evaluar y cuantificar el EEIMD (Warren et al. 1999), las manifestaciones histológicas y bioquímicas son

fundamentales para entender la etiología y los fenómenos fisiológicos relacionados con el daño muscular.

1.3.2.1 Manifestaciones histológicas

Se han observado directamente, mediante microscopía electrónica y óptica, numerosas alteraciones ultraestructurales provocadas por el EEIMD. Una de las más características es la disrupción del disco-Z (Friden y Lieber 1991), debido al sobreestiramiento de los sarcómeros. También son evidentes disrupciones en las bandas A e I (Manfredi et al. 1991), en el retículo sarcoplasmático y en los túbulos T (Takekura et al. 2001), así como alteraciones mitocondriales (Magalhães et al. 2013), de la matriz extracelular y de la red capilar (Stauber et al. 1990). Además, en muestras de tejido muscular afectado por el EEIMD aparecen fibras con su morfología alterada, por ejemplo: atrofiadas, angulares, hipercontraídas e hinchadas (Sayers y Hubal 2007). Pueden encontrarse también fibras miofagocitadas o necróticas, así como acumulaciones de mononucleares en los intersticios endo y perimisiales (Armstrong et al. 1983, McCully y Faulkner 1985). Curiosamente, los estudios en los que se analiza la morfología y capilarización en músculos sometidos a EEIMD son muy escasos (Yu et al. 2013). El artículo **II** de esta tesis aborda con profundidad las alteraciones inducidas por el EEIMD en la morfofuncionalidad e histopatología de las fibras del músculo sóleo.

Es importante tener en cuenta que, en este tipo de daño muscular, a diferencia del de origen traumático, la gran mayoría de fibras permanecen totalmente viables y sanas. Solo un pequeño porcentaje de las fibras musculares se verán seriamente afectadas o comprometidas (Armstrong et al. 1983). Esta es una de las principales motivaciones que llevó a la publicación del artículo **I**, en el que se desarrolló un método

de evaluación histopatológica para la semicuantificación de este tipo de daño muscular en sujetos entrenados.

1.3.2.2 Manifestaciones bioquímicas

Además de las citadas alteraciones histológicas, también han sido descritos los aspectos bioquímicos característicos del EEIMD. Tras la realización de ejercicio excéntrico extenuante (EEE) se han observado aumentos en la concentración plasmática de proteínas como la creatina quinasa (CK), mioglobina, troponina-I, aspartato aminotransferasa y lactato deshidrogenasa (Nosaka y Clarkson 1996). Estas proteínas provienen directamente de las fibras musculares dañadas como consecuencia de alteraciones en sus membranas plasmáticas.

1.3.2.3 Respuesta inflamatoria

A las pocas horas de la realización de ejercicio excéntrico extenuante hay una respuesta inflamatoria, observándose una elevación en sangre de varias citoquinas pro-inflamatorias, tales como la interleuquina-1 (IL-1), interleuquina-6 (IL-6) y el factor de necrosis tumoral α (TNF-α) (Evans y Cannon 1991), así como la presencia de monocitos (Malm et al. 1999). De hecho, la presencia de células inflamatorias (mayormente neutrófilos y macrófagos) en el tejido muscular ha sido descrita desde a partir de los 45 minutos (Fielding et al. 1993) hasta los 14 días después de la realización del EEE (Round et al. 1987). Esta respuesta inflamatoria resulta esencial para la limpieza de la *debris* y la reparación del tejido y, al mismo tiempo, puede ser responsable de la exacerbación el daño muscular (Tidball 1995).

1.3.2.4 Disrupción de la homeostasis del calcio

Como se ha apuntado anteriormente, el daño en la membrana celular producido por las fuerzas mecánicas puede alterar la permeabilidad de las células al Ca^{2+}. Así, han sido descritos incrementos en la concentración intracelular de Ca^{2+} tanto a nivel citosólico (Lynch et al. 1997) como a nivel mitocondrial (Duan et al. 1990). Además del papel que juega el Ca^{2+} en la fisiología de la contracción muscular, altas concentraciones de este ion producen *swelling* (hinchazón) mitocondrial, induciendo la liberación del citocromo c (Brustovetsky et al. 2002) y llevan a la sobre-activación de unas proteasas dependientes de calcio llamadas calpaínas. Entre otros substratos, estas proteasas degradan elementos citoesqueléticos y proteínas mitocondriales (Vissing et al. 2008). De hecho, ha sido descrita una afectación transitoria de la eficiencia y función mitocondrial tras la realización de ejercicio excéntrico extenuante (Magalhães et al. 2013). Sin embargo, a pesar de la evidente conexión entre el EEIMD y las mitocondrias, su relación no ha sido estudiada aún con profundidad. Este último punto es una de las razones que motivó la realización del artículo **III**.

1.3.3 EEIMD y red vascular

Además de afectar a las miofibras, el EEE también puede alterar otros componentes del tejido muscular, como las células endoteliales que conforman la red vascular. Como en cualquier otro tejido, el entramado capilar del músculo juega un papel clave en el mantenimiento de la homeostasis tisular, el suministro de oxígeno y nutrientes y la recogida de productos metabólicos secundarios. Resulta curioso que, a pesar de la relevancia del lecho capilar en la función muscular y en la

regeneración, son pocos los estudios que han analizado los efectos del EEE sobre la microvasculatura muscular (Stauber et al. 1990; Chen et al. 2003; Kano et al. 2004). En todos ellos se observaron alteraciones de menor o mayor grado a nivel capilar. Uno de los principales objetivos del artículo **II** es aportar más información a este aspecto poco estudiado del EEE, en tanto que el artículo **III** presenta algunos resultados que refuerzan, indirectamente, estos hallazgos.

1.3.4 Tratamiento del EEIMD

El tratamiento del EEIMD ha generado gran interés en las últimas décadas. Por ello, se han desarrollado una gran variedad de terapias, como la suplementación de antioxidantes, carbohidratos y proteínas, la utilización de anti-inflamatorios no esteroideos, la crioterapia, masajes, electroestimulación, etc. Sin embargo, a pesar de su extendido uso en la práctica clínica, la eficacia científica de todas estas terapias es cuanto menos dudosa. El problema reside no tanto en el principio teórico en el que se basan (si bien en algunos casos, como el uso abusivo de antiinflamatorios o antioxidantes, podría alterar los procesos adaptativos que facilitan la reparación del daño muscular) como en la inconsistencia en la dosis, frecuencia y/o intensidad con la que se aplican, dificultando así su meta-análisis. Para una extensa revisión sobre el tratamiento y prevención del EEIMD, ver Howatson y van Someren (2008).

Entre los tratamientos utilizados y propuestos hasta ahora en el EEIMD no se encuentra la HHI, a pesar de su uso en otros ámbitos de la actividad física (como la mejora del rendimiento deportivo). En el

próximo apartado (1.4 Hipoxia) se expondrán las razones que motivaron su aplicación como tratamiento del EEIMD.

1.3.5 Músculo sóleo

Por su función y posición anatómica, el músculo sóleo es especialmente susceptible a las contracciones excéntricas (Warren y Palubinskas 2007). Se trata de un músculo eminentemente postural, constituido en su totalidad por fibras oxidativas, siendo la mayoría fibras lentas (SO, *Slow oxidative*) (≈80%) y el resto por fibras rápidas oxidativas (FOG, *Fast Oxidative Glycolytic*) (≈20%), repartidas de manera relativamente homogénea (Wang y Kernell 2001). Cada tipo de fibra posee una serie de características morfológicas y metabólicas que determinan sus capacidades y funciones. La Tabla 1 (siguiente página) muestra un resumen de las principales características de los principales tipos de fibras.

Debido a las diferencias fundamentales en morfología y función de los diferentes fenotipos de fibras musculares, resulta imprescindible su correcta caracterización y tipificación en los análisis histológicos. En este estudio, la mayoría de resultados histomorfométricos discutidos hacen referencia a las fibras SO, por ser con diferencia el fenotipo dominante en el músculo sóleo. Tal y como se observa en la Tabla 1, las fibras SO muestran una generación de fuerza relativamente baja a cambio de una alta resistencia a la fatiga, por lo que son aptas para la realización de ejercicios prolongados de baja intensidad (específicamente, posturales), como los que lleva a cabo el músculo sóleo. Como consecuencia, estas fibras están altamente irrigadas y presentan una sección transversal media pequeña, a fin de facilitar la

difusión de oxígeno, fundamental para el metabolismo oxidativo. El aporte de oxígeno es crucial, en consecuencia, para el mantenimiento prolongado de la actividad contráctil en este tipo de fibras (Egan y Zierath 2013).

Tabla 1. Características morfológicas y metabólicas de los diferentes tipos de fibras.

	Tipo de fibra		
	SO	FOG	FG
Nomenclatura alternativa	Tipo I	Tipo IIa	Tipo IIb
Velocidad de contracción	Lenta	Rápida	Rápida
Resistencia a fatiga	Alta	Alta	Baja
Metabolismo	Oxidativo	Oxidativo	Anaeróbico
[Mioglobina]	Alta	Intermedia	Baja
Producción de fuerza	Baja	Intermedia	Alta
Densidad mitocondrial	Alta	Intermedia	Baja
Densidad capilar	Alta	Alta	Baja
Tipo de ejercicio predominante	Prolongado, baja intensidad	Duración media, alta intensidad	Corto, explosivo

Tabla adaptada de Egan y Zierath (2013). El músculo sóleo se compone aproximadamente de un 80% de fibras SO y un 20% de FOG, sin presencia de fibras FG. La nomenclatura alternativa responde a la isoforma dominante de la cadena pesada de la miosina. Para la naturaleza histológica de este trabajo resulta más adecuada la nomenclatura basada en el tipo de contracción y metabolismo de la fibra SO: *Slow oxidative* (lentas oxidativas), FOG: *Fast oxidative glycolytic* (rápidas oxidativas glicolíticas); FG: *Fast glycolytic* (rápidas glicolíticas).

1.4 Hipoxia

La hipoxia hipobárica se define como el descenso del aporte de oxígeno a los tejidos debido a una disminución de la presión parcial de oxígeno (PO_2), causada por una caída de la presión atmosférica (en la naturaleza, esta situación se da en las grandes alturas).

La exposición aguda o crónica a la hipoxia hipobárica puede causar una serie de efectos deletéreos en humanos no aclimatados, tales como la pérdida de masa muscular, un incremento en la producción de especies reactivas del oxígeno (ROS) y una disfunción de la fosforilación oxidativa mitocondrial (Clanton 2007; Favier et al. 2010; Chen et al. 2012). Además, la exposición a la hipoxia hipobárica puede derivar en una patología característica, llamada comúnmente *mal de altura*, cuyos síntomas más habituales son cefalea, náuseas, fatiga, disnea, palpitaciones, etc., y que en los casos más graves puede producir la aparición de edemas pulmonares y cerebrales (West 2012).

Con el fin de evitar esta serie de complicaciones, en 1934 el ucraniano Nikolai Sirotinin (1896-1977) realizó los primeros experimentos en el campo de la pre-aclimatación, proponiendo que unos pocos días en altitud moderada podrían incrementar la tolerancia a subsecuentes exposiciones a hipoxia. Es interesante destacar que, desde la década de los 30, se desarrolló en Rusia y demás países de la antigua Unión Soviética una gran cantidad de estudios relativos a los efectos a la exposición a la hipoxia hipobárica (revisado por Serebrovskaya, 2002), tanto a nivel fisiopatológico como terapéutico (Xi y Serebrovskaya 2012). Desgraciadamente, debido a la situación política establecida durante la Guerra Fría y a la barrera idiomática, la mayor parte de estas

investigaciones no vieron la luz en el mundo occidental hasta hace relativamente pocos años.

1.4.1 Acotando la intermitencia

El concepto de aclimatación o hormesis evoca forzosamente la idea de *intermitencia*: un estímulo *a priori* estresante o perjudicial, aplicado repetidamente en dosis bajas o sub-letales y acumulativas, puede desencadenar una respuesta adaptativa beneficiosa para el organismo (Calabrese 2010). Sin embargo, en el contexto de la hipoxia, la palabra "intermitencia" siempre debe matizarse: a día de hoy (último trimestre de 2016), el motor de búsqueda *Pubmed* devuelve 1482 artículos que incluyen las palabras *Intermittent* e *Hypoxia* en sus títulos. Entre ellos, pueden encontrarse desde estudios referentes a la apnea obstructiva del sueño (OSA, *Obstructive Sleep Apnea*), en la que se producen numerosos ciclos hipóxicos normobáricos de corta duración (de segundos a unos pocos minutos) hasta estudios sobre mineros andinos, con ciclos que pueden llegar a varias semanas ininterrumpidas de exposición a hipoxia hipobárica. Resulta evidente, en consecuencia, que es necesario delimitar el uso de "intermitente" referido al uso de la hipoxia como herramienta (Viscor et al. 2014). Es generalmente aceptado que, cuando se utiliza la hipoxia con objetivos terapéuticos, de mejora del rendimiento deportivo o como parte de un protocolo de aclimatación, las exposiciones deben constar de un número de ciclos limitado (entre uno y quince) y de una intensidad moderada (menos de 4500 m) (Navarrete-Opazo y Mitchell 2014). Incluso así, la diversidad de posibles protocolos es enorme, lo que dificulta la comparación entre diferentes estudios. La Figura 3 muestra el balance entre estos factores (ciclos, intensidad) y algunas de las respuestas fisiológicas generadas.

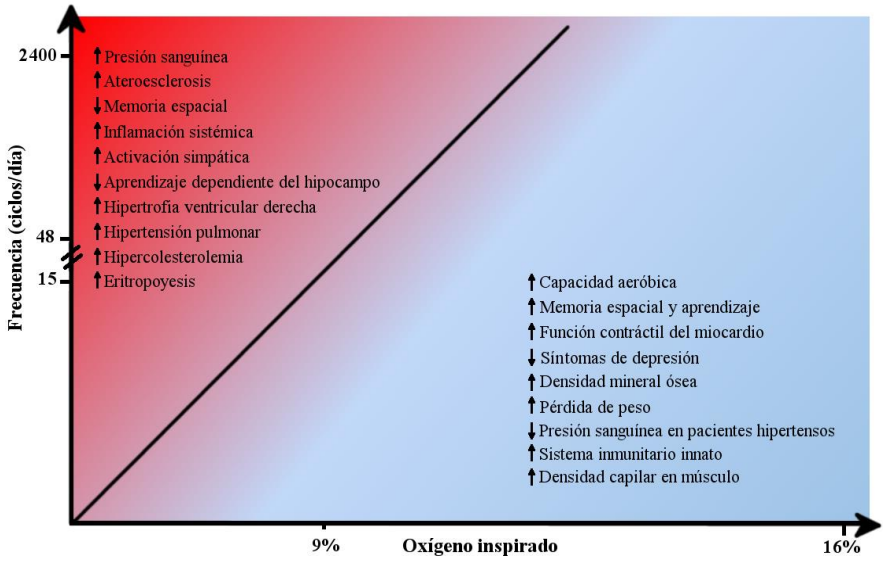


Figura 3. Resumen de las respuestas patológicas y adaptativas en función del porcentaje de oxígeno inspirado (intensidad) y ciclos diarios (frecuencia). Nótese que, además de estos factores, también son importantes la duración de cada uno de los ciclos y la duración total del protocolo. *Figura adaptada de Navarrete-Opazo y Mitchell (2014).*

1.4.2 Efectos beneficiosos derivados de la exposición a hipoxia intermitente

Tal y como se ha explicado en el punto anterior, en dosis, frecuencia, duración e intensidad adecuadas, la hipoxia puede desencadenar una serie de adaptaciones beneficiosas para el organismo. Sin embargo, esta cantidad de factores y su heterogeneidad a la hora de construir programas de exposición a hipoxia pueden conducir a la obtención de resultados contradictorios, por lo que existe cierta controversia en la comunidad científica en cuanto a los efectos negativos y/o positivos que puede provocar la hipoxia. Entre los efectos beneficiosos descritos, se encuentran mejoras en la capacidad de aprendizaje y memoria espacial (Lu et al. 2009), efectos ansiolíticos y antidepresivos (Rybnikova

et al. 2008), refuerzo del sistema inmunitario (Serebrovskaya et al. 2011), mejoras de la funcionalidad contráctil cardíaca (Béguin et al. 2005) y de la capacidad aeróbica (Rodríguez et al. 1999).

1.4.2.1 Respuestas a la hipoxia crónica e intermitente en el músculo esquelético

Para el desarrollo de esta tesis, resultan especialmente relevantes las respuestas generadas por la hipoxia en el tejido muscular esquelético. Además, se da la particularidad de que, en individuos sanos, el músculo esquelético es el único tejido en el que existen regularmente episodios de hipoxia intermitente sin que haya una reducción de la PO_2 arterial. Este fenómeno es debido a que el músculo ejercitante tiene una gran demanda metabólica que no siempre puede ser satisfecha por el suministro de oxígeno capilar, generando una situación de hipoxia local y transitoria a nivel miofibrilar. Debido a esta particularidad, resulta especialmente interesante la realización de ejercicio aeróbico en situaciones de hipoxia sistémica, pudiendo existir una relación sinérgica o aditiva[1].

A diferencia de la exposición crónica a la hipoxia, pocos estudios han analizado los efectos de la hipoxia intermitente a nivel ultraestructural. En el primer caso, se da una pérdida de la masa muscular debido a una

[1]Existen numerosos programas de entrenamiento en altitud, divididos en *Living low – Training high* y *Living high – Training low*, en los que el entrenamiento se realiza en altitud, regresando a nivel del mar a continuación (primer caso) o en los que los atletas viven durante un determinado periodo de tiempo en altitud, pero realizan las sesiones de entrenamiento a menor altitud (segundo caso). Una vez más, la variedad de protocolos y la gran cantidad de factores que entran en juego (duración, intensidad y frecuencia de los estímulos) dificultan enormemente la comparación entre diferentes programas y generan controversia en el ámbito científico. Para más información, ver algunas revisiones (Jacobs 2013; Levine y Stray-Gundersen 1997; McLean et al. 2014; Wilber 2013).

reducción en la sección transversal de las fibras (FCSA, *Fibre Cross-Sectional Area*) (Green et al. 1989). Aunque se ha sugerido que esta respuesta es una forma de deterioro muscular, no debe descartarse la posibilidad de que se trate de una respuesta adaptativa controlada: al disminuir la masa muscular, disminuye también la demanda de oxígeno por parte del músculo. Además, la reducción de la FCSA aumenta indirectamente la densidad capilar (CD, *Capillary Density*) del músculo, sin que sea necesario un proceso angiogénico, facilitando la difusión del oxígeno al disminuir la distancia de difusión del oxígeno a las mitocondrias (Panisello et al. 2008). Se ha descrito también, especialmente en alpinistas (cuando hay que añadir a la hipoxia la realización de ejercicio extenuante), una disminución de la densidad del volumen mitocondrial (Howald et al. 1990). Sin embargo, como sucede a nivel sistémico y en otros tejidos, la exposición intermitente puede desencadenar respuestas totalmente diferentes e incluso contrarias. Estas respuestas son especialmente evidentes (a veces exclusivas) cuando tal exposición se realiza junto a sesiones de ejercicio aeróbico. Como bien apuntó Lundby et al. (2009), es probable que para que el músculo genere una respuesta adaptativa a la hipoxia sea necesario comprometer su homeostasis de oxígeno. Este compromiso puede no ser suficientemente intenso si el músculo se encuentra en reposo, especialmente teniendo en cuenta, como se ha citado anteriormente, que es un tejido habituado a episodios hipóxicos durante su funcionamiento normal. A modo de ejemplo, la Figura 4 muestra los efectos de seis semanas de entrenamiento aeróbico a baja (*low*) y alta (*high*) intensidad en condiciones de normoxia (Nor) o hipoxia (Hyp) en los niveles de mRNA de proteínas y parámetros clave en la respuesta a la hipoxia y la fisiología muscular.

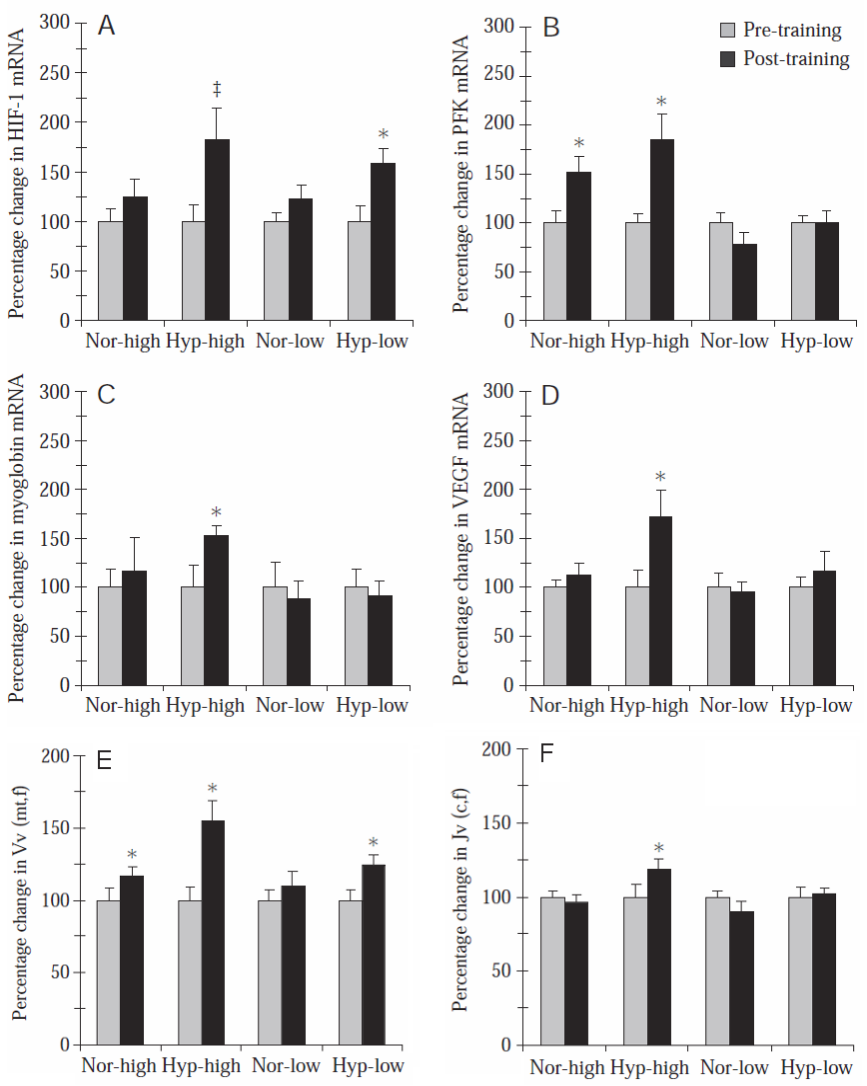


Figura 4. Efectos de seis semanas de entrenamiento en normoxia (Nor) o Hipoxia (Hyp) un 25% por debajo del umbral anaeróbico (*low*) o en el umbral anaeróbico (*high*) en los niveles de mRNA codificadores de *Hypoxia Inducible Factor 1* (HIF-1) (A), fosfofructoquinasa (PFK) (B), mioglobina (C), *Vascular Endothelial Growth Factor* (VEGF) (D); y de la densidad del volumen mitocondrial (E) y de capilares (F). *Figura adaptada de Hoppeler y Vogt (2001).* *, $p<0.05$ *vs.* Pre-training; ‡, $p<0.05$ *vs.* Hyp.

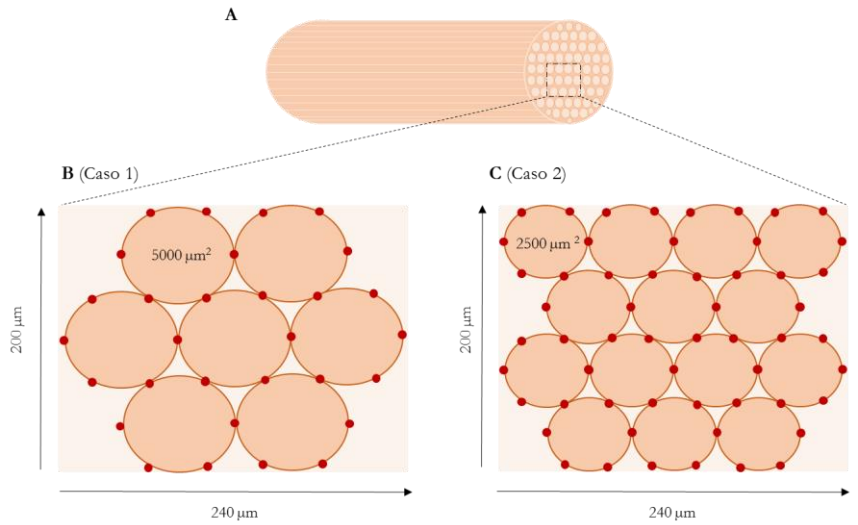
Tal y como puede observarse en la Figura 4A, la hipoxia induce la expresión de HIF-1 (*Hypoxia Inducible Factor* 1), independientemente de

la intensidad del co-estímulo. Este factor de transcripción es el principal modulador de la respuesta a la hipoxia (Semenza 2009), activando la transcripción de numerosos genes con el objetivo de producir las adaptaciones celulares necesarias para contrarrestar la disminución del aporte de oxígeno al tejido. Entre estas adaptaciones, destacan la mejora del transporte del oxígeno (vía aumento del hematocrito o de la mioglobina, Figura 4C), inducción de la formación de nuevos capilares mediante la expresión de VEGF (*Vascular Endothelial Growth Factor,* Figura 4D), aumentando la CD (Figura 4F) y el incremento de enzimas glicolíticas como la fosfofructoquinasa (PFK, Figura 4B). Además, como se ve reflejado en la Figura 4E, en dosis adecuadas la hipoxia puede incluso inducir un aumento de la densidad mitocondrial. Otros autores han encontrado niveles incrementados de citrato sintasa utilizando un protocolo de *Training high – Living low* (Melissa et al. 1997).

1.4.2.2 Adaptaciones de la red capilar a hipoxia crónica e intermitente en el músculo esquelético

Debido a la importancia de la microvasculatura en la homeostasis tisular, resultan especialmente relevantes las adaptaciones de la red capilar a diferentes estímulos hipóxicos. Una vez más, aun hoy en día no existe pleno consenso sobre los efectos de la hipoxia en la vascularización muscular. Así, Hoppeler et al. (1990), en un estudio llevado a cabo en alpinistas que realizaron una expedición a más de 5000 m durante 8 semanas, describió un aumento de la CD en el músculo vasto lateral, pero no en el cociente entre el número de capilares y el número de fibras (C/F). Junto a la reducción en la FCSA, Hoppeler y sus colaboradores llegaron a la conclusión de que el aumento en la CD no era debido a un fenómeno de angiogénesis (en tal caso, habría

aumentado el índice C/F) sino consecuencia de la disminución del tamaño de las fibras (para una mejor comprensión de los parámetros de capilarización, ver Figura 5). Este hallazgo estaba en concordancia con estudios en roedores de finales de los 80 (Snyder et al. 1985, Poole y Mathieu-Costello 1989). Sin embargo, años más tarde, una nueva aproximación llevada a cabo por Deveci et al. (2001) reveló diferencias en la respuesta a la hipoxia crónica en función del músculo analizado. Así, músculos activos y oxidativos incluso en reposo, como el diafragma y el sóleo, exhibieron incrementos en el índice C/F tras 3 semanas de exposición a hipoxia, a diferencia de músculos relativamente inactivos, como el tibial anterior (TA) y el *extensor digitorum longus*. Además, un análisis más minucioso, en el que las secciones transversales de los músculos eran subdivididas según la proporción de los diferentes fenotipos de fibras, reveló incrementos en el C/F en el córtex del músculo TA, pero no en la zona central. El mismo autor, un año más tarde, llevó a cabo un estudio similar, con una exposición más prolongada (6 semanas), detectando angiogénesis en todos los músculos analizados (diafragma, sóleo, TA) (Deveci et al. 2002). Estudios en nuestro laboratorio, en los que se utilizó un modelo de HHI, arrojaron resultados similares en músculos activos, remarcando la importancia de la actividad muscular en la respuesta angiogénica inducida por la hipoxia (Panisello et al. 2007, 2008). Consecuentemente, la inclusión de protocolos de ejercicio durante la exposición a hipoxia emergió como una interesante estrategia para inducir la formación de nuevos capilares en el músculo esquelético.



	Caso 1	Caso 2
Área microfotografía (mm²)	0,048	0,048
Capilares	30	55
CD (capilares / mm²)	625	1146
Fibras	7	14
FD (fibras / mm²)	146	292
C/F	4,28	3,93
NCF (capilares / fibra)	6	6
FCSA (µm²)	5000	2500
CCA (capilares por 1000 µm² de FCSA)	1,2	2,4

Figura 5. *A)* Sección transversal de músculo esquelético. *B y C)* Esquematización de una microfotografía. Valores y formas son arbitrarios, mostrándose solo a modo de ejemplo. Obsérvese como, a pesar de duplicar la CD en el Caso 2, el índice C/F muestra una ligera disminución, extrayéndose dos conclusiones: 1) El aumento de la CD ha sido una consecuencia de la reducción de la FCSA. Al reducirse el tamaño medio de las fibras, caben más capilares en un área determinada. El NCF, sin embargo, es el mismo, por lo que no se ha producido angiogénesis. 2) A su vez, la ausencia de formación de nuevos capilares no excluye una mejora en la capilarización del músculo. Así, a pesar de no aumentar el NCF ni el C/F, sí que se observa un mayor CCA, indicando que cada 1000 µm² de fibra se encuentra irrigado por el doble de capilares. Resulta fundamental, por lo tanto, la comprensión de estos parámetros para una interpretación adecuada de los resultados histomorfométricos en este y otros estudios. CD: densidad capilar, FD: densidad de fibras, C/F: capilares por fibra, NCF: número de capilares por fibra, FCSA: área de la sección transversal de la fibra.

A principios de los 90, Desplanches et al. (1993) describieron un incremento en el índice C/F y en el FCSA en sujetos sometidos a un protocolo de ejercicio en hipoxia durante 3 semanas. Alrededor de una década más tarde, otros autores obtuvieron resultados similares tanto en humanos (Geiser et al. 2001, Vogt et al. 2001) como en roedores (Olfert et al. 2001).

Es importante destacar que, en el caso de los estudios con humanos, la exposición a hipoxia se produce exclusivamente durante la realización del ejercicio, en tanto que en el estudio de Olfert y colaboradores, los animales se encontraban estabulados en condiciones de hipoxia crónica. Ahora bien, paralelamente a estas publicaciones surgieron otros estudios con resultados divergentes, en los que no se encontraron mejoras en la capilarización del músculo (Terrados et al. 1988; Melissa et al. 1997; Green et al. 1999; Masuda et al. 2001). Como se ha resaltado a lo largo de esta sección, la enorme variabilidad de los protocolos de hipoxia y ejercicio (intensidad, duración, tipo, frecuencia), de los sujetos (sexo, edad, condición física) y las condiciones experimentales (temperatura, biopsia, músculo analizado, análisis de las microfotografías) dificultan la obtención de respuestas concluyentes no solo respecto a la respuesta vascular a la hipoxia y/o ejercicio, sino también en el metabolismo oxidativo, rendimiento deportivo o VO_2 máxima. La Tabla 2 sintetiza algunas de las alteraciones (a veces contradictorias) inducidas por diferentes protocolos de entrenamiento en altitud a nivel bioquímico.

1.4.2.3 Efecto de la hipoxia en la modulación de la función mitocondrial

Las adaptaciones descritas hasta ahora carecen de sentido si no se analiza lo que se encuentra al otro lado del capilar. Y es que todo el

entramado vascular tiene como principal función aportar oxígeno al principal productor de energía del organismo: la mitocondria. Este orgánulo, a fin de sintetizar ATP, utiliza el oxígeno como aceptor final de electrones en la fosforilación oxidativa. Consecuentemente, las mitocondrias son muy sensibles a los niveles de oxígeno disponible y presentan una notable plasticidad y capacidad de adaptación a la hipoxia, ejercicio, y a la combinación de ambos estímulos.

De nuevo, un breve repaso bibliográfico arroja una gran variedad de resultados: se han reportado incrementos en la densidad de volumen mitocondrial inducidos por el ejercicio en hipoxia significativamente mayores que los hallados en ejercicio en normoxia (Geiser et al. 2001, Vogt et al. 2001), incrementos de magnitud similar al ejercicio en normobaria (Desplanches et al. 1993), incrementos significativamente mayores en la actividad de la citrato sintasa (CS), pero sin cambios en la densidad de volumen mitocondrial (Melissa et al. 1997; Green et al. 1999), y menor o similar incremento de la CS en entrenamiento en altitud que a nivel del mar (Terrados et al. 1988; Bakkman et al. 2007). En cualquier caso, la funcionalidad y eficacia mitocondrial son independientes, aunque no excluyentes, del volumen mitocondrial, por lo que son necesarios estudios que aborden los efectos de la hipoxia y ejercicio sobre procesos de biogénesis, bioenergética y dinámica mitocondrial.

Tabla 2. Principales estudios bioquímicos en los que se comparan los efectos del entrenamiento aeróbico a nivel del mar con los del entrenamiento aeróbico en altitud en un modelo *Training high – Living low*, omitiendo los trabajos en los que los sujetos permanecían en hipoxia a lo largo de todo el protocolo. Se han incluido estudios con hipoxia normobárica e hipobárica. En el primer caso, se expresa con su equivalente en metros de altitud. Nótese la gran variedad de protocolos de entrenamiento y exposición a hipoxia.

Estudio	Modelo	Entrenamiento	Hipoxia	Resultados en el grupo hipóxico *vs.* Nivel mar
Terrados et al. (1988)	Humano	Bicicleta 4-5 ses/sem x 105-150 min/ses 3-4 sem	2300 m	↑CD ↓PFK ↓LDH ↔CS
Zoll et al. (2006), Ponsot et al. (2006)	Humano	Atletismo 7 ses/sem 6 sem	3000 m	↑GLUT4 ↑PFK ↑PGC-1α ↑CS ↑SOD ↑COX ↔ETC
Terrados et al. (1990)	Humano	Ergómetro (pierna) 3-4 ses/sem x 30 min/ses 4 sem	2300 m	↑CS ↑Mioglobina ↓LDH ↔CD ↔FCSA
Desplanches et al. (1993)	Humano	Bicicleta 5 ses/sem x 45 min/ses 3 sem	5700 m	↑Densidad mitocondrial ↑C/F ↑FCSA
Melissa et al. (1997), Green et al. (1999)	Humano	Ergómetro (pierna) 3 ses/sem x 30 min/ses 8 sem	3200 m	↑CS ↔SDH ↔PFK ↔Densidad mitocondrial ↔CD ↔FCSA
Masuda et al. (2001)	Humano	Bicicleta 3 ses/sem x 60 min/ses 8 sem	2500 m	↔CS ↔Mb ↔C/F

Tabla 2. Continuación

Estudio	Modelo	Entrenamiento	Hipoxia	Resultados en el grupo hipóxico *vs.* Nivel mar
Geiser et al. (2001), Vogt et al. (2001)	Humano	Bicicleta 5 ses/sem x 30 min/ses 6 sem	3850 m	↑Densidad mitocondrial ↑CD ↑Mb ↑VEGF
Bakkman et al. (2007)	Humano	Bicicleta 4 ses/sem x 30 min/ses 4 sem	3000 m	↓CS ↓COX
Schmutz et al. (2010)	Humano	Bicicleta 5 ses/sem x 30 min/ses 6 sem	4000 m	↑Densidad mitocondrial ↑C/F
Gonchar et al. (2005)	Roedor	Natación 5 ses/sem x 30 min/ses 4 sem	4000 m	↑SOD ↑CAT ↓TBARS
Saxena et al. (2012)	Roedor	Natación 6 ses/sem x 60 min/ses 2 sem	10 mg/kg $CoCl_2$[1]	↑GLUT1 ↔GLUT4 ↔CS ↑SDH ↑PFK ↑COX ↔LDH ↑PGC-1α

Ses: sesiones, sem: semana, CD: densidad capilar, PFK: fosfofructoquinasa, LDH: lactato deshidrogenasa, CS: citrato sintasa, GLUT4: transportador de glucosa-4, PGC-1α: co-activador 1α del receptor gamma activado del proliferador de peroxisomas, SOD: superóxido dismutasa, COX: citocromo oxidasa c, ETC: cadena transportadora de electrones, FCSA: área de la sección transversal de la fibra, C/F: capilares por fibra, SDH: succinato deshidrogenasa, Mb: mioglobina, VEGF: factor de crecimiento vascular endotelial, CAT: catalasa, TBARS: substancias reactivas al ácido tiobarbitúrico, GLUT1: transportador de glucosa-1. ↑: aumento, ↓: disminución, ↔: sin cambios.

[1]El $CoCl_2$ (Cloruro de cobalto) se utiliza para mimetizar los efectos de la hipoxia, estabilizando HIF.

1.5 Relación entre la hipoxia intermitente y el EEIMD

Tal y como se ha explicado en puntos anteriores, tanto la red vascular como la mitocondrial se ven alteradas por el EEE, causando una disrupción en el aporte y consumo de oxígeno y nutrientes. Además, los procesos de reparación tisular son metabólicamente muy costosos, aumentando en gran medida la demanda de oxígeno (Tandara y Mustoe 2004), y la disfunción mitocondrial inducida por el ejercicio excéntrico puede comprometer aún más el limitado aporte de O_2. Esta disfunción puede ser responsable, adicionalmente, de un incremento en la producción de ROS (Magalhães et al. 2013). En consecuencia, restablecer o mejorar la red vascular, y por lo tanto el aporte de oxígeno, así como la funcionalidad mitocondrial, podría ser beneficioso para la recuperación del EEIMD. A pesar de ello, han recibido poca atención en el ámbito del EEIMD y como potencial diana terapéutica.

Capilares y mitocondrias se encuentran unidos por un elemento central, el oxígeno. Los capilares, como proveedores; las mitocondrias, como consumidoras. Tanto los primeros como las segundas son sensibles a los niveles de O_2 y capaces de adaptarse a variaciones en su disponibilidad. En consecuencia, manipular los niveles de O_2 (por ejemplo, mediante altitud simulada) es una manera de modular estos dos elementos simultáneamente. Así, mediante el uso de la HHI, sola o en combinación con ejercicio aeróbico, se podría aumentar la CD (con o sin neovascularización), mejorando el aporte de oxígeno y nutrientes, facilitando la recogida de *debris* y de metabolitos secundarios. La modulación de la homeostasis mitocondrial podría derivar en una red

más saludable y eficiente bioenergéticamente, con menor producción de ROS y mayor defensa contra la señalización apoptótica.

En base a todo lo expuesto hasta este punto nace la hipótesis de la presente tesis: si la exposición a HHI, especialmente cuando se compromete la homeostasis del oxígeno (por ejemplo, durante el ejercicio) es capaz de desencadenar respuestas fisiológicas adaptativas y beneficiosas en el músculo esquelético, tanto a nivel morfológico como metabólico, las mismas respuestas podrían darse en músculos sometidos a EEE, atenuando o modulando positivamente las alteraciones causadas por el EEIMD. La Figura 6 muestra un esquema simplificado de las interrelaciones que existen entre el EEIMD y la HHI.

Por lo tanto, el objetivo de este estudió fue evaluar si la HHI es capaz de inducir alteraciones que puedan resultar beneficiosas para la recuperación del EEIMD sin comprometer la histomorfología muscular en el sóleo de ratas entrenadas.

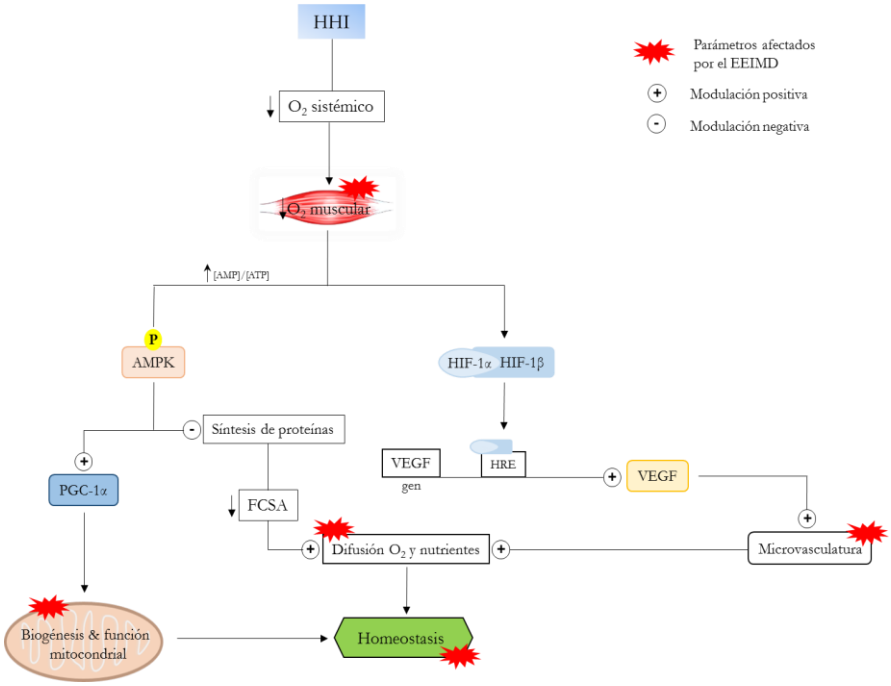


Figura 6. Interrelaciones simplificadas entre el EEIMD y la HHI. El EEIMD (representado por el estallido rojo) daña la microvasculatura, comprometiendo el aporte de oxígeno y nutrientes al músculo, así como a la función mitocondrial, debido a una pérdida de equilibrio de la homeostasis del Ca^{2+}. Estas alteraciones acaban por comprometer la homeostasis de las miofibras, induciendo, en el peor de los casos, a procesos de apoptosis o necrosis. Siguiendo la hipótesis planteada en esta tesis, la aplicación de HHI podría modular positivamente estas alteraciones mediante las vías indicadas. HHI: hipoxia hipobárica intermitente, AMPK: AMP quinasa, PGC-1 α: co-activador 1α del receptor gamma activado del proliferador de peroxisomas, FCSA: área de la sección transversal de la fibra, HIF-1: factor inducible por la hipoxia-1, VEGF: factor de crecimiento endotelial vascular, HRE: elementos de respuesta hipóxica

2.

Objetivos

1. Confirmar la presencia de daño muscular inducido por un protocolo de ejercicio excéntrico extenuante en ratas de laboratorio entrenadas.

2. Diseñar una herramienta metodológica capaz de evaluar a nivel histopatológico el daño muscular inducido por ejercicio excéntrico extenuante en ratas entrenadas.

3. Analizar la morfometría, composición fibrilar, capilarización y capacidad oxidativa del músculo sóleo de ratas entrenadas sometidas a un protocolo de ejercicio excéntrico extenuante.

4. Analizar si la hipoxia hipobárica intermitente, con o sin ejercicio aeróbico ligero, puede revertir alteraciones inducidas por el ejercicio excéntrico extenuante en la morfometría, capilarización y capacidad oxidativa del músculo sóleo de ratas entrenadas.

5. Analizar la expresión de proteínas marcadoras de biogénesis, dinámica, bioenergética y estrés oxidativo mitocondrial en el músculo sóleo de ratas sometidas a un protocolo de inducción de daño muscular mediante ejercicio excéntrico extenuante.

6. Analizar si la hipoxia hipobárica intermitente, con o sin ejercicio aeróbico ligero, puede revertir alteraciones inducidas por el ejercicio excéntrico extenuante en la expresión de proteínas marcadoras de biogénesis, dinámica, bioenergética y estrés oxidativo mitocondrial en el músculo sóleo de ratas entrenadas.

3.

Informe del director

La doctora Teresa Pagès Costas y el doctor Joan Ramon Torrella Guio, como directores de la Tesis Doctoral presentada por David Rizo Roca, hacen constar que el doctorando ha participado activamente en los artículos que forman esta memoria, tal como queda reflejado en el orden y composición del equipo de autores de cada uno de ellos. El doctorando ha tenido un papel fundamental en el diseño experimental y el tratamiento de los datos. También ha tenido un importante papel en el proceso de difusión y publicación de los resultados y conclusiones, es decir, en la redacción de los manuscritos y en el proceso de revisión por pares.

Los factores de impacto de las revistas donde se han publicado, aceptado y enviado los artículos que conforman esta tesis son los siguientes:

Artículo I

Título de la publicación: A semiquantitative scoring tool to evaluate eccentric exercise-induced muscle damage in trained rats.

Autores: Rizo-Roca D, Ríos-Kristjánsson JG, Núñez-Espinosa C, Ascensão A, Magalhães J, Torrella JR, Pagès T, Viscor G.

Revista: European Journal of Histochemistry

Año: 2015 Volumen: 59 Número: 4 Páginas: 1-7

DOI: 10.4081/ejh.2015.2544

JCR Impact Factor (2015): 2.421 JCR 5 Years I.F.: 1.818

Participación del doctorando: Participación en el cuidado de los animales, en la aplicación de los protocolos de entrenamiento e

inducción al daño muscular, así como en el muestreo de tejidos. Responsable de la preparación, tinción y análisis de las muestras. Autor intelectual de la herramienta desarrollada en el artículo. Tratamiento estadístico y gráfico de los datos. Redacción del manuscrito.

Artículo II

Título de la publicación: Intermittent hypobaric hypoxia combined with aerobic exercise improves muscle morphofunctional recovery after eccentric exercise to exhaustion in trained rats.

Autores: Rizo-Roca D, Ríos-Kristjánsson JG, Núñez-Espinosa C, Santos-Alves E, Gonçalves IO, Magalhães J, Ascensão AA, Pagès T, Viscor G, Torrella JR.

Revista: Journal of Applied Physiology

Año: 2016 (En prensa) Volumen: n/d Número: n/d Páginas: n/d

DOI: 10.1152/japplphysiol.00501.2016

JCR Impact Factor (2016): n/d JCR 5 Years I.F.: 3.421

Participación del doctorando: Participación en el cuidado de los animales, en la aplicación de los protocolos de entrenamiento, inducción al daño muscular, y exposición a hipoxia hipobárica, así como en el muestreo de tejidos. Responsable de la preparación, procesamiento y análisis de las muestras. Tratamiento estadístico y gráfico de los datos. Redacción del manuscrito.

Artículo III

Título de la publicación: Modulation of mitochondrial biomarkers by intermittent hypobaric hypoxia and aerobic exercise after strenuous eccentric exercise in trained rats.

Autores: Rizo-Roca D, Ríos-Kristjánsson JG, Núñez-Espinosa C, Santos-Alves E, Magalhães J, Ascensão AA, Pagès T, Viscor G, Torrella JR.

Revista: Applied Physiology Nutrition and Metabolism (*actualmente en segunda revisión*)

Año: n/d Volumen: n/d Número: n/d Páginas: n/d

DOI: n/d

JCR Impact Factor (207): n/d JCR 5 Years I.F.: 2.789

Participación del doctorando: Participación en el cuidado de los animales, en la aplicación de los protocolos de entrenamiento, inducción al daño muscular, y exposición a hipoxia hipobárica, así como en el muestreo de tejidos. Responsable de la preparación, procesamiento y análisis de las muestras. Tratamiento estadístico y gráfico de los datos. Redacción del manuscrito.

4.

Publicaciones

Artículo I

A semiquantitative scoring tool to evaluate eccentric exercise-induced muscle damage in trained rats. (2015). Rizo-Roca D[1], Ríos-Kristjánsson JG[1], Núñez-Espinosa C[1], Ascensão A[2], Magalhães J[2], Torrella JR[1], Pagès T[1], Viscor G[1]. European Journal of Histochemistry 59(4): 1-7.

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A semiquantitative scoring tool to evaluate eccentric exercise-induced muscle damage in trained rats

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Abstract

Unaccustomed eccentric exercise is a well-documented cause of exercise-induced muscle damage. However, in trained subjects muscle injury involves only light or moderate tissue damage. Since trained rats are widely used as a model for skeletal muscle injury, here we propose a semiquantitative scoring tool to evaluate muscle damage in trained rats. Twenty male Sprague-Dawley rats were trained for two weeks following a two-week preconditioning period, and randomly divided into two groups: control rats (CTL; n=5) and rats with eccentric exercise-induced muscle damage (INJ; n=15). Injured rats were sacrificed at three time points: 1, 3 and 7 days post injury (n=5 each). Transverse sections from the right soleus were cut (10 µm) and stained with haematoxylin-eosin. Samples were evaluated by two groups of observers (four researchers experienced in skeletal muscle histopathology and four inexperienced) using the proposed tool, which consisted of six items organised in three domains: abnormal fibre morphology, necrotic/(re) degenerating fibres (*muscle fibre domain*), endomysial and perimysial infiltration (*inflammatory state domain*) and endomysium and perimysium distension (*interstitial compartment domain)*. We observed the expected time course in the six evaluated items. Furthermore, agreement among observers was evaluated by measuring the Intraclass Correlation Coefficient (ICC). Within the experienced group, items from the *muscle fibre* and *interstitial compartment* domains showed *good* agreement and the two items from the *infiltration compartment domain* showed *excellent* agreement. In conclusion, the proposed tool allowed quick and correct evaluation of light to moderate muscle damage in trained rats with good agreement between observers.

Introduction

Several muscle injury models have been developed in order to better understand the underlying mechanisms involved in muscle damage assessment and recovery. These various models can be classified as either contraction-induced, resulting from eccentric contractions or strenuous exercise; or trauma-induced, due to exposure of the muscle to a toxin, laceration or crush injury.[1] Among the contraction-induced models, the eccentric exercise-induced muscle damage model is one of the most widely used in physiology laboratories.[2,3] Unlike models of extreme physical trauma, in eccentric exercise-induced muscle damage only a relatively small proportion of fibres are affected, with most of the muscle cells remaining healthy and functional.[4-7] Moreover, some training programmes can also reduce the amount of damage produced by eccentric exercise-induced muscle damage. It is well known that prior bouts of eccentric contractions provide a protective effect against eccentric exercise-induced muscle damage but, although numerous hypotheses have been proposed and tested, the unifying mechanism remains unclear.[8,9] In terms of the effect of non-specifically eccentric training, such as level running, few studies have been conducted. In a study by Schwane and Armstrong,[10] the level running group showed a certain degree of protection from further eccentric exercise-induced damage. Another study found that, after downhill running, endurance-trained rats had lower serum creatine kinase activity than sedentary rats,[11] while Koh and colleagues demonstrated that lengthening contractions are not necessary to induce protection from eccentric muscle damage.[12] Some of the mechanisms responsible for the damage induced by eccentric exercise, such as loss of calcium homeostasis and the inflammatory response,[13] are known to be regulated by endurance training.[14,15] Moreover, endurance training can increase the slow oxidative fibre population through fibre-type conversion from fast to slow.[16] Given that fast fibres are more susceptible to eccentric exercise-induced muscle damage than slow fibres,[17] trained rats could be more protected against eccentric contraction due to their fibre type composition. Furthermore, in level running the soleus muscle (a predominantly slow muscle) also performs eccentric contractions during the gait cycle, probably developing some adaptations characteristic of specific eccentric training.[18]

Currently, in order to evaluate the impact of eccentric contraction-induced injuries, a wide variety of non-invasive functional and biochemical measurements are usually performed, including maximal voluntary contraction torque and range of motion, swelling, ratings of soreness, maximum isometric force production, blood levels of myofibre protein and inflammatory cytokines and T2 signal intensity.[19] In animal research (especially in rodent models) muscle biopsies and excisions are common, allowing histological approaches that can provide valuable information to better understand the injury phenomena. For the reasons mentioned above, moderate but appreciable damage is induced in endurance-trained subjects. Thus, we thought it necessary to develop an appropriate support tool to evaluate the muscle damage induced by eccentric exercise in trained rats, and here we present a sensitive histopathological semiquantitative tool that allows rapid evaluation of light to moderate muscle damage. At the histopathological level, features of muscle damage include fibre necrosis, swelling, an atrophic and sharp appearance, sarcomere and Z-line disruption, infiltration by phagocytic or inflammatory cells (neutrophils, macrophages, lymphocytes), interstitial oedema, enlarged interstitial area and extracellular matrix disruption.[20] Since muscle damage is always followed by muscle regeneration (in healthy subjects), features of myofibre regeneration are also taken into

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Key words: Histopathology; haematoxylin-eosin; histochemical method.

Contributions: DRR, JRT, TP, GV, study conception and design; DRR, JGRK, CNE: care and training of experimental animals, data collection; DRR, JGRK, CNE, AA, JM, JRT, TP, GV: data analysis and interpretation; DRR, JRT, manuscript drafting; DRR, JRT, JM, TP, GV, manuscript revision. All authors read and approved the final manuscript.

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account, including: small fibres within the intrafascicular area of a mature myofibre, round myofibres, central or internalised nuclei and basophilic cytoplasm.[21,22] We used most of these well-known features to design a scoring method to evaluate muscle damage in the soleus muscle of trained rats, based on three major categories: histopathological abnormalities in the myofibres, the inflammatory state and interstitial compartment distension.

Materials and Methods

Animals and experimental design

Twenty male Sprague-Dawley run-trained rats were used for this study. All animals were maintained at an average temperature of 23°C under a light-dark cycle of 12 h/12 h with food and water *ad libitum*. The animals were randomly divided into two experimental conditions: trained rats that did not suffer muscle injury before sampling (Control, CTL; n=5) and trained rats that were submitted to an eccentric exercise-induced muscle damage protocol and sacrificed 1, 3 or 7 days after muscle injury (Injured, INJ t01, t03 and t07 respectively; n=5 each group).

All procedures were performed in accordance with the internal protocols of our laboratory, which were authorized by the University of Barcelona's Ethical Committee for Animal Experimentation and ratified, in accordance with current Spanish legislation, by the *Departament de Medi Ambient i Habitatge* of the Catalan Government (*Generalitat de Catalunya*).

Training protocol

All animals were trained under environmental conditions (21±2°C) on a flat treadmill (LE 8710; Panlab, Barcelona, Spain). After a two-week preconditioning protocol in which the duration and intensity of the exercise were gradually increased, the training protocol began (Figure 1). This training period consisted of two daily running sessions for two weeks at a speed of 45 cm s^{-1} over 35 min. According to Rodrigues *et al.*,[23] a rat running at a speed of 45 cm s^{-1} in a treadmill develops an exercise intensity of 90-100% of its VO_{2max}. Due to the high intensity of exercise, it was scheduled to occur in two bouts with an interval of 6 h for recovery between sessions.

Eccentric exercise-induced muscle damage protocol

One, three or seven days after completion of the training period, skeletal muscle damage was induced in the INJ group by eccentric muscle contraction exercise, as described by Armstrong *et al.*,[3] consisting of downhill running at 50 cm s^{-1} down an incline of 15° until exhaustion. The protocol was applied twice on the same day: one session in the morning and one in the afternoon, with a rest period of 4 h between the end of the first session and the beginning of the second one.

Muscle sampling

Under animal's anaesthesia, the right soleus muscles were excised, immediately frozen in pre-cooled isopentane and then stored in liquid nitrogen. After dissecting out other tissues not involved in the present study, rats were killed by exsanguination.

Light microscopic observations of histopathological changes

Soleus muscles were placed in an OCT embedding medium (Tissue-Tek®; Sakura, Alphen aan den Rijn, The Netherlands) at 22°C and serial transverse sections (10 µm) of the equatorial region were cut using a cryostat (model CM3050S; Leica, Wetzlar, Germany) and stained with haematoxylin-eosin (HE) to evaluate the histopathological features of muscle damage. Stained samples were observed under a light microscope (BX61, Olympus, Tokyo, Japan) and photographed using a coupled camera (DP70, Olympus). To avoid sampling bias, and although the soleus muscle is homogeneous in fibre types, three pictures were taken from different areas and evaluated separately.

Structure of the proposed semi-quantitative tool

Three domains of histopathologic abnormality were chosen as the main elements of the scoring system: i) muscle fibre; ii) inflammatory state; and iii) interstitial compartment. Within each domain, and taking into account their importance, different items were determined considering their frequency of occurrence (items with a very low rate of occurrence were discarded) and physiological relevance (items strongly related to eccentric exercise-induced muscle damage). The three domains were analysed independently to avoid bias in the relative importance of each one. All items were scored as 0, 1 or 2, as a wider range of scores would lead to a more confusing and slower tool. With just three values, which generically represent *absence of damage* (0), *moderate damage* (1) and *severe damage* (2), the classification of each item is more intuitive and faster without losing accuracy.

Three pictures for each sample each covering an area of 0.55 mm^2 (approximately 120 fibres per picture) were analysed. Three measurements (one per picture) were taken for each item and subject, but only the highest score was considered. This criterion was applied to avoid *score dilution*. As stated above, muscle damage in trained rats is moderate and usually focused in a few fibres. Consequently, large areas of the samples remain undamaged and would receive a low score. If all the scores

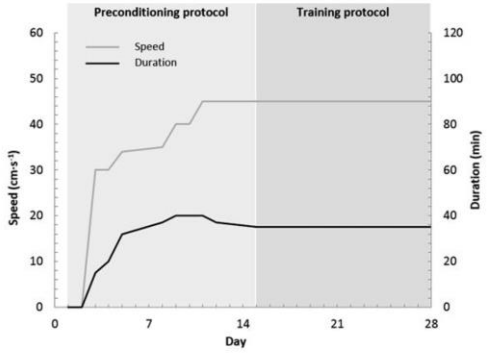

Figure 1. The exercise schedule for the preconditioning and training protocol, with two sessions per day, except for the first 4 days. During the first two sessions, the animals were placed on the treadmill, but did not run.

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were considered, given enough pictures, even rats that showed great but focused damage would have obtained a score close to 0.

Table 1 shows the chosen domains and items and their threshold for the different scores. These thresholds were established experimentally using CTL samples. An explanation for each domain and item is given in the following sections.

Muscle fibre domain

Healthy, uninjured muscle tissue exhibits relatively uniform polygonal myofibres that contain many peripherally placed nuclei. Intact sarcolemma and non-fragmented sarcoplasm are also characteristics of normal myofibres.

This domain was divided into two items based on their severity: i) Fibres with abnormal morphology (*abnormal morphology* item), which may or may not have retained their functionality. Abnormal fibre morphology included fibre atrophy (small, angulated or rounded fibres), swollen appearance or fibre splitting. ii) Severely damaged fibres that were undergoing necrosis or myophagocytosis (*necrotic/(re)degenerating fibres* item). Necrotic and degenerating fibres were identified by the presence of infiltrating inflammatory cells (myophagocytosis), fragmented sarcoplasm, dark staining (hypercontracted fibres) and pale staining (necrotic fibres), while regenerating fibres were, in the early stages, represented by small basophilic myotubes and later by *bluish-stained* myofibres with central nuclei.[24]

Given that haematoxylin and eosin was the only staining protocol used, differentiating between necrotic and regenerating fibres was sometimes difficult. Additionally, regenerative processes are usually preceded by necrosis. For these reasons, necrotic, degenerating and regenerative fibres were included as a single item.

Extracellular inflammatory domain

The inflammatory response is well studied in eccentric exercise-induced muscle damage and is characterized, besides several biochemical-related consequences, by leukocyte infiltration.[25] Although neutrophils, macrophages and lymphocytes are involved in this response, their differentiation would be time and resource consuming, as several antibodies would be needed in order to identify each cell type. Consequently, the inflammatory domain took into account all leukocytes together, but was divided into two items according to their localization: i) perimysial infiltration; and ii) endomysial infiltration.

Interstitial compartment

In physiological conditions, muscle fibres are tightly packed together into fascicles separated by scant connective tissue. The extracellular matrix is a very dynamic compartment and responds to mechanical stress,[26] and can be disrupted as a consequence of the eccentric stimulus, leading to an inflammatory response[27] and fibrotic process. Therefore, we evaluated the distension of the interstitial compartment, divided into the items i) endomysium; and ii) perimysium.

Statistical methods

All samples were evaluated by two groups of observers: the first group comprised four researchers experienced in skeletal muscle histology, and the second group comprised four researchers who were inexperienced in this field of study. In order to assess the degree of agreement among observers, the Intraclass Correlation Coefficient (ICC) was calculated using MedCalc for Windows ver. 15 (MedCalc Software, Ostend, Belgium). This measure is used to demonstrate consensus and agreement among observational ratings provided by multiple observers or instruments, 1 being the maximum value for an ICC measure.[28] In our study, the data was modelled assuming that the same observers would evaluate all samples, although these observers may be a subset of a larger set of observers. ICC values are presented with a 95% confidence interval (95% CI). The level of agreement can be interpreted as follows: <0.40 = poor; 0.40-0.59 = fair; 0.60-0.74 = good; and ≥0.75 = excellent. These cut-offs, although arbitrary, are commonly used.[29]

Histological scores were compared using a Kruskal-Wallis test followed by Dunn's multiple

Table 1. Structure of the scoring tool used to evaluate eccentric exercise-induced muscle damage.

Domains and items	Score	Definition
Muscle fibre		
Abnormal morphology	0	<4 fibres
Small, rounded or angular fibres, splitting	1	4 to 7 fibres
and hypertrophied fibres were considered abnormal	2	>7 fibres or an entire fascicle
Necrotic/(re)degenerating	0	Absent
Basophilic, light stained, central nuclei	1	1 to 2 fibres
and myophagocytosed fibres	2	>2 fibres
Extracellular inflammatory state		
Endomysial infiltration	0	<6 cells
Small, mononuclear cells found in the endomysium	1	one cluster or ≥6 cells
	2	>1 cluster or an entire fascicle infiltrated
Perimysial infiltration	0	≤10 cells
Small, mononuclear cells found in the perimysium	1	>10 cells
	2	>2 clusters or widely diffused
Interstitial compartment		
Endomysium distension	0	Tight space
Space between individual muscle fibres	1	Moderately distended
	2	Completely distended
Perimysium distension	0	Tight space
Space between fascicles	1	Moderately distended
	2	Completely distended

Table 2. Intra-class coefficients with 95% confidence intervals evaluated by two groups of researchers (n=4 each group).

Domain	Item	ICC (95% CI) Experienced	ICC (95% CI) Inexperienced
Muscle fibre	Abnormal morphology	0.71 (0.55-0.85)	0.38 (0.20-0.63)
	Necrotic/(re)degenerating	0.63 (0.44-0.79)	0.41 (0.17-0.66)
Extracellular inflammatory state	Endomysial infiltration	0.82 (0.70-0.91)	0.52 (0.28-0.73)
	Perimysial infiltration	0.78 (0.65-0.89)	0.49 (0.23-0.72)
Interstitial compartment	Endomysium distension	0.69 (0.52-0.83)	0.28 (0.02-0.56)
	Perimysium distension	0.67 (0.49-0.82)	0.37 (0.10-0.66)

ICC, Intra-class coefficients; CI, confidence interval.

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comparison post hoc test. Statistical significance was considered as $P<0.05$.

Results

Application of the proposed semi-quantitative tool

Soleus cross-sections from all groups were evaluated using the proposed tool (Table 1). As expected, the CTL group received the lowest score for each item while INJ t01 and t03 received the highest (Figure 2). When compared to CTL rats, the INJ t01 group had a significantly higher score for the following items: abnormal morphology ($P=0.0188$); necrotic and (re)degenerating fibres ($P=0.0082$), endomysial and perimysial infiltration ($P=0.0087$ and $P=0.0049$, respectively), and perimysium distension ($P=0.0343$). INJ t03 rats followed a similar pattern, with significantly higher scores in abnormal morphology ($P=0.0048$), necrotic and (re)degenerating fibres ($P=0.0382$), and endomysial infiltration ($P=0.0029$). Finally, INJ t07 did not differ significantly from the other groups, receiving for each item a mean score between the CTRL and INJ t01/t03 groups. Figure 3 shows representative pictures of each group.

Agreement among observers on scoring data

Six items were separately evaluated by four experienced and four inexperienced researchers in skeletal muscle physiology and morphology. In the experienced group, abnormal morphology, necrotic/(re)degenerating fibres, and endomysium and perimysium distension items showed *Good* agreement (ICC≥0.60), while the two items regarding infiltration (endomysial and perimysial infiltration) showed *excellent* agreement (ICC≥0.75). On the other hand, the group of four inexperienced researchers only showed *poor* and *fair* agreement (abnormal fibres and interstitial compartment items; and necrotic/(re)degenerating fibres and extracellular inflammatory state items, respectively). ICC values for each item are shown in Table 2. Representative examples of the use of the proposed tool by the two groups of observers are shown in Figure 4.

Discussion

We developed the proposed tool in order to quickly and simply evaluate and understand the amount of muscle damage in trained rats. Although there are already numerous related semiquantitative measurement tools, none of them is sufficiently sensitive to detect/evaluate light to moderate damage. For example, Takekura *et al.*[30] used a model in non-trained rats to evaluate different features, such as necrotic and spreading fibres, scoring from - (no changes observed) to ++++ (up to 8% of fibres affected). Their lowest value for damage (score +) was up to 2%. In contrast, in our trained rats this value would represent an important amount of damage (score 2).

Other methods use the percentage of injured fibres to evaluate muscle damage, for

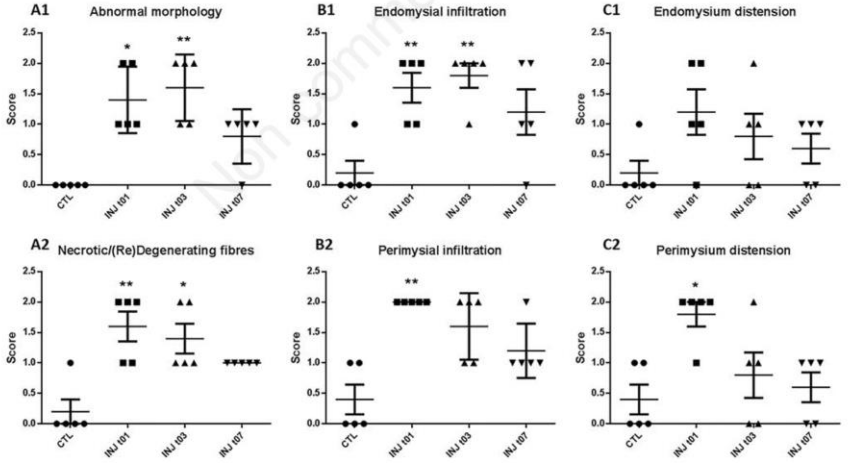

Figure 2. Scatter plot in which each symbol (dots, squares and triangles) represents the given score for a subject. Mean and SEM are represented. Asterisks (one or two) represent significant differences vs CTL ($P<0.05$ and $P<0.01$, respectively). A1 and A2, muscle fibre domain items; B1 and B2, extracellular inflammatory domain items; C1 and C2, interstitial compartment items.

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example, McCormack *et al.*[31] used this method when evaluating muscle injury in a model of ischaemia/reperfusion, Koh *et al.*[12] applied it after stimulating the peroneal nerve to evaluate the damage produced in extensor *digitorum longus* by repeated lengthening contractions. However, given the small number of injured fibres present in trained animals, evaluating injury based on the percentage of damaged fibres will not provide reliable results. Wedderburn *et al.*[32] used a scoring system involving four domains and 16 items (contrasting with our three domains and six items), which represents a more exhaustive way of evaluating the damage. Their tool is adequate for clinical and diagnostic purposes but is highly time-consuming, contrasting with the rapidity of our method, when applied to a model of trained animals.

Therefore, we here propose a semiquantitative approach that offers a compromise between reliability and speed, and allows samples to be classified in whole numbers as scores (0, 1 or 2). The proposed tool was found to be able to discriminate between healthy and damaged samples. For all items, the CTL group received a mean score below 1, significantly

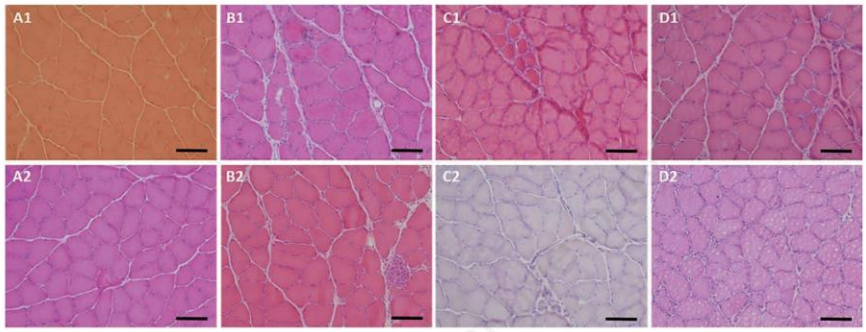

Figure 3. Representative microphotographs of CTL rats (A1 and A2), INJ t01 (B1 and B2), INJ t03 (C1 and C2) and INJ t07 (D1 and D2). Degeneration and myophagocytosis appear in pictures B2, C1 and C2. Endomysial infiltration is evident in B1, C1 and D1. Scale bars: 100 µm.

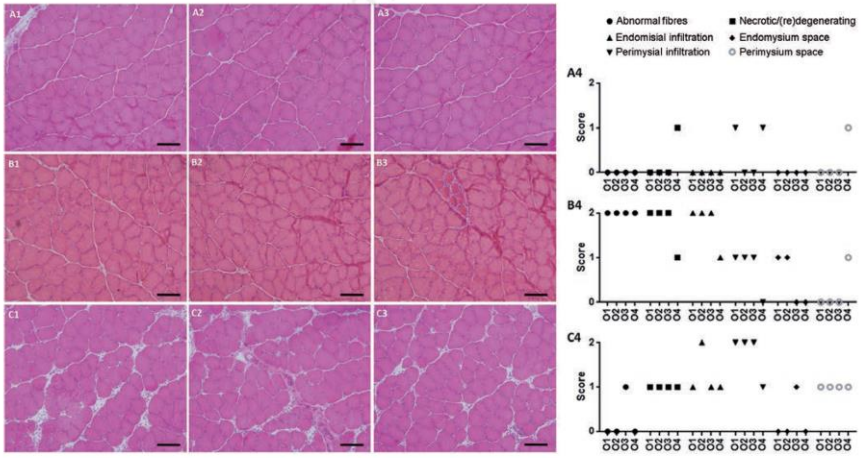

Figure 4. Microphotographs of evaluated pictures. A4, B4 and C4 show the respective scores given by four experienced observers (O1-O4). Only the highest score from each picture was ultimately taken into account in order to better represent great anomalies such as those found in B3. Scale bars: 100 µm.

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lower than the scores received by the INJ t01 group (except for the Endomysium distension item). Furthermore, for all items, the tool showed the time course described in the literature following exercise-induced muscle damage: an early acute inflammatory and degenerative phase (highest scores are in the t01 and t03 groups) followed by a later stage (t07) with diminished signs of degeneration and inflammation.

Regarding the *interstitial compartment* domain, both endomysium and perimysium distension items showed the same time course as the previous domains, although without statistical differences when the different time points were compared, with the exception of Perimysium distension in the INJ t01 group. There was much interindividual variance among subjects, with each of the three possible scores co-existing within each group. Thus, although the trend was clear, there were no significant differences.

Inter-rater reliability

A representative example of scoring using the proposed semiquantitative tool (Table 1) is shown in Figure 4. Good agreement between the four observers was apparent in most items.

Regarding the degree of agreement among the four observers, the items belonging to the *inflammatory state* domain (endomysial and perimysial infiltration) obtained the highest ICC (Table 2), which qualified as *excellent*. The fact that mononuclear infiltrating cells are easily identifiable, as well as the easily distinguishable features of score 0 and 2 (Table 1), are key factors explaining this high agreement. The lowest ICC (0.63), which still qualified as *good*, was obtained for the item necrotic/(re)degeneration. This could be explained by the fact that some of its features are slightly subjective. For example, cytoplasm coloration, namely basophilic or pale staining, is an important marker used to classify a fibre for this item, but its evaluation depends, to some degree, on observer sensitivity. With regards to the Abnormal fibres item, *good* agreement (ICC=0.71, close to the *excellent* threshold) was obtained. The *interstitial compartment* domain (endomysium and perimysium distension), despite being purely qualitative and subjective (these items were evaluated as *not distended*, *moderately distended* and *completely distended*) also showed *Good* agreement (ICC=0.69 and 0.67). Although the ICC is a reliable statistical test for assessing agreement,[30] we decided to add an additional group of observers to definitively rule out the possibility that the consensus occurred by chance. This additional group of four observers comprised researchers who were inexperienced in skeletal muscle histopathology. Effectively, this group demonstrated *poor* and *fair* levels of agreement for all items.

In conclusion, the proposed semiquantitative scoring tool offers a quick, simple, sensitive and understandable method for evaluating eccentric exercise-induced muscle damage in trained rats by using a simple, widely used and highly standardised procedure such as haematoxylin-eosin staining. The scoring system showed the expected time course of muscle injury when tested on samples of trained rats. Moreover, it generated enough agreement to be generally used by researchers who need to assess light to moderate muscle damage in rats. Potential limitations must be taken into account: application of the tool is restricted to research purposes, and the threshold between scores should be empirically adjusted depending on the model and degree of muscle damage.

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Artículo II

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Intermittent hypobaric hypoxia combined with aerobic exercise improves muscle morphofunctional recovery after eccentric exercise to exhaustion in trained rats

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Running head: aerobic exercise and hypoxia in muscle damage recovery

ABSTRACT

Unaccustomed eccentric exercise leads to muscle morphological and functional alterations, including microvasculature damage, the repair of which is modulated by hypoxia. Here we present the effects of intermittent hypobaric hypoxia and exercise on recovery from eccentric-induced muscle damage (EEIMD). Soleus muscles from trained rats were excised pre- (CTRL) and 1, 3, 7 and 14 days after a double session of EEIMD protocol. A recovery treatment consisting of one of the following protocols was applied one day after the EEIMD: passive normobaric recovery (PNR), a 4-hour daily exposure to passive hypobaric hypoxia at 4000m (PHR) or hypobaric hypoxia exposure followed by aerobic exercise (AHR). EEIMD produced an increase in the percentage of abnormal fibers compared with the CTRL; and affected the microvasculature by decreasing capillary density (CD, capillaries per mm^2) and the capillary-to-fiber ratio (CF). After 14 days, AHR exhibited a CD and CF similar to the CTRL (789 and 3.30 vs. 746 and 3.06) and significantly higher than PNR (575 and 2.62) and PHR (630 and 2.92). Furthermore, VEGF expression showed a significant 43% increase in AHR when compared with PNR. Moreover, after 14 days, the muscle fibers in AHR had a more oxidative phenotype than the other groups, with significantly smaller cross-sectional areas (AHR: 3745; PNR: 4502; PHR: 4790 μm^2), higher citrate synthase activity (AHR: 14.8; PNR: 13.1; PHR: 12 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) and a significant 27% increment in PGC-1α levels compared with PNR. Our data show that hypoxia combined with exercise attenuates or reverses the morphofunctional alterations induced by EEIMD.

NEW & NOTEWORTHY

Our study provides new insights into the use of intermittent hypobaric hypoxia combined with exercise as a strategy to recover muscle damage induced by eccentric

exercise. We analyzed the effects of hypobaric exposure combined with aerobic exercise on histopathological features of muscle damage, fiber morphofunctionality, capillarization, angiogenesis and the oxidative capacity of damaged soleus muscle. Most of these parameters were improved after a 2-week protocol of intermittent hypobaric hypoxia combined with aerobic exercise.

KEYWORDS

Intermittent hypobaric hypoxia, aerobic exercise, soleus fiber morphofunctionality, eccentric exercise-induced muscle damage.

INTRODUCTION

Strenuous unaccustomed eccentric exercise alters muscle function and structure. It is well known that eccentric exercise-induced muscle damage (EEIMD) leads to delayed-onset muscle soreness, swelling, loss of peak contraction force and increased myofibrillar protein concentrations in blood (e.g., myoglobin, creatine kinase, troponin-I) (33, 64). Histological and ultrastructural changes in myofibers are also evident, such as scattered fiber necrosis and atrophy, inflammatory cell infiltration and extracellular matrix enlargement (3, 70). Myophagocytosis of necrotic fibers, increased production of ROS, damaged mitochondria and activation of satellite cells are also features of the muscle damage produced by unaccustomed eccentric contractions (62). In addition to these alterations, unaccustomed eccentric exercise also damages the muscle microvasculature, impairing its microcirculation and hemodynamics (13, 17, 38, 57). Such disruption of the microvasculature leads to a lack of oxygen and nutrients, which is exacerbated by the high energy-demanding repair processes that take place at the damaged site. Thus, the restoration of the microvasculature is an essential step in the recovery from muscle damage.

Sport scientists and professionals are in a constant search of new methods to accelerate muscle recovery. Several treatments have been developed, such as cryotherapy, antioxidant, carbohydrate and anti-inflammatory supplementation, massage and exercise, but in most cases the evidence is equivocal, probably due to the lack of a consensus regarding the dose, intensity and frequency of these treatments (32). Most of these interventions aim to reduce delayed onset muscle soreness, inflammation or oxidative stress (32). However, little attention has been given to the microvasculature as a potential therapeutic target, which is quite surprising considering the importance of the endothelial cells and capillary network in inflammation (28), ischemic damage (27) and the supply of energetic substrates and oxygen to tissues. Thus, the enhancement of the microvasculature could be a field worth studying. Since hypoxia is a master regulator of the capillary bed, interventions including hypoxia protocols could have interesting effects on muscle recovery after eccentric exercise (42). Despite the widespread popularity of altitude training, neither the role of hypoxia nor the role of exercise in hypoxia have been studied in the recovery from muscle damage.

The physiological effects and adaptive responses to distinct models of hypoxia exposure in healthy subjects have been well studied (51, 66, 81), and some of them have been suggested to improve physical performance in elite athletes (4, 78). Despite some controversy (30), increased erythropoiesis and aerobic capacity (19, 69) and improved lactate threshold (9) are among the described benefits of controlled hypoxia exposure. These benefits have led to the development of several training options that combine periods of living and/or training at sea level and altitude (5). Moreover, some specific tissue responses to hypoxia have been reported in skeletal muscle since this tissue has a high plasticity and can develop an adaptive response when exposed to hypoxic conditions. When the exposure to hypoxia is too acute, too severe or long lasting, there is a loss of muscle mass (20), increased production of reactive oxygen species (ROS)

89 (15, 47) and impaired mitochondrial oxidative phosphorylation (12, 47). However,
90 when applied at adequate levels of intensity and duration or with distinct patterns of
91 exposure, hypoxia can trigger beneficial adaptations, particularly in the most oxygen-
92 demanding organs and tissues (55). As reported by Panisello et al. (60, 61), sedentary
93 rats exposed to intermittent hypobaric hypoxia (IHH) showed significant increases in
94 capillary density and the capillary-to-fiber ratio in the myocardium and diaphragm. In
95 contrast, these adaptations were not found in the tibialis anterior, a skeletal muscle with
96 little oxygen demand in confined animals. As pointed out by Lundby et al. (45),
97 functional and structural adaptations of skeletal muscle in response to hypoxic stimuli
98 only seem to occur when its oxygen homeostasis is challenged. Studies in which
99 exposure to intermittent hypoxia (from minutes to hours) was combined with exercise
100 protocols reported increased mitochondrial volume density (23) and citrate synthase
101 activity (26, 50) and enhanced antioxidant capacity (25) in hindlimb muscles. These
102 changes are mainly modulated through PGC-1α (peroxisome proliferator-activated
103 receptor gamma coactivator 1-alpha), a master regulator of mitochondrial biogenesis
104 (44). Moreover, the combination of exercise and hypoxia is a powerful angiogenic
105 stimulus, mainly through the upregulation of pro-angiogenic factors (such as VEGF,
106 PDGF and PGC-1α) and the downregulation of anti-angiogenic factors, such as
107 thrombospondin-1 (7, 59), improving muscle capillarization. Moreover, it has been
108 reported that hypoxia and exercise increase the number of circulating endothelial
109 progenitor cells (1, 58); these are involved in the neovasculogenesis and regeneration of
110 blood vessels (63).

111 In addition to all these potential benefits of hypoxia and exercise in the muscle
112 microvasculature, some *in vitro* studies have reported that hypoxia promotes the
113 proliferation of cultured myogenic satellite cells (11, 41). Indirect evidence has also
114 been found in *in vivo* models. Nielsen et al. (56) reported that resistance training with
115 blood flow restriction leads to increased proliferation of myogenic stem cells in the
116 trained muscle. Accordingly, the use of hypoxia exposure strategies to counteract the
117 deleterious impact of some models of exercise on skeletal muscle is a matter that
118 deserves to be better analyzed.

119 Therefore, the purpose of the present study was to explore whether exposure to IHH,
120 alone or combined with aerobic exercise, can modulate or improve different aspects of
121 muscle morphofunctionality, microvasculature and metabolism during the recovery
122 from muscle damage. We hypothesize that the exposure to IHH, especially when
123 combined with aerobic exercise, will positively regulate and enhance the
124 microvasculature and oxidative capacity, leading to a modulation of the
125 histomorphological changes induced by EEIMD.

126

127 **MATERIAL AND METHODS**

128 ***Animals***

129 Seventy-eight male Sprague-Dawley rats were used for this study. All animals were
130 maintained at an average temperature of 23°C under a light-dark cycle of 12 h/12 h with
131 food and water *ad libitum*. All animals were trained twice daily for a month before
132 being randomly assigned to different experimental conditions. Rats assigned to the
133 control group (CTRL, n=6) were euthanized 3 days after their last training session. The
134 other animals underwent an EEIMD protocol and then were divided into three
135 experimental groups, starting the following day: 1) rats that passively recovered in
136 normoxic conditions (PNR, passive and normoxic recovery); 2) rats that passively
137 recovered following a protocol of IHH exposure (PHR, passive and hypoxic recovery);
138 and 3) rats that actively recovered following IHH exposure combined with light aerobic
139 exercise performed immediately after exposure in a treadmill (AHR, active and hypoxic
140 recovery). Animals from these three groups were euthanized immediately after the last
141 intervention at four different time points (n=6 each): 1, 3, 7 and 14 days after the
142 EEIMD protocol (t01, t03, t07 and t14, respectively). Figure 1 summarizes the
143 experimental design.

144 All procedures were performed in accordance with the internal protocols of our
145 laboratory, which were authorized by the University of Barcelona's Ethical Committee
146 for Animal Experimentation and ratified, in accordance with current Spanish legislation,
147 by the *Departament de Medi Ambient i Habitatge* (*Generalitat de Catalunya*).

148 ***Aerobic training protocol***

149 All animals were trained at room temperature (21 ± 2°C) on a treadmill (LE 8710,
150 Panlab, Barcelona, Spain). After a 10-day preconditioning phase, in which the duration
151 and intensity of the running exercise were gradually increased in order to habituate the
152 animals to the treadmill and the new activity, the speed of the treadmill was set at 45
153 $cm \cdot s^{-1}$ and the duration of exercise at 35 min. The animals trained twice daily (with a
154 recovery interval of 6 h) for 2 weeks. This daily double-session of running allowed rats
155 to achieve the physiological adaptations to endurance exercise while optimizing the
156 duration of the whole protocol.

157 ***Eccentric exercise-induced muscle damage protocol***

158 Skeletal muscle damage was induced by eccentric exercise consisting of downhill
159 running with a decline of 15° and at 50 $cm \cdot s^{-1}$ until exhaustion (3). This protocol began
160 3 days after the end of the training period, and was applied twice on the same day: one
161 session in the morning and one in the afternoon, with a resting period of 4 h between the
162 end of the first session and the beginning of the second one.

163 ***Intermittent hypobaric hypoxia exposure***

164 IHH sessions started the day after the EEIMD protocol and were performed using a
165 hypobaric chamber with a volume of about 450 L, providing space for three rat cages. A
166 relative vacuum was created by a rotational vacuum pump (TRIVAC D5E, Leybold,
167 Köln, Germany) with its air-flow rate regulated at the inlet by a micrometric valve.

Inner pressure was controlled by two differential sensors (ID 2000, Leybold, Köln, Germany) driving a diaphragm pressure regulator (MR16, Leybold, Köln, Germany). The target pressure of 462 torr (equivalent to 4,000 m of altitude) was achieved steadily over about 15 min. Once this pressure had been reached, the chamber pressure was maintained and regulated for 4 h. At the end of the session, pressurization to normal barometric pressure was gradually restored over 15 min. The total number of days of hypobaric hypoxia exposure varied according to the sampling schedule. Animals assigned to t01 were in the hypobaric chamber for a single session, whereas t14 animals were subjected to 2 weeks of daily exposure. Animals had *ad libitum* access to food and water kept in open-air reservoirs inside the hypobaric chamber during the hypoxia sessions.

Aerobic exercise recovery protocol

AHR rats were enrolled in a recovery exercise program consisting of a daily session of aerobic exercise immediately after a hypobaric hypoxia session. These rats were placed on a treadmill to run in accordance with a program of low impact exercise. The exercise session lasted 20 min with a gradual increase in speed until reaching 30 $cm \cdot s^{-1}$ and a gradual increase in inclination from 0° to 5°.

Plasma and muscle sampling

The same day after the last intervention, soleus muscles were excised, rinsed in saline solution, immediately frozen in pre-cooled isopentane and stored in liquid nitrogen until further analysis. Blood samples were obtained from the vena cava, placed in EDTA tubes and centrifuged at 1,300 *g* for 10 minutes. Plasma was collected and stored at -80°C until assayed.

Histochemical procedures

Soleus muscles were placed in an OCT embedding medium (Tissue-Tek®, Sakura Finitek Europe, Zoeterwoude, The Netherlands) at -22°C and serial transverse sections (12–16 µm) were cut using a cryostat (Leica CM3050S, Wetzlar, Germany). The following histochemical assays were performed: 1) succinate dehydrogenase (SDH) to identify aerobic and anaerobic fibers (50); 2) myofibrillar adenosine triphosphatase (mATPase) following pre-incubation in alkaline (pH 10.7) and acid (pH 4.2) media to differentiate between slow and fast fibers (8); 3) endothelial adenosine triphosphatase (eATPase) to reveal muscle capillaries (22); and 4) hematoxylin-eosin (H-E), to evaluate the histopathological features of muscle damage.

Morphofunctional measurements

All morphofunctional measurements were performed on microphotographs obtained with a light microscope (BX61, Olympus, Tokyo, Japan) connected to a digital camera (DP70, Olympus, Tokyo, Japan) at a magnification of 20x. All the parameters listed below were empirically determined from windows of transverse cross section tissue of approximately $5.5 \cdot 10^5$ μm^2 from three different zones or muscle fields (lateral, central

and medial) in each sample using an image analyzing software (ImageJ; Rasband 1997–2014). After testing for the absence of differences between the three muscle fields from each sample, the data obtained from all fields were considered together so that the sample size was large enough.

All fibers were typified as slow oxidative (SO) or fast oxidative glycolytic (FOG). The following parameters were counted or calculated: fiber density (FD), capillary density (CD) and capillary-to-fiber ratio (C/F=CD/FD). The fiber cross-sectional area (FCSA) and the circularity shape factor ($SF=4\cdot\pi\cdot FCSA/perimeter^2$), which is an estimation of the circular morphology of the fiber (with a value of 1 for a perfect circle) were measured. The total number of fibers analyzed in each muscle ranged from 200 to 400. The index expressing the relationship between the number of capillaries per fiber and the fiber cross-sectional area ($CCA=number\ of\ capillaries\ per\ fiber\cdot 10^3/FCSA$) was also calculated. This index is considered a measure of the number of capillaries per 1,000 μm^2 of FCSA.

Histopathological evaluation

Transverse cross sections of soleus muscle were stained with H-E and used for histopathological examination. The area with more muscle damage features was microphotographed and the percentage of abnormal or damaged fibers (small rounded, angular, necrotic or undergoing myophagocytosis) was calculated. Additionally, two domains or features of muscle damage (mononuclear infiltrates and connective tissue enlargement) were semiquantitatively assessed according to a modified version of a previously published procedure by our laboratory (68). Briefly, each domain was subdivided in two items regarding their location (endomysial or perimysial) and to each item was given a score from 0 to 2, being 0 a score that would be expected in healthy samples and 2 in damaged ones. Thus, the maximum score for a domain was 4. Thresholds between scores were established using PNR rats as reference, as the scoring tool was intended to be applied only in a model of eccentric exercise in trained rats.

Skeletal muscle damage plasma markers quantification

For the quantitative detection of creatine kinase-MM (CK-MM) and myoglobin in plasma samples, a commercially available ELISA (Life Diagnostics Inc., PA, USA) was used according to the manufacturer instructions.

PGC-1α, GLUT1 and VEGF protein semiquantification

A subset of four animals from each group was used to measure the protein content of PGC-1α, Glucose transporter 1 (GLUT1) and VEGF. Thirty milligrams of soleus muscle were homogenized in ice-cold lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.5% DOC and 0.1% SDS) supplemented with protease and phosphate inhibitor cocktails (P8340 and P5726, Sigma) and centrifuged at 10,000 *g* for 10 min. Protein concentration of the collected supernatants was determined using the bicinchoninic acid assay (Thermo Scientific, IL, USA). Equivalents amounts of muscle

protein were separated by SDS/PAGE (10%) and transferred onto PVDF membranes (Millipore, MA, USA). Membranes were blocked with non-fatty dry milk and incubated with anti-PGC-1α (sc-5816) and anti-VEGF (sc-152), from Santa-Cruz (Santa Cruz, CA, USA), and with anti-GLUT1 (ab40084, Abcam, Cambridge, UK). After incubation with the corresponding secondary antibodies (Santa Cruz, CA, USA) membranes were photographed using a ChemiDoc XRS+ imaging system and analyzed with Image Lab software (both from Bio-Rad Laboratories). Ponceau-S staining was used as loading control. Results are expressed as percentage of the PNR group within each sampling point.

Citrate synthase enzymatic assay

Citrate synthase activity was measured as a marker of mitochondrial content and oxidative capacity. Approximately 10 mg of soleus muscle were homogenized in 100 mL of ice-cold medium containing 75 mM of Tris-HCl, 2 mM of $MgCl_2$ and 1 mM of EDTA (pH 7.6). Based on the method of Srere (74), the homogenate was centrifuged at 11,000 *g* and the supernatant was used to measure spectrophotometrically the enzymatic activity.

Statistics

Data are reported as means ± SE in tables and text. Most figures are represented as box-and-whisker plots. The box represents the interquartile range and shows the first and the third quartile separated by the median. The mean is represented by a black dot and whisker end points represent the minimum and maximum values. Parametrical data were analyzed using a one-way or two-way ANOVA tests followed by Holm-Sidak *post hoc* test or using a Student's *t*-test when appropriate. Non-parametrical data (namely the histopathological evaluation of the muscle damage) were analyzed using ANOVA on ranks followed by the Dunn's multiple comparison procedure. In all cases, a P-value < 0.05 was considered as statistically significant.

RESULTS

Animal characteristics

Table 1 shows the following animal characteristics: Body weight (BW), heart/BW and soleus/BW ratios, and Fulton index calculated as the weight ratio of right ventricle and left ventricle plus septum. The BW of all the animals gradually increased over time, although AHR animals did it more slowly. In fact, at day 14 the weight of AHR was significantly lower than PNR ($P < 0.05$). No statistical differences between treatments were found in the heart/BW ratio, although at day 14 a non-significant decrease in heart/BW ratio was observed in PNR and PHR, especially when compared to AHR. However, the Fulton index, a marker of right ventricular hypertrophy, was significantly increased at day 14 in PHR compared with both CTRL and AHR, suggesting pulmonary arterial hypertension. The soleus/BW ratio remained without statistical differences throughout time in all groups.

Muscle damage assessment

In order to confirm plasma signs of EEIMD, concentrations of CK-MM and myoglobin were measured. As shown in Fig. 2A and Fig. 2B, a significant increase in both CK-MM and myoglobin was found after 24 h in PNR ($P < 0.01$ and $P < 0.001$, respectively), PHR ($P < 0.05$) and AHR ($P < 0.05$ and $P < 0.01$, respectively) animals. The plasma concentration of CK-MM significantly decreased in the subsequent days in all experimental groups, with the exception of AHR t03 rats. Myoglobin concentration followed the same behavior in PNR and PHR groups, with significant lower concentrations in t03, t07 and t14 than in t01 ($P < 0.001$ in all cases). In AHR animals, however, myoglobin concentration was not found significantly decreased at any temporal point when compared to t01. Furthermore, AHR and PNR groups exhibited significantly higher myoglobin than PHR animals at t14 ($P < 0.001$). Moreover, Fig. 2C and Fig. 2D show histological features of EEIMD found in PNR t03 rats, as highlighted by arrows (mononuclear infiltrations) and asterisks (fibers undergoing myophagocytosis or with abnormal shape).

Figure 3 shows the histopathological evaluation of the right soleus muscle. With the exception of AHR t14, all other groups showed a significant increase in the percentage of abnormal fibers after the EEIMD protocol (regardless of treatment and time) (Fig. 3A). The AHR t14 animals did not have significantly more abnormal fibers than CTRL animals (6.0 ± 3.0 and 2.3 ± 1.8, respectively) but had fewer abnormal fibers than PHR animals (22.1 ± 10.5, $P < 0.01$) at day 14. No other significant differences were found between treatments, although a remarkably large number of abnormal fibers was observed in AHR animals at day 3 (24.3 ± 12.2). Nevertheless, this was drastically attenuated on subsequent days (8.5 ± 5.1, $P < 0.05$ at day 7, $P < 0.01$ at day 14).

A similar trend was observed when evaluating mononuclear infiltrates and connective tissue (Fig. 3B). All groups reached their peak score for both domains on day 3, although on day 7 PHR and AHR animals showed a significant decrease in the mononuclear infiltrates domain ($P < 0.01$). However, after day 7, each group showed divergent trends. PNR animals had similar scores for both domains at t14, while PHR animals showed a significant re-peak for mononuclear infiltrates ($P < 0.01$), reaching a total score close to that of day 3, and AHR animals presented significantly lower scores than at t03. Furthermore, at t14, AHR animals had a lower score for the connective tissue enlargement domain than any other group ($P < 0.01$) and a lower score in the mononuclear infiltrate domain than PHR animals ($P < 0.05$).

Skeletal muscle fiber type composition, cross-sectional area and shape

Myosin ATPase staining of soleus cross-sections showed that the fiber type composition remained unaltered under all conditions and at all time points (83% of SO fibers and 17% of FOG on average) (Fig. 4). The FCSA of both SO and FOG fibers was also measured. As shown in Fig. 5A, no significant differences were found in SO cross-sectional areas in the first 7 days for any group. However, 14 days after the EEIMD session, both PNR and PHR animals showed a significantly increased mean FCSA

when compared with AHR animals (4502 ± 483 and 4790 ± 365 vs. 3745 ± 360 μm^2, $P < 0.01$ and $P < 0.001$, respectively) or the CTRL group (3537 ± 394, $P < 0.01$ and $P < 0.001$, respectively). These differences were also statistically significant when compared with their own previous time points (days 1, 3 and 7). AHR was the only group with unchanged fiber size throughout the recovery protocol. Furthermore, a statistically significant interaction was found between treatment and time ($P < 0.05$). On the other hand, the mean FCSA of FOG fibers (Fig. 5B) did not differ significantly between the different treatments and time points. Regarding fiber shape, both types of fiber kept a constant circularity (0.80 ± 0.01 on average) in all groups (Fig. 5C and Fig. 5D).

Total muscle and individual fiber capillarization and angiogenesis

Endothelial ATPase staining of soleus transverse cross-sections revealed a significant decrease in the CD (Fig. 6A) 3 and 7 days after the EEIMD protocol in all groups when compared with CTRL animals (746 ± 71 capillaries per mm^2). In the case of PNR and PHR animals, this decrease was also significant after 14 days. Specifically, PNR animals showed a gradual decrease in CD over time (649 ± 38 at t03, 613 ± 67 at t07 and 575 ± 45 at t14). PHR animals kept a constant CD from t03 (641 ± 52), t07 (648 ± 81) to t14 (630 ± 65). In AHR animals there was a significant decrease in CD at t03 (640 ± 28) and t07 (625 ± 33), but this trend was significantly reversed by day 14 (789 ± 42). Moreover, at t14, this value was significantly higher than the value for the PNR and PHR groups.

In order to analyze the relationship between the number of capillaries and fiber size, the C/F ratio was calculated (Fig. 6B). PNR animals exhibited a steady significant decrease throughout the time course of the protocol (CTRL, 3.06 ± 0.10; t01: 2.66 ± 0.15, t03: 2.57 ± 0.16; t07: 2.52 ± 0.23; t014: 2.62 ± 0.18). In contrast, the C/F in PHR animals showed a completely different trend over time. After a significant decrease on the first day (2.57 ± 0.19), the C/F ratio gradually recovered from t03 (2.66 ± 0.20) to t07 (2.69 ± 0.29) and achieved a significantly higher value than t03 and t07 by t14 (2.92 ± 0.19). Moreover, at t14 it was also higher than in PNR animals. Finally, AHR showed a significant decrease in the C/F ratio during the first 7 days when compared to CTRL animals (t01: 2.64 ± 0.19; t03: 2.68 ± 0.20; t07: 2.63 ± 0.22), which was completely reversed after 14 days (3.30 ± 0.10).

In order to analyze the capillarization of individual fibers, the CCA of SO and FOG fibers was calculated (Fig. 6C and D). Regarding SO fibers, PNR animals showed a significant decrease in CCA at t07 and t14 (t07: 1.98 ± 0.12; t14: 1.69 ± 0.19) when compared with CTRL animals (2.26 ± 0.16). In clear contrast, PHR and AHR animals were already showing a significant decrease in CCA at t03 (1.83 ± 0.14 and 1.88 ± 0.14, respectively). After a non-significant increase by t07, at t14 PHR animals still showed a significantly reduced CCA (1.78 ± 0.09) compared with CTRL animals at t01 and t07, while AHR animals exhibited a significantly higher CCA (2.18 ± 0.14) than at t03. Furthermore, the CCA in AHR animals was also significantly higher than in PNR and

PHR animals at the same time point. The CCA of FOG fibers exhibited a similar trend throughout the protocol, although there were fewer statistical differences. The CCA in PNR animals showed a gradual decrease over time, with a significantly lower value at t14 (2.01 ± 0.15) than at t01 (2.45 ± 0.23) and t03 (2.37 ± 0.10). At t03, the CCA was significantly higher than in the other groups. The CCA of PHR animals was significantly lower at t03 (1.87 ± 0.13) than in CTRL animals (2.40 ± 0.30) and at t07 (2.32 ± 0.27). Likewise in SO fibers, ARH animals were able to recover from the initial decrease in CCA. In accordance with data from the PHR group, at t03 the CCA in ARH animals was significantly lower (2.06 ± 0.17) than in PNR animals, but had recovered by t14, reaching a value significantly higher than that in PNR animals (2.51 ± 0.30 vs. 2.01 ± 0.15).

In all the above mentioned parameters there was a statistically significant interaction between treatment and time ($P < 0.001$).

In addition to the histological characterization of the capillarization, the levels of VEGF were measured at the end of the protocol. As shown in Fig. 7A, both PHR and AHR animals showed significantly ($P < 0.01$) higher expression of VEGF (154 ± 18 and 143 ± 16, respectively) than PNR animals.

Skeletal muscle oxidative capacity

Citrate synthase activity and PGC-1α content were analyzed as markers of mitochondrial oxidative capacity. Although no changes were found at t03, a significant drop in citrate synthase activity was observed in all groups at t07 and t14 (Fig. 8A). Moreover, no significant differences between treatments were observed until t14, when AHR rats showed significantly higher citrate synthase activity than PNR and PHR animals (14.8 ± 0.86, 13.1 ± 0.44 and 12 ± 0.78, respectively).

Similarly, PGC-1α expression was higher in AHR animals at t07 and t14 when compared with PNR animals (Fig. 8B). Furthermore, a non-significant, but notable, increase was observed at t03. In PHR rats, PGC-1α expression was similar to PNR at all time points with the exception of t07, at which there was a large and significant increase.

Protein expression of GLUT1

GLUT1 protein expression was used as a hypoxic marker at the end of the protocol. AHR rats exhibited significantly higher levels of GLUT1 than PNR animals (127 ± 15.4 vs. 100 ± 11.5, $P < 0.01$), but not than PHR rats (113 ± 16.7) (Fig. 7B).

DISCUSSION

Intermittent hypoxia in combination with exercise is known to induce beneficial adaptations in skeletal muscle and has been increasingly used in athlete training (5). Furthermore, hypoxia seems to be closely involved in wound healing, as the hypoxic environment that results from the destruction of the blood vessels at the damaged site

406 activates the HIF signaling cascade (54). Accordingly, among the downstream targets of
407 the HIF signaling pathway, numerous genes are involved in tissue repair, angiogenesis
408 and the regulation of metabolism, such as VEGF and GLUT1 (79). However, whether
409 or not systemic IHH exposure can improve recovery from muscle damage remains
410 unknown. In fact, to the best of our knowledge, this is the first paper discussing the
411 effects of IHH exposure on rat skeletal muscle morphofunctional, capillarization and
412 metabolic parameters after a double session of eccentric exercise-induced muscle
413 damage.

414 ***Exercise-induced muscle damage protocol***

415 A downhill running-based eccentric exercise-induced muscle damage protocol applied
416 to trained rats was used in the present paper. This is a well-known and widely used
417 model for inducing muscle damage in rats (3). Nevertheless, in order to produce clear
418 muscle damage, the protocol was applied twice on the same day, as a single session was
419 not severe enough to significantly raise the plasma levels of myoglobin and creatine
420 kinase-MM (CK-MM) (data not shown). Although an attenuation of the muscle damage
421 induced by eccentric exercise has been associated with the repeated bout effect (16, 49),
422 some studies have revealed that endurance training also provides a certain degree of
423 protection (35, 40, 71). Nevertheless, two sessions of downhill running applied to
424 trained rats resulted in a significant increase in CK-MM and myoglobin in the plasma
425 (Fig. 2). Furthermore, microphotographs of soleus muscle transverse cross-sections
426 stained with H-E also exhibited features of muscle damage, such as necrotic and
427 atrophied fibers, mononuclear infiltrations and connective tissue enlargement (Fig. 2C
428 and D). Therefore, appreciable, although moderate, muscle damage was induced by the
429 eccentric exercise protocol. However, in this paper, the focus was on the effects of the
430 recovery treatments on muscle morphometry and metabolism rather than on the
431 regeneration process itself.

432 ***Animal characteristics***

433 One of the main concerns regarding the recovery protocols studied here is the potential
434 deleterious effects of hypoxia, namely loss of muscle mass and pulmonary
435 hypertension. To address these issues, excised soleus muscle and heart were weighed
436 and the Fulton index was calculated. However, as shown in Table 1, no significant
437 differences were found in the soleus weight/BW ratio between the different treatments
438 at t14, although a slight decrease was observed in PHR animals. On the other hand, the
439 Fulton's index, an expression of right ventricular hypertrophy and thus a marker of
440 pulmonary arterial hypertension, was significantly increased in this hypoxic group.
441 Although a well-known consequence of chronic hypoxia, this finding is quite surprising
442 in a model of intermittent and moderate hypoxia exposure. In fact, intermittent and
443 moderate hypoxia has been shown to reduce systemic arterial pressure in hypertensive
444 patients (72) and to induce cardioprotective effects (48, 82). Nevertheless, this potential
445 pathological effect was not found when IHH was combined with aerobic exercise
446 (AHR), although the hypoxic-induced right ventricular hypertrophy could be disguised

447 by a left ventricular hypertrophy, a typical adaptation to endurance training (65).
448 Furthermore, the AHR animals had a significantly lower BW and a clear, but not
449 significant, increase in the heart weight/BW ratio, signs of beneficial adaptations to
450 endurance exercise. Therefore, although some minor undesirable effects seem to be
451 induced by IHH, these were completely overcome by its combination with aerobic
452 exercise.

453 ***Histopathological evidence***

454 As previously mentioned, slight but appreciable damage was observed in soleus
455 samples after the EEIMD protocol. Moreover, as described in the literature (77), a peak
456 in the percentage of abnormal fibers, mononuclear infiltrates and connective tissue
457 enlargement was observed within the first 72 h (t03). Interestingly, at the end of the
458 protocol (t14) AHR animals showed overall faster recovery compared with the other
459 groups. In fact, at this time point these animals had a similar percentage of abnormal
460 fibers to that in CTRL animals and showed no evidence of enlarged connective tissue.
461 The skeletal muscle extracellular matrix is of great relevance in the force transmission
462 and repair processes (24). Moreover, it is disrupted after EEIMD, which contributes to
463 the inflammatory response (75). Thus, more rapid readjustment of the extracellular
464 matrix distension could improve overall recovery (34). However, despite the observed
465 positive impact of IHH in combination with aerobic exercise, IHH alone resulted in
466 different outcomes. In fact, despite being similar to CTRL animals, at t14 PHR animals
467 had a significantly higher percentage of abnormal fibers and a higher score for
468 mononuclear infiltrates when compared with AHR. This is consistent with data reported
469 by Chaillou et al. (10) in which an impairment in the regeneration of notexin-induced
470 muscle damage was evident in rats exposed to chronic hypobaric hypoxia.

471 ***Muscle fiber morphometry, capillarization and metabolism***

472 Interesting data were found regarding fiber morphometry, capillarization and
473 metabolism, particularly in the last phase of the recovery protocol (t14). This late
474 response is not surprising as the effects of hypoxia exposure are dose-dependent (55)
475 and myofiber phenotypic changes require complex time-consuming genetic, metabolic
476 and cellular adjustments. Interestingly, these late changes were only present in AHR,
477 but not in PHR animals.

478 At the end of the recovery protocol, PNR and PHR animals had slow oxidative fibers
479 with a significantly larger FCSA, while AHR maintained a constant mean fiber size
480 until t14 (Fig. 5). The increase in the FCSA of the passive (non-exercised) groups (PNR
481 and PHR) was probably a consequence of the normal growth of the animals, as FCSA
482 increases with age and BW (43). In addition to a lower BW, it is important to note that
483 AHR animals underwent daily aerobic exercise, an important modulator of the muscle
484 fiber phenotype. Specifically, endurance training prompts the soleus muscle to develop
485 a more oxidative metabolism (6) through several adaptations, such as an increase in the
486 activity of oxidative enzymes, mitochondrial density and fiber capillarization (21). The
487 latter can be achieved by an increase in the total number of capillaries and angiogenesis,

but also through fiber size modulation. In fact, for a given capillary density, smaller fibers have a better capillary supply than larger ones, which is reflected by a higher CCA index. In fact, it is widely known that oxidative fibers are smaller than anaerobic fibers (80), and thus have more capillaries per unit of transverse cross-sectional area. Moreover, a reduction in fiber size in some muscles of rats exposed to IHH has also been reported (60, 61). Therefore, it is not surprising that AHR animals had a smaller FCSA than those from the other two groups. Furthermore, analysis of the soleus muscle capillarization was consistent with this assumption. After the EEIMD protocol, all groups showed a decrease in capillarization parameters, such as the CD and C/F ratio. To the best of our knowledge, such a large and rapid decrease has not been reported by others, although previous studies have reported endothelial damage and alterations in muscle microcirculation and capillary structure after strenuous eccentric exercise (39). At least in part, this apparent reduction could be due to the nature of the staining technique, which is based on the endothelial ATPase activity (22). Damaged endothelial cells could exhibit impaired eATPase activity, which decreases staining and could lead to an undercount of the number of capillaries. In any case, these results suggest an impairment in the microvascular network that may have led to compromised oxygen delivery. At the end of the protocol, the capillarization in AHR animals was similar to that in CTRL animals and significantly different from all other groups (Fig. 6). This group not only had a high CD and CCA (fiber size-related parameters that could be explained by their reduced mean FCSA), but also an increased C/F ratio, suggesting an angiogenic phenomenon. To further elucidate this possibility, we analyzed the protein expression of VEGF at this time point. Accordingly, VEGF expression was significantly increased compared with PNR animals, which confirmed an angiogenic process. Interestingly, a similar response was also found in PHR animals. In fact, despite having a similar CD and CCA to PNR animals, the C/F ratio as well as VEGF expression were significantly higher. Although it is well known that chronic hypoxia, exercise or a combination of both are important stimuli for VEGF-mediated angiogenesis (7, 18, 67), whether or not IHH is able to induce angiogenesis has remained unclear (14). Data from the present study support the notion that intermittent hypobaric hypoxic treatment (IHH) *per se* was able to induce an angiogenic response, although it was not intense enough to maintain the pace of fiber growth.

Taken together, these data suggest that the soleus of rats exposed to IHH and aerobic exercise conserve the characteristic small, highly capillarized, fatigue-resistant muscle fibers typical of slow-twitch postural muscles. Accordingly, increased levels of PGC-1α in AHR animals after the first week of exposure to IHH were also found. This coactivator factor plays a major role as a regulator of energy metabolism, stimulating mitochondrial biogenesis and oxidative metabolism (44), and as a modulator of angiogenesis through VEGF HIF-independent regulation (2, 59). Our results are consistent with previous studies reporting PGC-1α modulation both by endurance training and hypoxia (2, 37, 76). Additionally, the increase in PGC-1α expression seems to have partially attenuated the decrease in oxidative capacity found at the end of the protocol in AHR animals, as the citrate synthase activity was significantly less

diminished than in the other groups (Fig. 7A). This enzyme is a well-known marker of mitochondrial density and oxidative capacity (31, 73), and thus the higher level of citrate synthase activity found in AHR animals reinforces the idea that soleus fibers kept a phenotype closer to that of CTRL animals. In contrast, animals from the other groups had larger, more poorly capillarized and fewer oxidative muscle fibers, which are suboptimal characteristics for a postural muscle. In fact, an undervascularized tissue would suffer from insufficient O_2 and energy substrates, potentially leading to mitochondrial and metabolic disturbances, and ultimately impairing the recovery from muscle damage.

Protein expression of HIF target genes

The importance of the physical activity as co-stimulus for IHH had been remarkable, as most of the positive results were exhibited by AHR animals. This differential response between PHR and AHR groups could be associated with the additional challenge imposed by aerobic exercise on the muscle oxygen homeostasis. Both VEGF and GLUT1 are highly upregulated by hypoxia through HIF (79). Although VEGF was increased in both groups exposed to IHH, GLUT1 was found upregulated only in AHR rats, indicating that IHH in combination with exercise triggered the HIF signaling cascade in a stronger way than the IHH alone. The light session of aerobic exercise increased the oxygen demand of the muscle, challenging the oxygen supply and thus activating the HIF pathway, as reflected by the increased levels of GLUT1. It is improbable, however, that the physical activity protocol alone would produce significant results. Before EEIMD, rats trained 35 min at 45 $cm \cdot s^{-1}$ twice a day, giving a daily distance ran of 1890 m. On the other hand, AHR rats only ran 360 m per day at reduced intensity (30 $cm \cdot s^{-1}$). Although several studies have demonstrated that reduced training volume is able to maintain the training-induced physiological adaptations in trained subjects (29, 46), it seems that is of fundamental importance to keep the training intensity (52). Thus, it is unlikely that the recovery running protocol of AHR rats, which had reduced volume, frequency and intensity, could maintain the physiological adaptations unless combined with an additional stimulus. In any case, although our results suggest that the observed results are at least partially HIF-dependent, the effects of aerobic exercise alone should be followed up in future studies in order to elucidate this point.

Fiber types

Despite the modulatory effects described above, no differences in fiber type distribution were found (Fig. 4). Not unexpectedly, the 2-week protocol of daily IHH exposure did not induce any kind of fiber type transition. This phenomenon requires longer exposure to chronic hypoxia and is more likely to occur in early stages of postnatal development, where a transition from SO towards FOG has been observed (36).

In conclusion, a decrease in capillarization and oxidative capacity was found 2 weeks after a double session of strenuous eccentric exercise that led to muscle damage.

However, a 2-week daily session of IHH combined with aerobic exercise reversed the signs of EEIMD, reinforcing and preserving the slow oxidative phenotype of the soleus fibers. Moreover, the percentage of abnormal fibers and the extent of connective tissue enlargement, correlating with a better capillary network and enhanced mitochondrial oxidative capacity, also decreased. These changes undoubtedly provide a better environment for muscle damage repair and reinforce the use of IHH combined with aerobic exercise as a useful therapeutic tool to counteract the deleterious effects perpetrated by EEIMD.

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FIGURE LEGENDS

Table 1. Relevant physiological parameters. Data are represented as mean ± standard deviation. Statistically significant differences are indicated according to the following codes: * vs. CTRL; † vs. t14 (for comparisons between different time points within the same treatment); *a* vs. PNR and *b* vs. PHR (for comparisons between different treatments at the same time point). One, two and three repeated symbols correspond to $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 1. Summary of the experimental design and preconditioning and training protocols. From the 4th day of the preconditioning protocol, as well as in the whole training protocol, there were two identical running sessions per day. *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 2. Validation of the EEIMD protocol in trained rats. *A-B*: Plasma concentration of the muscle damage biomarkers CK-MM and myoglobin. Statistically significant differences are indicated according to the following codes: * vs. CTRL; † vs. t14, ‡ vs. t07 and § vs. t03 (for comparisons between different time points within the same treatment); ▌for comparisons between different treatments at the same time point. One, two and three repeated symbols correspond to $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. *C-D*: Representative microphotographs of soleus muscle of trained rats after 3 days of the EEIMD protocol. Features of muscle damage are indicated by asterisks (*) (fibers undergoing myophagocytosis) and arrows (mononuclear infiltrates). Black scale bar represents 100 µm.

Figure 3. Effect of IHH and exercise after the EEIMD protocol on the histopathological evaluation of the soleus muscle. *A*: Percentage of abnormal fibers in selected damaged fields. *B*: Semiquantitative evaluation of two aspects of muscle damage: mononuclear infiltrates and connective tissue enlargement. A score according to its severity, ranging from 0–2, was given to its endomysial and perimysial components, giving a maximum score of 4 for each aspect. Statistically significant differences are indicated according to the following codes: * vs. CTRL; † vs. t14, ‡ vs. t07 and § vs. t03 (for comparisons between different time points within the same treatment); ▌for comparisons between different treatments at the same time point. One, two and three repeated symbols correspond to $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. *C-E*: representative microphotographs of soleus muscle at the end of the protocol (t14) of rats treated by PNR (*C*), PHR (*D*) and AHR (*E*). Black scale bar represents 100 µm. *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 4. Effect of IHH and exercise after the EEIMD protocol on fiber type distribution. *A*: Percentage of slow oxidative (SO) and fast oxidative glycolytic (FOG) fibers in the soleus muscle. *B*: Representative myosin ATPase staining with basic (pH 10.7) pre-incubation revealing slow (light-stained) and fast (dark-stained) fibers. Black scale bar represents 100 µm.

Figure 5. Effect of IHH and exercise after the EEIMD protocol on soleus muscle fiber size and shape. *A*: Mean FCSA of SO fibers. *B*: Mean FCSA of FOG fibers. C: Mean circularity of SO fibers. *D*: Mean circularity of FOG fibers. Statistically significant differences are indicated

according to the following codes: * vs. CTRL; † vs. t14 (for comparisons between different time points within the same treatment). ❚ for comparisons between different treatments at the same time point. One, two and three repeated symbols correspond to $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 6. Effect of IHH and exercise after the EEIMD protocol on soleus muscle capillarization. *A*: Capillary density. *B*: Capillary-to-fiber ratio. C: Mean CCA of SO fibers. *D*: Mean CCA of FOG fibers. Statistically significant differences are indicated according to the following codes: * vs. CTRL; † vs. t14, ‡ vs. t07, § vs. t03 (for comparisons between different time points within the same treatment). ❚ for comparisons between different treatments at the same time point. One, two and three repeated symbols correspond to $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 7. Effects of the recovery treatments on protein expression of HIF downstream targets VEGF (A) and GLUT1 (B) 14 days after the EEIMD protocol. All samples (n=4 per group) were run on the same gel. Bands were normalized to Ponceau-S staining (a corresponding representative band is shown). Normalized bands were expressed as percentage of the PNR group. The bands shown are not necessarily arranged in the same order as on the gel. ❚ $P < 0.05$, ❚❚ $P < 0.01$. *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 8. Effect of IHH and exercise after the EEIMD protocol on the oxidative capacity and mitochondrial content of soleus muscle. *A*: Citrate synthase activity normalized to the muscle protein content. *B*: Protein expression of the biogenesis marker PGC-1α. Each time point corresponds to one gel (n=4 per recovery treatment) and no comparisons were made between different days. Bands were normalized to Ponceau-S staining (a corresponding representative band is shown). Normalized bands were expressed as percentage of the PNR group. The bands shown are not necessarily arranged in the same order as in the gel. Statistically significant differences are indicated according to the following codes: * vs. CTRL; † vs. t14, ‡ vs. t07, § vs. t03 (for comparisons between different time points within the same treatment). ❚ for comparisons between different treatments at the same time point. One, two and three repeated symbols correspond to $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Table 1. Relevant physiological parameters.

		t01			*t03*			*t07*			*t14*		
	CTRL	**PNR**	**PHR**	**AHR**	**PNR**	**PHR**	**AHR**	**PNR**	**PHR**	**AHR**	**PNR**	**PHR**	**AHR**
Body weight (g)	374±21	377±25 †††	373±30 †††	364±21 †††	378±16 †††	393±10 †††	366±25 †††	408±12 **†††	406±24 *†††	395±19 †	475±34 ***	453±36 ***	430±31 **a
Heart/Body weight (mg/g)	2.68±0.14	2.79±0.16	2.83±0.10	2.78±0.17	2.86±0.22	2.78±0.16	2.81±0.19	2.86±0.15	2.77±0.10	2.81±0.18	2.55±0.27	2.51±0.09	2.80±0.20
Fulton index	0.32±0.04	0.32±0.04	0.34±0.03 ††	0.32±0.04	0.32±0.02	0.27±0.02 †††	0.31±0.05	0.32±0.03	0.33±0.07 ††	0.33±0.05	0.34±0.05	0.40±0.07 *	0.33±0.05 b
Soleus/Body weight (mg/g)	0.43±0.04	0.46±0.07	0.48±0.07	0.48±0.04	0.48±0.03	0.43±0.04	0.49±0.03	0.46±0.05	0.45±0.02	0.43±0.03	0.45±0.04	0.40±0.05	0.45±0.03

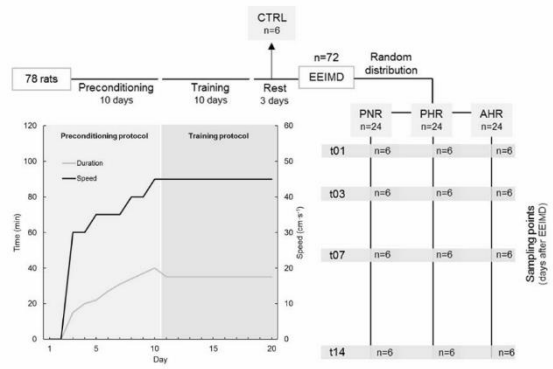
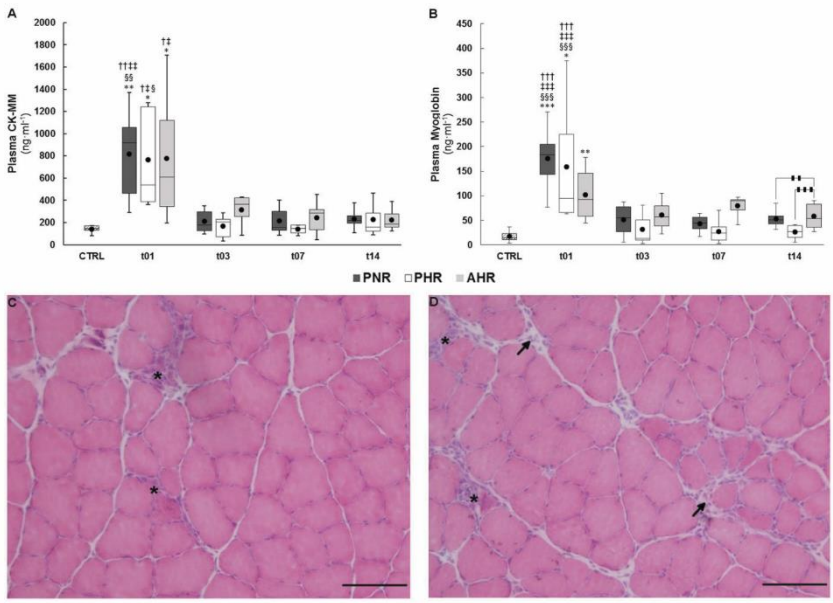


Figure 1.



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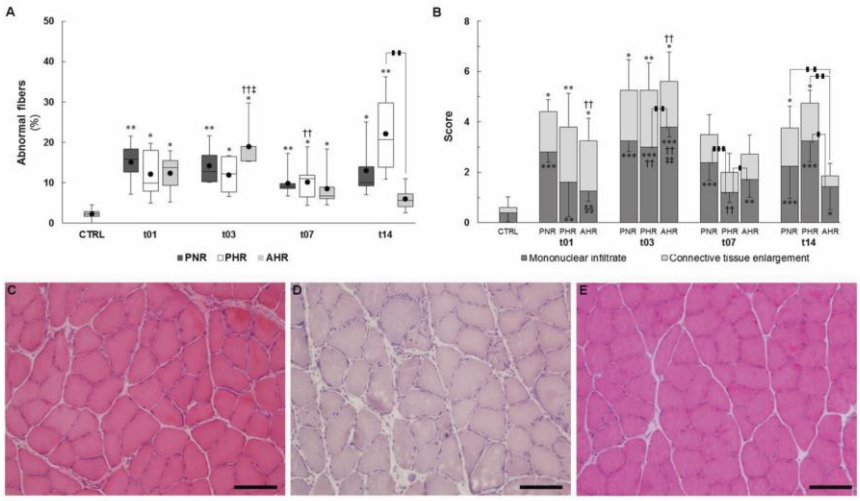

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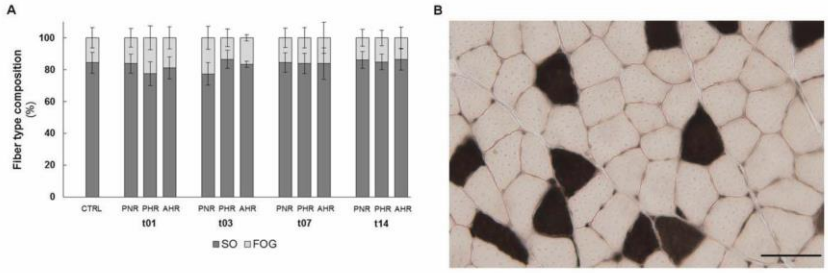

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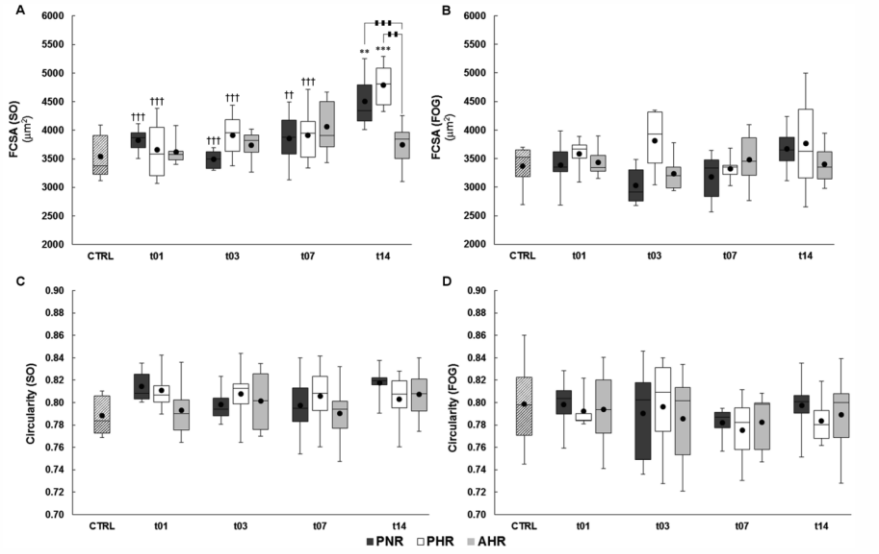

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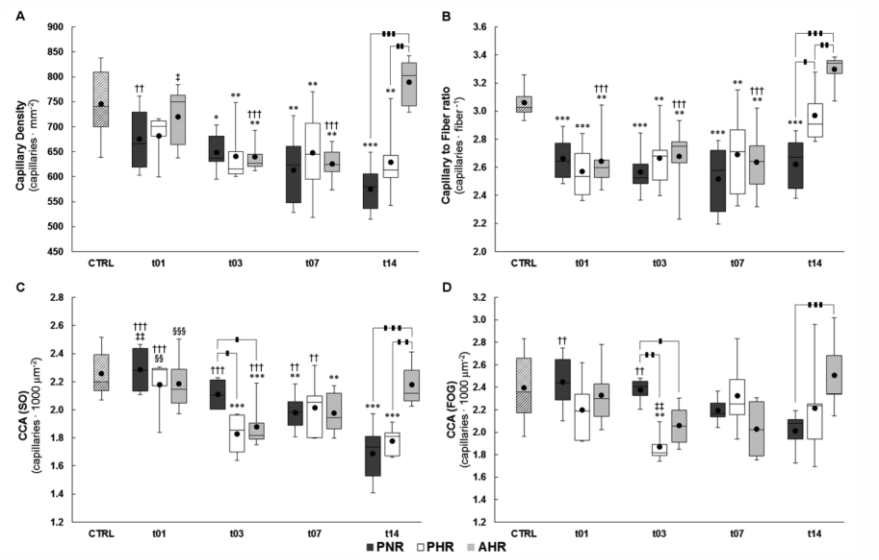

Figure 6.

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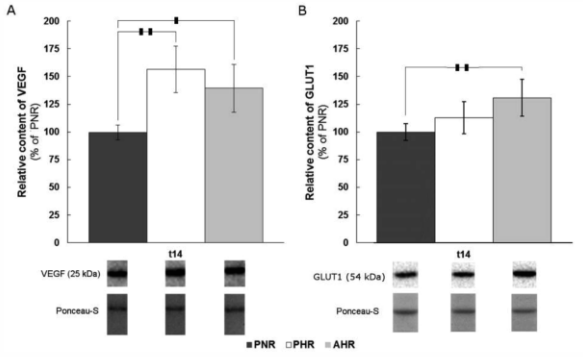

Figure 7.

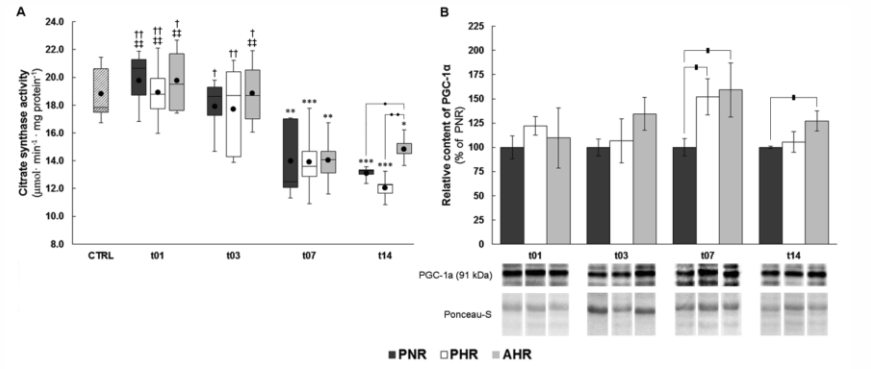

Figure 8.

Artículo III

Modulation of mitochondrial biomarkers by intermittent hypobaric hypoxia and aerobic exercise after strenuous eccentric exercise in trained rats. (En segunda revision*). Rizo-Roca D[1], Ríos-Kristjánsson JG[1], Núñez-Espinosa C[1,2], Santos-Alves E[3], Magalhães J[3], Ascensão AA[3], Pagès T[1], Viscor G[1], Torrella JR[1]. Applied Physiology Nutrition and Metabolism.

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* Las partes en rojo corresponden a los cambios introducidos tras la primera revisión.

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Modulation of mitochondrial biomarkers by intermittent hypobaric hypoxia and aerobic exercise after eccentric exercise in trained rats

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Keyword:	intermittent hypoxia, mitochondrial biomarkers, eccentric exercise, muscle damage

Modulation of mitochondrial biomarkers by intermittent hypobaric hypoxia and aerobic exercise after eccentric exercise in trained rats

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ABSTRACT

Unaccustomed eccentric contractions induce muscle damage, calcium homeostasis disruption and mitochondrial alterations. Since exercise and hypoxia are known to modulate mitochondrial function, we aimed to analyze the effects on eccentric exercise-induced muscle damage (EEIMD), in trained rats, of two recovery protocols based on: 1) intermittent hypobaric hypoxia (IHH) and 2) IHH followed by exercise. The expression of biomarkers related to mitochondrial biogenesis,

dynamics, oxidative stress and bioenergetics was evaluated. Soleus muscles were excised before (CTRL) and 1, 3, 7 and 14 days after an EEIMD protocol. The following treatments were applied one day after the EEIMD: passive normobaric recovery (PNR), four-hour daily exposure to passive IHH at 4000m (PHR) or IHH exposure followed by aerobic exercise (AHR). Citrate synthase activity had reduced 7 and 14 days after application of the EEIMD protocol. However, this reduction was attenuated in AHR rats at day 14. PGC-1α and Sirt3 and TOM20 levels had decreased after 1 and 3 days, but the AHR group exhibited increased expression of these proteins, as well as of Tfam, by the end of the protocol. Mfn2 greatly reduced during the first 72 h, but returned to basal levels passively. At day 14, AHR rats had higher levels of Mfn2, OPA1 and Drp1 than PNR animals. Both groups exposed to IHH showed a lower p66shc(ser^{36})/p66shc ratio than PNR animals, as well as higher complex IV subunit I and ANT levels. These results suggest that IHH positively modulates key mitochondrial aspects after EEIMD, especially when combined with aerobic exercise.

INTRODUCTION

Unaccustomed eccentric contractions induce muscle damage with subsequent swelling, delayed-onset muscle soreness, inflammation, leakage of intracellular proteins, increased production of reactive oxygen species (ROS), myophagocytosis of necrotic fibers and a decrease in maximal force generation (Schwane et al. 1983, Armstrong et al. 1983, Teague and Schwane 1995, MacIntyre et al. 1996, Close et al. 2004). Several mechanisms that explain the etiology of eccentric exercise-induced muscle damage (EEIMD) have been proposed. Although the precise order and sequence of the events are still a matter

of scientific debate, it is clear that the early events that lead to EEIMD include: the overstretching and disruption of sarcomeres, failure in the excitation-contraction coupling and disruption of the cytoskeletal elements involved in force transmission (Proske and Morgan 2001). After these initial events, inflammatory cells start accumulating in the damaged area, myofibrillar proteins degrade, ROS production increases and calcium homeostasis is disrupted (Clarkson and Sayers 1999, Sayers and Hubal 2007). Specifically, an increased concentration of intracellular calcium following eccentric contractions has been found in both human and rodent models (Duan et al. 1990, Fridén and Lieber 1996, Sonobe et al. 2008), probably as a consequence of the disruption of the muscle and sarcoplasmic reticulum membranes (Chen et al. 2007). This increase in intracellular calcium concentration activates calpain, a calcium-dependent non-lysosomal protease that cleaves cytoskeletal and mitochondrial proteins (Vissing et al. 2008, Kar et al. 2010). Despite some controversy (Molnar et al. 2006), eccentric exercise and calcium overload have been associated with impaired mitochondrial function, namely decreased respiratory control ratio (RCR), complex V activity, repolarization and maximal energization, mitochondrial swelling, and mitochondrial permeability transition pore (mPTP) opening (Rattray et al. 2011, Magalhães et al. 2013b), which is related to the activation of apoptosis cascade signaling. However, little is known about the effects of eccentric exercise on mitochondrial dynamics, such as fission and fusion, biogenesis and the expression of electron transporter chain complexes.

Because mitochondria are the main powerhouse of the cell and play an important role in oxidative stress, apoptosis signaling and calcium

homeostasis, they have emerged as an interesting target for the prevention and treatment of muscle pathologies, including EEIMD. It has been demonstrated that non-pharmacological tools such as endurance training and hypoxia exposure, which are well-known mitochondrial modulators, are effective at improving mitochondrial function. For instance, Rattray et al. (2013) found attenuated mitochondrial calcium overload and preserved RCR and mPTP Ca^{+2} sensitivity in the vastus lateralis of male rats after an acute bout of eccentric exercise using a downhill training protocol, while increased subsarcolemmal mitochondrial biogenesis has been found in the same muscle of humans submitted to hypoxic training (Desplanches et al. 2014). Furthermore, Magalhães et al. (2013a, 2014) reported the positive modulation of rat cardiac mitochondrial energetics and signaling by endurance training and intermittent hypobaric hypoxia (IHH). Similarly, exercise-induced mitochondrial cardioprotective effects have also been found in a rat model of doxorubicin-induced cardiotoxicity (Ascensão et al. 2012b, Marques-Aleixo et al. 2015). Specifically, these studies showed that adequate doses of intermittent hypoxia and exercise can induce beneficial mitochondrial adaptive responses, such as increased efficiency of ATP production, reduced susceptibility to mitochondrial permeability transition pore (mPTP) opening, decreased caspase-3 activity and Bcl-2/Bax ratio and higher resistance against anoxia/reoxygenation stress. This positive modulation, produced by both endurance exercise and intermittent hypoxia, could be, at least partially, due to an increase of mitochondrial biogenesis and dynamics, as well as reduced oxidative stress.

Thus, the main goal of the present study was therefore to analyze the effects of intermittent hypobaric hypoxia, alone or combined with aerobic exercise, in different markers of mitochondrial biogenesis, dynamics, oxidative stress and energetics after strenuous eccentric exercise. Because muscle damage is especially relevant in athletes, all the experiments were conducted in aerobic trained rats.

MATERIAL and METHODS

Animals

Fifty-two male Sprague-Dawley rats were used in this study. During the experimental protocol, animals were maintained at an average temperature of 23°C under a light-dark cycle of 12 h/12 h with food and water *ad libitum*. All animals underwent training on a treadmill for a month. Three days after the last training session, four rats were euthanized (CTRL group). The remaining rats were submitted to an EEIMD protocol and randomly divided into three experimental groups the next day: 1) PNR (passive and normoxic recovery, n=16), rats that passively recovered from EEIMD in normoxic conditions; 2) PHR (passive and hypoxic recovery, n=16), rats that passively recovered from EEIMD following a protocol of IHH exposure; and 3) AHR (active and hypoxic recovery, n=16), rats that performed a light running session on a treadmill immediately after IHH. Animals from these three groups were euthanized immediately after the last intervention at four different time points (n=4 each): 1, 3, 7 and 14 days after the EEIMD protocol (t01, t03, t07 and t14, respectively). Fig. 1 shows a schematization of the experimental design.

All procedures were performed in accordance with the internal protocols of our laboratory, which were authorized by the University of Barcelona's Ethical Committee for Animal Experimentation and ratified, in accordance with current Spanish legislation, by the Departament de Medi Ambient i Habitatge (Generalitat de Catalunya).

Aerobic training protocol

All rats underwent an aerobic training protocol. All training sessions were carried out at room temperature (21 ± 2°C) on a treadmill (LE 8710, Panlab, Barcelona, Spain). The aerobic training protocol consisted of an adaptation phase (5 days/week during two weeks), in which the duration and intensity of the running exercise were gradually increased in order to habituate the animals to the treadmill and the new activity, and then two weeks (5 days/week) in which the speed of the treadmill was set at 45 $cm \cdot s^{-1}$ and the duration of the exercise at 35 min. After the fourth day of the adaptation phase, and until the end of the training protocol, the animals trained twice daily (with a recovery interval of 6 h). This daily double session of running allowed rats to adapt physiologically to the endurance exercise and optimized the duration of the whole protocol.

Eccentric exercise-induced muscle damage protocol

Skeletal muscle damage was induced by eccentric exercise consisting of downhill running with a decline of 15° and at 50 $cm \cdot s^{-1}$ until exhaustion (Armstrong et al. 1983). This protocol began three days after the end of the training period and was applied twice on the same day: one session in the morning and one in the afternoon, with a resting period of 4 h

between the end of the first session and the beginning of the second session.

Intermittent hypobaric hypoxia exposure

IHH sessions started the day after the EEIMD protocol and were performed using a hypobaric chamber with a volume of about 450 L. A relative vacuum was created with a rotational vacuum pump (TRIVAC D5E, Leybold, Cologne, Germany) whose air-flow rate was regulated at the inlet by a micrometric valve. Inner pressure was controlled by two differential sensors (ID 2000, Leybold, Cologne, Germany), which drove a diaphragm pressure regulator (MR16, Leybold, Cologne, Germany). The target pressure of 462 torr (equivalent to 4,000 m of altitude) was achieved steadily over a period of about 15 min. Once this pressure had been reached, the chamber pressure was maintained and regulated for 4 h. At the end of the session, normal barometric pressure was gradually restored over a period of 15 min. The total number of days of hypobaric hypoxia exposure varied according to the sampling schedule. Animals assigned to t01 were in the hypobaric chamber for a single session, whereas t14 animals were subjected to two weeks of daily exposure. Animals had *ad libitum* access to food and water kept in open-air reservoirs inside the hypobaric chamber during the hypoxia sessions.

Running exercise recovery protocol

AHR rats were enrolled in a recovery exercise program consisting of a daily session of aerobic exercise immediately after a hypobaric hypoxia session according to the sampling schedule. These rats were placed on a treadmill to run in accordance with a low-impact exercise program. The exercise session lasted 20 min with a gradual increase in speed until

they reached 30 $cm \cdot s^{-1}$ and a gradual increase in inclination from 0° to 5°.

Plasma and muscle sampling

After the last intervention, rats were euthanized by heart excision under urethane anesthesia (5 $ml \cdot kg^{-1}$ from a 30 $g \cdot dL^{-1}$ solution). Soleus muscles, which are widely used in EEIMD studies due to its susceptibility to eccentric contractions and are recruited during aerobic exercise, were excised, rinsed in saline solution, immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Blood samples were obtained from the vena cava, placed in EDTA tubes and centrifuged at 1,300 *g* for 10 minutes. Plasma was collected and stored at -80°C until assayed.

Citrate synthase activity assay

Approximately 10 mg of soleus muscle were homogenized in 100 mL of ice-cold medium containing 75 mM of Tris-HCl, 2 mM of $MgCl_2$ and 1 mM of EDTA (pH 7.6). In accordance with Srere's method (1969), the homogenate was centrifuged at 11,000 *g* and the supernatant used to measure enzymatic activity spectrophotometrically.

Creatine kinase and myoglobin biomarkers

A commercially available ELISA (Life Diagnostics Inc., PA, USA) was used for the quantitative detection of creatine kinase isoenzyme MM (CK-MM) and myoglobin in plasma samples, in accordance with the manufacturer's instructions.

Western blotting

Thirty milligrams of soleus muscle were homogenized in ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% DOC and 0.1% SDS) supplemented with protease and phosphate inhibitor cocktails (P8340 and P5726, Sigma) and centrifuged at 10,000 *g* for 10 min. The protein concentration of the collected supernatants was determined using the bicinchoninic acid assay (Thermo Scientific, IL, USA). Equivalent amounts of muscle protein were separated by SDS/PAGE (10%) and transferred onto PVDF membranes (Millipore, MA, USA). Membranes were blocked with nonfat dry milk and incubated with anti-peroxisome proliferator-activated receptor gamma co-activator protein (PGC-1α) (sc-5816), anti-mitochondrial transcription factor A (Tfam) (sc-23588), anti-translocase of the outer membrane 20 (TOM20) (sc-11415), anti-Mitofusin 2 (Mfn2) (sc-50331) and anti-adenine nucleotide translocator (ANT) (sc-9299) from Santa Cruz (CA, USA); with anti-Optic atrophy 1 (OPA-1) (ab119685) and anti-OXPHOS Western blotting cocktail (ab110413) (containing antibodies against CI, CII, CIII, CIV and CV subunits: anti-NDUFB8, anti-CII-30 kDa, anti-CIII-Core protein 2, anti-CIV subunit I and anti-CV alpha subunit, respectively) from Abcam (Cambridge, UK); with anti-dynamin-related protein 1 (Drp-1) (#8570), anti-Bax (#2772), anti-Bcl-2 (#2870), anti-sirtuin 3 (SIRT3) (#2627) and anti-p66sch (#2432) from Cell Signaling (Danvers, MA, USA); and with anti-p66shc(pSer36) (6E10) from Calbiochem Merck Millipore (Darmstadt, Germany). After incubation with the corresponding secondary antibodies (Santa Cruz, CA, USA), membranes were photographed using a ChemiDoc XRS+ imaging system and analyzed with Image Lab software (both from Bio-

Rad Laboratories). Ponceau-S staining was used to normalize differences in protein loading or transfer, and the final data were expressed as the percentage variation of the control values (% CTRL) or PNR values (% PNR).

Statistics

The results were reported as mean ± SE for each experimental group. Data were analyzed using a one-way ANOVA test followed by the Holm-Sidak *post hoc* test or Student's *t*-test when appropriate. A p-value < 0.05 was considered statistically significant. Regarding the Western blot results, statistical comparisons were only tested when samples were within the same membrane. Samples belonging to each temporal point (t01, t03, t07 or t14) were always loaded into the same gel, thereby allowing statistical comparisons. Additional gels containing CTRL and PNR t01-t14 samples were run in order to statistically evaluate the time course of the analyzed proteins. All statistical analyzes were carried out with the statistical package SigmaPlot (Systat Software Inc.).

RESULTS

Biomarkers of muscle damage

Plasma levels of CK-MM and myoglobin were assessed in order to confirm the validity of the EEIMD protocol. As shown in Fig. 2, both CK-MM and myoglobin significantly increased in plasma after a double session of strenuous eccentric exercise ($p < 0.001$).

Citrate synthase activity

As shown in Fig. 3, no significant differences were observed during the first 72 h either among treatments or *versus* the CTRL group. At t07 and

t14, however, all groups showed a significant decrease in CS activity when compared with their counterparts at t01 and t03 and when compared to CTRL animals. Furthermore, AHR rats exhibited higher CS activity than PNR and PHR animals at t14.

Protein expression of mitochondrial biogenesis markers

The content of two key promoters of mitochondrial biogenesis was assessed. As seen in Fig. 4A, PGC-1α content significantly decreased one day after the EEIMD protocol (CTRL vs. PNR t01). AHR animals showed increased levels of PGC-1α compared to PNR rats at all time points, and these were statistically significant at t07 and t14. PHR rats showed a similar trend to AHR animals, with a significant increase at t07. A non-significant decrease in Tfam content (Fig. 4B) was found at t03 (CTRL vs. PNR, $p = 0.06$). AHR animals showed a significant 1.6-fold increase over PNR rats at both t07 and t14, while PHR rats showed a significant decrease at t07 and an increase at t14. Since mitochondrial biogenesis is accompanied by an increase in the protein import machinery, we also analyzed the content of TOM20 (Fig. 4C). The protein content was significantly reduced at t03 and t14 (CTRL vs. PNR). Both AHR and PHR groups exhibited higher levels of TOM20 than PNR at t14.

Protein expression of mitochondrial dynamics markers

Since mitochondrial dynamics play a key role in mitochondrial function, we semi-quantified the content of fusion (Mfn2, OPA-1) and fission (Drp-1) proteins. Strenuous eccentric exercise produced a significant 2-fold and 3-fold reduction in Mfn2 expression at t01 and t03 (CTRL vs. PNR), which partially reversed on the following days, t07 and t14 (Fig.

283 5A). Although the PNR group recovered its Mfn2 levels faster than the
284 AHR group at t07, at t14 AHR rats showed significantly higher levels
285 of Mfn2 than PNR animals. As shown in Fig. 5B, OPA-1 did not show
286 significant alterations over time, although a decreasing trend was
287 observed ($p = 0.08$ at t14, CTRL vs. PNR). Both PHR and AHR groups
288 had significantly higher levels of OPA-1 than PNR at t07 and t14. The
289 EEIMD protocol significantly increased fission protein Drp-1 levels, by
290 up to 100% of the control at all temporal points (CTRL vs. PNR) (Fig.
291 5C). At t14, moreover, AHR rats showed expression levels 1.6-fold
292 higher than PNR and PHR groups.

293 **Apoptotic signaling**

294 As observed in Fig. 6A, the content of the pro-apoptotic protein Bax
295 significantly increased seven days after the EEIMD protocol (CTRL vs.
296 PNR). The same trend was observed before (t03) and after (t14),
297 although without statistical significance ($p = 0.09$ and $p = 0.06$,
298 respectively). The combination of exercise and hypoxia showed a non-
299 significant trend towards attenuation of this increase at t07.
300 Furthermore, a significant reduction in the levels of anti-apoptotic
301 protein Bcl-2 was observed at t03 (CTRL vs. PNR). AHR animals
302 showed higher levels of Bcl-2 than PNR ($p < 0.05$) and PHR ($p < 0.01$)
303 groups at t14 (Fig. 6B). The Bax/Bcl-2 ratio significantly increased in
304 PNR t03 rats when compared to CTRL and PNR t01 ($p < 0.01$) (Fig.
305 6C). AHR animals showed a non-significant reduction at t07 when
306 compared to PNR ($p = 0.06$).

Protein expression of mitochondrial oxidative stress markers

Protein levels of Sirt3, a pivotal regulator of oxidative stress, were assessed (Fig. 7A). A significant reduction one and three days after the EEIMD protocol ($p < 0.01$) was observed (CTRL vs. PNR). Sirt3 returned to its basal levels in PNR rats at t07, while AHR animals showed significantly higher levels than the other groups at t07 ($p < 0.01$) and t14 ($p < 0.05$).

The protein content of p66shc, in addition to its phosphorylated form (p66shc-pSer36) and their ratio, was used as the protocol endpoint measurement of mitochondrial oxidative stress (Fig. 7B-D). Both groups exposed to IHH exhibited a significant lower p66shc(pSer^{36})/p66shc ratio than PNR rats, due to either a reduction in its phosphorylated form, as in the case of PHR rats ($p < 0.05$ vs. PNR), or an increase in its total form (AHR, $p < 0.05$ vs. PNR).

Protocol endpoint measurement of electron transport chain proteins and ANT

The content of electron transport chain (ETC) complexes I-IV and ATP synthase subunits and ANT was semi-quantified (Fig. 8) two weeks after the application of the EEIMD protocol. IHH induced a significant increase in the levels of complex I and IV synthase subunits, as well as those of ANT (PHR vs. PNR, $p < 0.05$). Despite the fact that AHR animals showed lower levels of complex I subunit than PHR rats, the former exhibited higher levels of complex IV subunit and ANT than PNR animals ($p < 0.05$).

DISCUSSION

Previous studies demonstrated that unaccustomed eccentric exercise induces an immediate and transient impairment of the mitochondrial function in skeletal muscle, such as decreased RCR, impaired calcium handling and mPTP opening (Rattray et al. 2011, Magalhães et al. 2013b). However, the effects of EEIMD on the protein expression of markers of mitochondrial biogenesis, dynamics and bioenergetics, or of apoptosis and oxidative stress, have not been addressed before. Here we present the changes that EEIMD induced in the expression of different related proteins and how IHH, alone or combined with aerobic exercise, modulated these alterations.

Muscle damage markers and citrate synthase activity

As expected, increased plasma levels of CK-MM and myoglobin were found after a double session of exhaustive downhill running, thus confirming the presence of muscle damage. Soleus muscle CS activity was found to be unaltered during the first 72 h. Immediate alterations in mitochondrial function after eccentric exercise have been reported in rats (Rattray et al. 2011, Magalhães et al. 2013b); as a consequence, a decrease in CS would be expected. In fact, CS activity has been found decreased, unaltered or even increased in both human and rats after acute exercise (Ji et al. 1988, Leek et al. 2001, Molnar et al. 2006). Furthermore, the application of a training protocol before the EEIMD could have conferred a protective phenotype, delaying the expected CS activity decrease. Indeed, CS activity decreased abruptly between days 3 and 7, thus suggesting that EEIMD could trigger a delayed response in the activity of certain enzymes, in addition to the immediate impairment found by other authors. At the end of the protocol, AHR rats showed

a partial reversal of this reduction. Other studies had already demonstrated that IHH, alone and combined with aerobic exercise, can upregulate CS activity in both humans (Melissa et al. 1997) and rats (Esteva et al. 2009). As detailed below, this upregulation could be explained by the findings obtained for mitochondrial biomarkers of biogenesis, dynamics and oxidative stress.

Mitochondrial biogenesis markers

Our results showed that acute eccentric exercise induced an immediate decrease in PGC-1α protein expression. This reduction in PGC-1α could lead to impaired mitochondrial biogenesis, which would explain the decrease observed in the mitochondrial content marker CS over the subsequent days. Interestingly, the groups exposed to IHH (PHR and AHR) showed increased levels of PGC-1α and its downstream transcription factor Tfam between seven and/or fourteen days after EEIMD, which would suggest mitochondrial biogenesis. The higher levels of TOM20 found at t14 in these groups reinforce this hypothesis, as this protein mediates in the mitochondrial protein import destined for the matrix, which is essential during mitochondrial biogenesis (Grey et al. 2000). Although exposure to chronic hypobaric hypoxia has usually been associated with decreased mitochondrial content and biogenesis (Horscroft and Murray 2014), IHH has already been demonstrated to induce the opposite response, at least in rat lung, although the mechanism remains unknown (Chitra and Boopathy 2014). This apparent discordance is not surprising, as it is becoming increasingly evident that the effects of hypobaric hypoxia are strongly dependent on the dose, intensity and frequency of the exposure (Navarrete-Opazo and Mitchell 2014).

385 **Mitochondrial dynamics markers**

386 An imbalance between fusion and fission proteins was induced by
387 eccentric exercise. During the first 72 h, the fission protein marker Drp-
388 1 drastically increased, while the expression of Mfn2 halved. This
389 imbalance towards mitochondrial fission could be due to the presence
390 of damaged mitochondria (Youle and van der Bliek 2012) and, together
391 with the decrease in mitochondrial biogenesis markers, suggests that
392 mitochondrial turnover was impaired by EEIMD. Two weeks
393 afterwards, the Drp-1 levels were still significantly higher than before
394 EEIMD. AHR rats exhibited even higher levels of Drp-1, but also of
395 both fusion proteins Mfn2 and OPA-1, which would suggest that the
396 higher rates of mitochondrial fission in this group were also
397 compensated by higher fusion frequency. These high rates of both
398 fusion and fission could indicate a more dynamic, adaptable
399 mitochondrial network. Furthermore, the maintenance of an
400 interconnected mitochondrial network is crucially important for
401 keeping mitochondria energetically active (Tondera et al. 2009).

402 **Apoptotic signaling**

403 In line with the literature (Sudo and Kano 2009), increased apoptotic
404 signaling was found three days after EEIMD, as reflected by the
405 Bax/Bcl-2 ratio. This increase in apoptotic signaling at t03 coincided
406 with the peak expression of Drp-1, which is involved in cytochrome *c*
407 release (Suen et al. 2008) and, therefore, in apoptotic signaling. IHH
408 with aerobic exercise seemed to confer a protective phenotype against
409 apoptotic signaling, as AHR rats showed a lower Bax/Bcl-2 ratio at t07
410 and t14 than PNR animals. Similar results were found in cardiac
411 mitochondria in a study carried out by Magalhães et al. (2014), in which

412 rats exposed to IHH and aerobic exercise showed decreased apoptotic
413 signaling.

414 **Mitochondrial oxidative stress markers**

415 Sirtuins are deacetylases that play an important role in aging and life
416 span regulation. Specifically, Sirt3 is mainly located in mitochondria, and
417 its function is to activate oxidative and antioxidant enzymes (Wu et al.
418 2014a). At the same time, downregulation of Sirt3 can be induced by
419 oxidative stress (Wu et al. 2014a). Thus, it is not surprising that after
420 strenuous eccentric exercise, which is known to induce oxidative stress
421 (Stagos et al. 2015), Sirt3 expression was found to be downregulated,
422 although it returned to CTRL levels after seven days.

423 As reported by other studies (Ascensão et al. 2012a), rats exposed to
424 IHH and aerobic exercise (AHR) showed higher levels of Sirt3, whose
425 upregulation has been associated with the activation of antioxidant
426 defenses (Kincaid and Bossy-Wetzel 2013). Furthermore, Sirt3 is a
427 target gene of PGC-1α, which was also found to be upregulated, and
428 contributes to the mitochondrial biogenesis mediated by PGC-1α
429 (Kong et al. 2010). In addition to its role in oxidative metabolism,
430 oxidative stress and mitochondrial biogenesis, it is also responsible for
431 the deacetylation and activation of OPA-1 (Samant et al. 2014), which
432 suggests that IHH with aerobic exercise not only upregulated the
433 content of this protein, but also increased its activity.

434 In addition to the increased levels of Sirt3 in AHR animals, rats exposed
435 to IHH, alone or with aerobic exercise, exhibited a reduction in the
436 p66shc(pSer^{36})/p66shc ratio. Since p66shc plays an important role in
437 oxidative response and is serine-phosphorylated by oxidative damage

(Migliaccio et al. 1999), the fact that AHR rats showed increased levels of total p66shc, but the same amount of serine^{36}-phosphorylated p66shc, could indicate that these animals triggered an adaptive response to oxidative stress, thereby showing good correlation with the increase in Sirt3. On the other hand, PHR rats showed lower levels of p66shc(pSer^{36}), which would suggest that their protective phenotype against oxidative stress was mediated by another mechanism.

Electron transport chain proteins and ANT

The content of complex I-V subunits and ANT were measured at the end of the protocol in order to assess whether the changes observed in mitochondrial markers of biogenesis, dynamics and oxidative stress affected ECT-related proteins. Surprisingly, the increase found in PGC-1α, Tfam and TOM20 did not align well with the results obtained from the analysis of the ETC complexes content. However, this apparent inconsistence could be a consequence of the sequence of the events involved in mitochondrial biogenesis, as there is a temporal delay between the gene and protein expression and the assembly of multi-subunit respiratory complexes (Hood 2001). Nevertheless, although no changes were observed in the protein content of the ATP synthase, IHH induced an increase in ANT, which is responsible for the exchange of ADP/ATP across the inner mitochondrial membrane (Fiore et al. 1998). Thus, even with similar levels of ATP synthase, rats exposed to IHH could benefit from a higher flux of substrate. Moreover, ANT is a component of the MPTP, which mediates in apoptosis signaling (Kinnally et al. 2011). Decreased ANT content has been linked to increased susceptibility to Ca^{2+}-induced MPTP opening (Oliveira and

Wallace 2006). Consequently, an increase in ANT levels could be interpreted as a more protective phenotype against apoptosis.

Interestingly, IHH also increased the content of complex IV, which may have an indirect antioxidant effect by reducing ROS generation in complexes I and III and reducing electron leakage (Chen et al. 2003a, Parise et al. 2005). This explains, at least partially, the reduced p66shc(pSer^{36})/p66shc ratio in PHR and AHR rats, as p66shc is mainly phosphorylated at serine-36 by H_2O_2 (Migliaccio et al. 1999), whose production is derived from the activity of complexes I and III.

It is important to remember, however, that mitochondrial protein content alone is not enough to determine mitochondrial health, does not provide information about energy production efficiency and does not relate to the activity of the complexes.

Aerobic exercise relevance and dose-dependent effect of IHH

Overall, most of these alterations were found to be attenuated or reversed in rats exposed to IHH (PHR and AHR). This counteraction of the deleterious effects induced by EEIMD was substantially more robust in AHR rats, which would suggest that aerobic exercise enhances the IHH response. In fact, the rationale behind the use of a light session of aerobic exercise was to further challenge the oxygen homeostasis in order to effectively trigger a hypoxic response in the soleus muscle, as in a resting state the oxygen demand could be easily handled even in a hypoxic environment. Thus, the recovery exercise protocol was more a *hypoxic enhancer* than an independent treatment itself, especially when applied to trained subjects. It is important to note that all rats were trained with two daily sessions of 35 min at 45 cm·s^{-1} (1.89 Km·day^{-1})

before the EEIMD protocol, while the exercise applied during the recovery consisted of a single daily session of 20 min at 30 $cm \cdot s^{-1}$ (0.36 $Km \cdot day^{-1}$). That was a reduction of a fifth of the training volume, while the intensity was decreased by 66%. Taking into account the importance of the training intensity in the maintaining or development of exercise-induced physiological adaptations (Mujika and Padilla 2000), it seems unlikely that the solely application of this exercise protocol could have induced the observed alterations in trained subjects.

Moreover, significant differences between rats exposed to IHH and normobaric rats were only found after seven and fourteen days, in agreement with the notion that the response to IHH is dose-dependent (Navarrete-Opazo and Mitchell 2014).

Conclusion

Our data showed that EEIMD downregulated the expression of mitochondrial biogenesis and fusion-related proteins (PGC-1α, Mfn2), upregulated mitochondrial fission markers (Drp-1) and induced an apoptotic response after three days of application. Although CS activity was not altered during the first three days, a significant decrease was found after the first and second weeks. IHH followed by aerobic exercise upregulated the protein content of mitochondrial biogenesis and dynamics markers and decreased the p66shc($pSer^{36}$)/p66shc ratio, thus suggesting a reduction in oxidative stress. Concordantly, Sirt3, which modulates all these processes directly or indirectly, was found to be upregulated. All these findings seemed to eventually result in increased CS activity, which would suggest that the changes found at protein expression level effectively lead to functional adaptation.

These findings open the door for further studies in athletes in the field of the treatment of the muscle damage. Thus, the application of IHH combined with exercise emerges as a new potential therapeutic tool. In fact, altitude training (being the hypoxia natural or simulated in hypobaric chambers) is already used in humans in order to induce cardioprotective adaptations, muscular adaptations and improve exercise performance, so its application in muscle damage seems like a logical further step in the field of the hypoxic training.

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752

FIGURE CAPTIONS

Figure 1. Schematic representation of the experimental design. *EEIMD*, eccentric exercise-induced muscle damage; *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 2. Validation of the EEIMD protocol in trained rats. A-B: Plasma concentration of the muscle damage biomarkers CK-MM and myoglobin. Asterisks (***) indicate $p < 0.001$ vs. CTRL. *PNR*, passive normoxic recovery.

Figure 3. Effect of IHH and exercise after the EEIMD protocol on the citrate synthase activity normalized to muscle protein content. Statistically significant differences are indicated according to the following codes: * vs. CTRL; *a* vs. t01, *b* vs. t03 (for comparisons between different time points within the same treatment); ▮ for comparisons between different treatments at the same time point. One, two and three repeated symbols correspond to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 4. Effect of EEIMD and the recovery protocols on the protein expression of different markers of mitochondrial biogenesis in the soleus muscle. *A*: relative content of PGC-1α. *B*: relative content of Tfam. *C*: relative content of TOM20. Bands were normalized to Ponceau-S staining (the corresponding well is shown). Normalized bands were expressed as a percentage of the CTRL group. The bands shown are not necessarily arranged in the same order as in the gel (in that case, a white space separates the bands and wells). Statistically

779 significant differences are indicated according to the following codes: *
780 vs. CTRL; ❙ for comparisons between different treatments at the same
781 time point. One, two and three repeated symbols correspond to $p <$
782 0.05, $p < 0.01$ and $p < 0.001$, respectively. *PNR*, passive normoxic
783 recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic
784 recovery.

785 **Figure** 5**.** Effect of EEIMD and the recovery protocols on the protein
786 expression of different markers of mitochondrial dynamics in the soleus
787 muscle. *A*: relative content of Mfn2. *B*: relative content of OPA-1. *C*:
788 relative content of Drp-1. Bands were normalized to Ponceau-S staining
789 (the corresponding well is shown). Normalized bands were expressed
790 as a percentage of the CTRL group. The bands shown are not
791 necessarily arranged in the same order as in the gel (in that case, a white
792 space separates the bands and wells). Statistically significant differences
793 are indicated according to the following codes: * vs. CTRL; *a* vs. t01, *b*
794 vs. t03 (for comparisons between different time points within the same
795 treatment); ❙ for comparisons between different treatments at the same
796 time point. One, two and three repeated symbols correspond to $p <$
797 0.05, $p < 0.01$ and $p < 0.001$, respectively. *PNR*, passive normoxic
798 recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic
799 recovery.

800 **Figure 6.** Effect of EEIMD and the recovery protocols on the protein
801 expression of different markers of apoptosis signaling in the soleus
802 muscle. *A*: relative content of Bax. *B*: relative content of Bcl-2. *C*:
803 Bax/Bcl-2 ratio. Bands were normalized to Ponceau-S staining (the
804 corresponding well is shown). Normalized bands were expressed as a
805 percentage of the CTRL group. The bands shown are not necessarily

arranged in the same order as in the gel (in that case, a white space separates the bands and wells). Statistically significant differences are indicated according to the following codes: * vs. CTRL; *a* vs. t01 (for comparisons between different time points within the same treatment); ❚ for comparisons between different treatments at the same time point. One and two repeated symbols correspond to $p < 0.05$ and $p < 0.01$, respectively. *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

Figure 7. Effect of EEIMD and the recovery protocols on the protein expression of different markers of mitochondrial oxidative stress in the soleus muscle. *A*: relative content of Sirt3. *B*: relative content of p66shc(pser^{36}) at the end of the recovery protocol. *C*: relative content of p66shc at the end of the recovery protocol. *D*: p66shc(pser^{36})/p66shc ratio at the end of the recovery protocol. Bands were normalized to Ponceau-S staining (the corresponding well is shown). Normalized bands were expressed as a percentage of the CTRL group (in *A*) or as a percentage of the PNR group (in *B-D*). The bands shown are not necessarily arranged in the same order as in the gel (in that case, a white space separates the bands and wells). Statistically significant differences are indicated according to the following codes: * vs. CTRL; *a* vs. t01 (for comparisons between different time points within the same treatment); ❚ for comparisons between different treatments at the same time point. One, two and three repeated symbols correspond to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. *PNR*, passive normoxic recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic recovery.

832 **Figure 8.** Effect of the recovery protocols on the expression of electron
833 transport chain complex subunits and ANT in the soleus muscle at the
834 end of the recovery protocol. *A*: relative content complex I NDUFB8
835 subunit. *B*: relative content of complex II 30 kDa subunit. *C*: relative
836 content of complex III core 2 subunit. *D*: relative content of complex
837 IV subunit I. *E*: relative content of ATP synthase subunit alpha. *F*:
838 relative content of ANT. Bands were normalized to Ponceau-S staining
839 (the corresponding well is shown). Normalized bands were expressed
840 as a percentage of the PNR. The bands shown are not necessarily
841 arranged in the same order as in the gel (in that case, a white space
842 separates the bands and wells). Symbol ▮ indicates statistical differences
843 between different treatments ($p < 0.05$). *PNR*, passive normoxic
844 recovery; *PHR*, passive hypoxic recovery; *AHR*, active hypoxic
845 recovery.

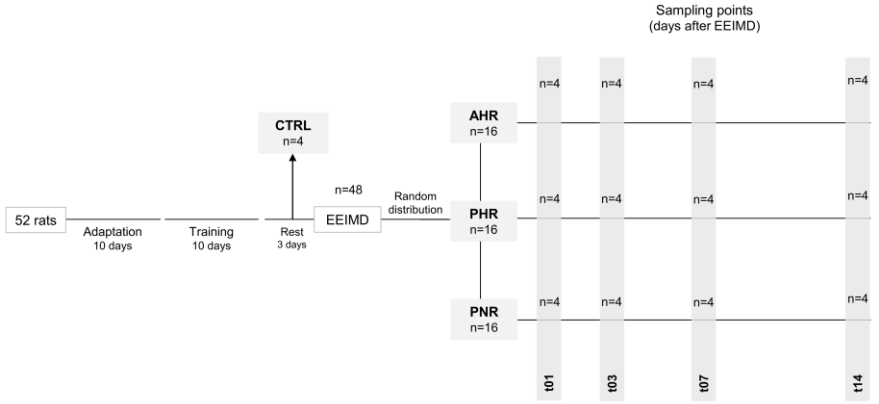


Figure 1.

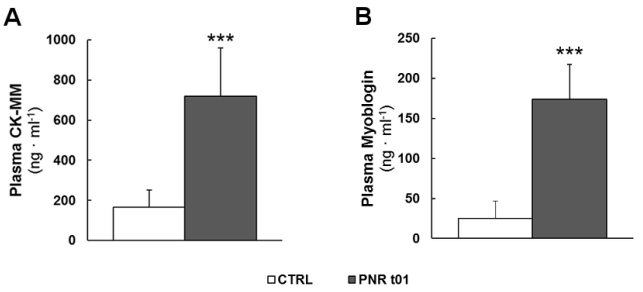


Figure 2.

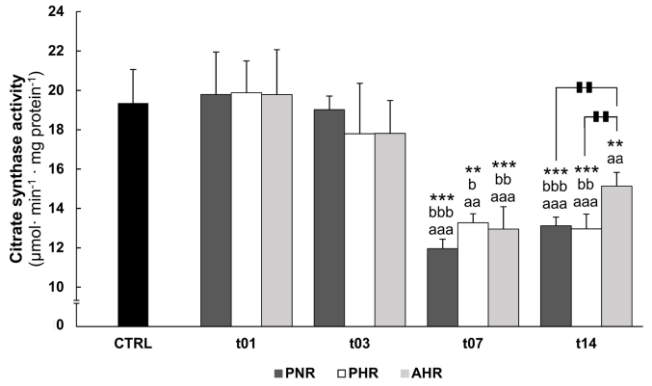
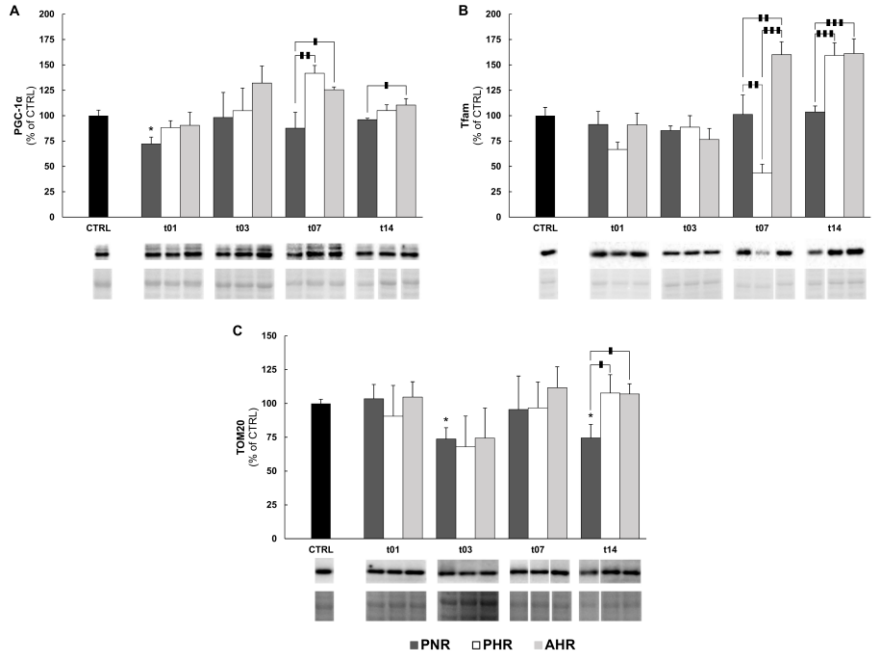


Figure 3.



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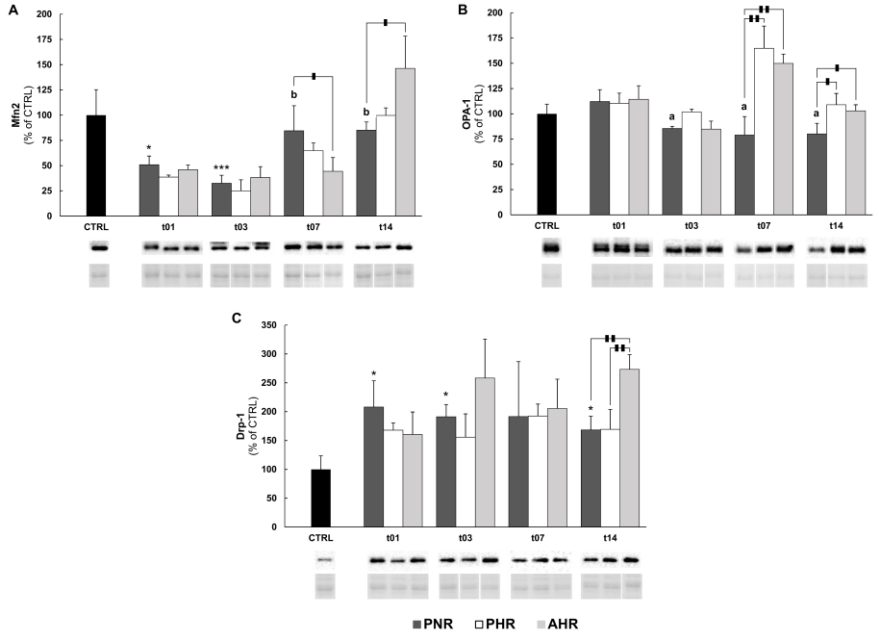

Figure 5.

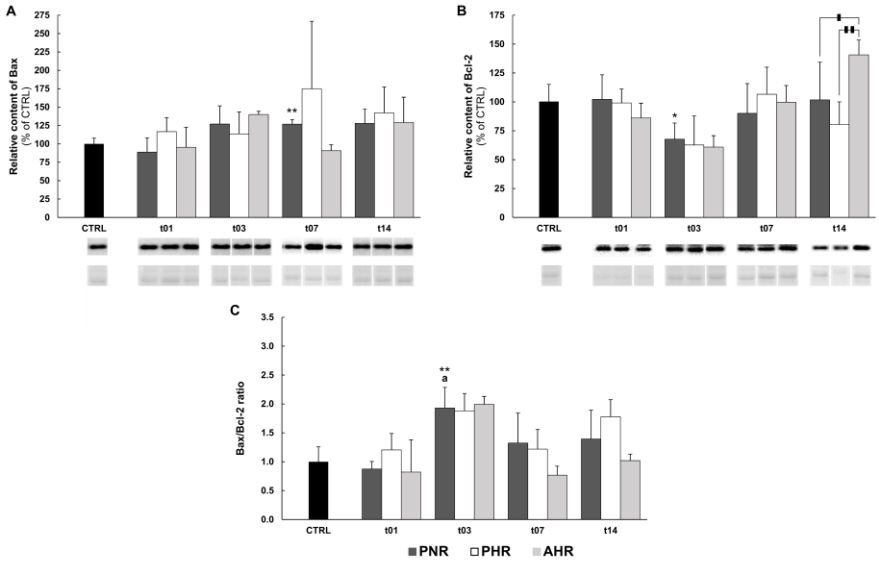

Figure 6.

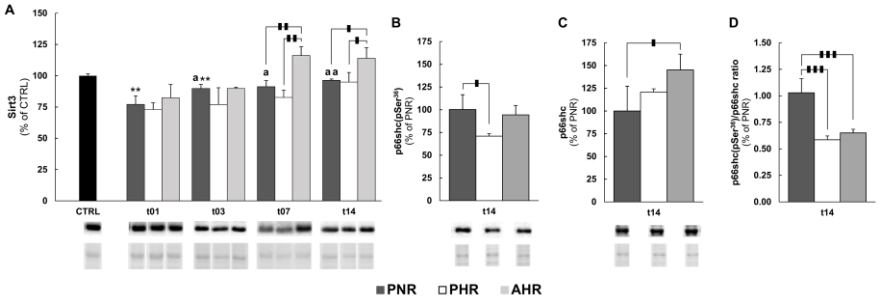

Figure 7.

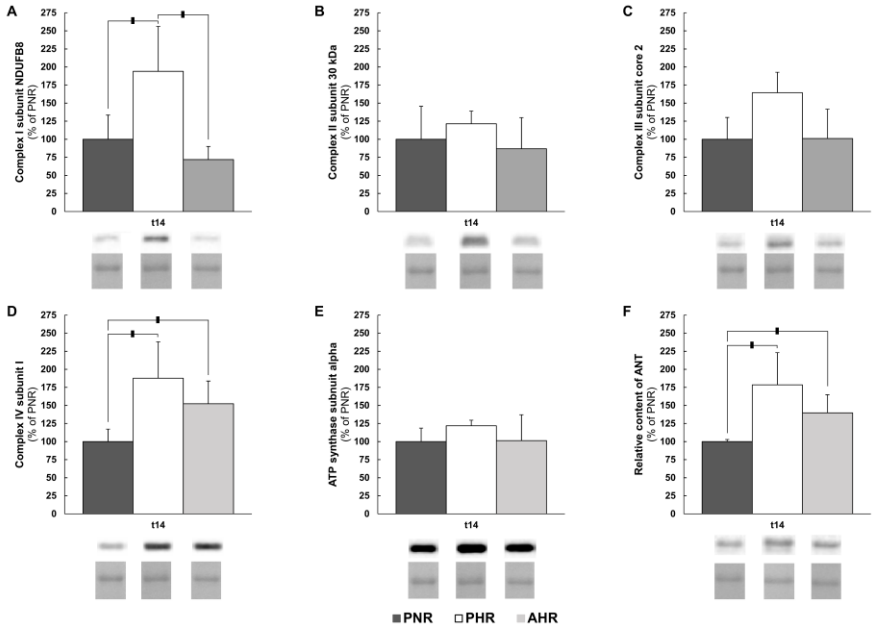
A
Complex I subunit NDUFB8
(% of PNR)
B
Complex II subunit 30 kDa
(% of PNR)
C
Complex III subunit core 2
(% of PNR)
D
Complex IV subunit I
(% of PNR)
E
ATP synthase subunit alpha
(% of PNR)
F
Relative content of ANT
(% of PNR)
275
250
225
200
175
150
125
100
75
50
25
0
t14
PNR
PHR
AHR

Figure 8.

5.

Discusión de los resultados

5.1 Validación del EEIMD

El primer paso en el desarrollo de este proyecto de investigación fue validar la presencia de EEIMD mediante un protocolo de carrera continua y extenuante en pendiente negativa (*downhill running*) en ratas entrenadas. Si bien este método ha sido ampliamente validado y aplicado repetidamente desde que Armstrong y colaboradores publicaran su ya clásico artículo a principios de los 80 (Armstrong et al. 1983), nuestro grupo se encontró con dificultades para aplicarlo en nuestro modelo animal, consistente en ratas Sprague-Dawley jóvenes-adultas entrenadas durante un mes[2]. De hecho, tal y como puede observarse en la Figura A1 del Anexo, los animales que realizaron una única sesión de carrera continua en pendiente negativa exhibieron concentraciones en plasma de mioglobina prácticamente iguales a las ratas control. Tal y como se argumenta en la *Introducción* del artículo **I**, aunque los episodios previos de ejercicio excéntrico son el principal método para prevenir o atenuar el EEIMD (Howatson y van Someren 2008), el entrenamiento aeróbico (sin pendiente) también puede inducir protección frente al EEIMD en el músculo sóleo (Schwane y Armstrong 1983, Isanejad et al. 2015). Es probable que el componente excéntrico presente en el ciclo de la marcha (*gait cycle*) durante la carrera en plano (sin desnivel), y adaptaciones propias del ejercicio aeróbico, tales como una mejor regulación de la homeostasis del Ca^{2+} y de la

[2] El protocolo de entrenamiento consistía en una fase de dos semanas de *adaptación*, en la que las ratas se habituaron al tipo de ejercicio requerido (carrera continua) y en la que la velocidad y duración aumentaban gradualmente, y otra fase de dos semanas en la que tanto la velocidad y duración se mantuvieron constantes (45 $cm \cdot s^{-1}$ durante 35 min, dos veces por día). A pesar de que el término *adaptación* podría sugerir una baja intensidad, si se observa la Tabla A1 del Anexo podrá comprobarse que durante la primera semana ya se alcanzan una velocidad y duración cercanas a la definitiva.

respuesta inflamatoria (Koh 2002; Inashima et al. 2003) sean responsables de este aparente fenotipo protector. Además, la contracción con alargamiento (característica del ejercicio excéntrico) no es un elemento indispensable para inducir protección frente al daño inducido por ejercicio (Koh y Brooks 2001).

En cualquier caso, una doble sesión del mismo protocolo (con un intervalo de 4 horas) sí resultó suficiente para inducir una elevación significativa en los niveles de mioglobina (Figura A1 del Anexo). La Figura 2A y 2B (**II**) muestran elevaciones significativas en plasma de creatina quinasa MM (CK-MM) y mioglobina (proteínas intramusculares liberadas al torrente sanguíneo debido al daño en las membranas celulares) 24 horas después de la aplicación del protocolo. Además, en los cortes histológicos que aparecen en la misma Figura 2C y 2D, correspondientes a ratas sacrificadas 3 días después del protocolo de carrera continua en pendiente negativa, se observa la presencia de infiltraciones por mononucleares y de fibras en proceso de miofagocitosis. Todos estos hallazgos confirman que el protocolo aplicado indujo daño muscular.

5.2 Semicuantificación del EEIMD en ratas entrenadas

A diferencia de los modelos de inducción de la lesión muscular mediante traumatismos, el daño inducido por ejercicio excéntrico afecta a una fracción relativamente pequeña de las fibras musculares (Armstrong et al. 1983). Esta fracción podría incluso ser menor en el caso de sujetos previamente entrenados, a pesar de ser sometidos a una doble sesión de ejercicio excéntrico, debido al ya comentado efecto

protector concedido por las adaptaciones fisiológicas al ejercicio aeróbico. Por todo ello, se decidió crear una herramienta que permitiera evaluar, de manera rápida y sencilla, la magnitud y componentes del daño muscular en cortes transversales del músculo sóleo teñidos mediante hematoxilina-eosina (HE).

5.2.1 Elementos evaluados

Para la construcción de la herramienta, se evaluaron 6 parámetros o ítems relevantes en la fisiopatología del EEIMD: 1) Fibras con morfología anormal, 2) Fibras necróticas o en degeneración, 3) Infiltraciones mononucleares en espacios endomisiales, 4) Infiltraciones mononucleares en espacios perimisiales, 5) Distensión del endomisio y 6) Distensión del perimisio. Estos ítems se agruparon en 3 dominios: de la fibra muscular (1 y 2); del estado inflamatorio extracelular (3 y 4); y del compartimento intersticial (5 y 6). Tal y como resume la Tabla 1 (**I**), cada elemento puede recibir una puntuación que oscila entre 0 y 2, en función de la magnitud de la alteración.

5.2.2 Objetividad y aplicación de la herramienta

Tras una breve explicación del funcionamiento de la herramienta, se facilitó la Tabla 1 (**I**) a 4 investigadores familiarizados con la histología del músculo esquelético y a 4 estudiantes de grado para que analizaran una serie de cortes histológicos de ratas entrenadas sin (CTRL) y con EEIMD (PNR). Las puntuaciones obtenidas se analizaron utilizando el coeficiente de correlación intraclase (ICC, *Intraclass Correlation Coefficient*). Este test estadístico permite evaluar el nivel de acuerdo o consenso entre diferentes observaciones, reportando valores comprendidos entre 0 (consenso inexistente) y 1 (máximo consenso), considerándose

valores superiores a 0,60 generalmente satisfactorios (Shrout y Fleiss 1979, Cicchetti 1994). Tal y como se muestra en la Tabla 2 (**I**), cuando la herramienta fue utilizada por investigadores con experiencia en el área, se generó un buen consenso en todos los ítems (>0,60), sugiriendo que las puntuaciones resultantes son independientes del observador y por lo tanto objetivas y replicables. Por el contrario, y a modo de *control negativo*, cuando la misma herramienta fue utilizada por estudiantes o investigadores no familiarizados con la histología se obtuvieron valores de ICC menores a 0,60.

Además, la aplicación del método permitió discernir claramente entre muestras CTRL y muestras PNR (**II**: Fig. 2).

5.2.3 Uso en investigación

Lejos de querer proporcionar unos criterios universales, el objetivo de esta publicación fue proporcionar una base adaptable a diferentes modelos de daño muscular, con diferentes grados de daño. Así, los *thresholds* para cada ítem son totalmente ajustables en función de la severidad de la lesión, y los propios elementos que componen cada dominio son susceptibles de ser ampliados o reducidos. Tal y como se muestra en el artículo **II**, en el que la herramienta fue utilizada para evaluar muestras de diferentes condiciones experimentales, la Tabla 1 (**I**) fue adaptada para obtener un mejor resultado (por ejemplo, evaluando los elementos de los dominios del estado inflamatorio y del compartimento intersticial independientemente de su localización endo o perimisial).

5.3 Efectos del EEIMD y HHI en el músculo sóleo

5.3.1 Evaluación histopatológica

Como era de esperar, tras aplicar el protocolo de EEIMD se produjo un aumento en el porcentaje de fibras anormales, en la cantidad de infiltraciones por mononucleares y un aumento del espacio extracelular (**II**: Fig. 3). A diferencia de los niveles de CK-MM y mioglobina en plasma, los parámetros histológicos se mantuvieron alterados incluso 14 días después de la inducción del daño. Esta discordancia ha sido repetidamente descrita en la bibliografía y es bien sabido, que los marcadores plasmáticos no correlacionan temporalmente con la evolución histológica ni funcional (Manfredi et al. 1991; Komulainen et al. 1994). La presencia de alteraciones histológicas hasta dos semanas después de la inducción del daño responde al curso natural de la reparación de cualquier daño tisular, en el que las fases iniciales de degeneración e inflamación van seguidas de fases de proliferación, remodelación y fibrosis (Prisk y Huard 2003).

En cuanto a los tratamientos aplicados, los hallazgos más interesantes se encontraron en el grupo AHR (HHI + ejercicio aeróbico ligero) en t14 (véase **II:** Fig. 1 para un esquema del diseño experimental utilizado en las publicaciones **II** y **III**). Este grupo exhibió un porcentaje de fibras anormales y un espacio intersticial parecidos al de las ratas CTRL, a diferencia de los otros grupos. Esto podría ser debido a que el ejercicio ejerce una regulación positiva sobre las metaloproteasas de la matriz extracelular (Rullman et al. 2009), enzimas encargadas de la degradación de diferentes tipos de colágeno, lo que evitaría su deposición excesiva (Riso et al. 2016).

5.3.2 Alteraciones en la histomorfología y red capilar

Ni el protocolo de EEIMD ni la exposición a HHI afectó la distribución del tipo de fibras (**II**: Fig. 4) ni a su forma o circularidad (**II**: Fig. 5C-D). Aunque el ejercicio excéntrico extenuante sí puede alterar la morfología de las fibras musculares (desde una forma redondeada a una altamente angular) (Sayers y Hubal 2007), el hecho de que solo un pequeño porcentaje de ellas estén afectadas impide que tales alteraciones se vean reflejadas a nivel estadístico. En el caso de la distribución de los tipos de fibras, se ha descrito que protocolos de larga duración de exposición crónica a hipoxia hipobárica pueden inducir una transición de fibras lentas a rápidas (Itoh et al. 1990). Sin embargo, tal y como habíamos demostrado en nuestro grupo de investigación, la exposición intermitente no induce esta transición (Panisello et al. 2008). Para la discusión de los resultados, la mayoría de estos hacen referencia a las fibras SO, por ser, con diferencia, las más abundantes. En cualquier caso, los resultados observados siguieron una tendencia similar en las fibras FOG.

Sí que se observaron cambios en la capilarización del músculo. La realización de una doble sesión de ejercicio excéntrico extenuante indujo una rápida disminución del número de capilares por mm^2 (CD) y del cociente entre el número de capilares y el número de fibras (C/F) (**II**: Fig. 6A-B). Esta rápida y evidente disminución fue relativamente sorprendente: aunque ya se habían descrito con anterioridad alteraciones en la microvasculatura de músculos sometidos a ejercicio excéntrico agudo (Kano et al. 2004, 2005), es difícil creer que estas alteraciones pudiesen haber causado una disminución tan brusca en la CD y C/F. Una explicación razonable a este fenómeno podría hallarse

en la naturaleza metodológica de la técnica de tinción de capilares utilizada. Esta técnica aprovecha la actividad de la ATPasa endotelial (eATPasa) presente en los endotelios capilares para revelar su presencia (Fouces et al. 1993). Así, podría ser que el daño endotelial inducido por el ejercicio excéntrico, ya descrito por Kano et al. (2004), redujera la actividad enzimática de la eATPasa e impidiera la tinción de todos los capilares. En cualquier caso, parece claro que la microvasculatura del músculo quedó alterada de un modo u otro, e incluso dos semanas después del EEIMD no exhibió signos de recuperación. Es más, tal y como se muestra en la Figura 6C (**II**), el número de capilares por fibra, relativizado a su área (CCA), disminuyó significativamente el día 14, a pesar de mantener CD y C/F en valores similares a los días previos. Esta reducción de la capilarización por unidad de área de la fibra fue debida, al menos en parte, por el incremento de la FCSA media (**II**: Fig. 5A). El incremento en la sección transversal media de las fibras puede responder a diferentes procesos fisiológicos: 1) el crecimiento normal del animal, todavía relevante en ratas jóvenes-adultas, que conlleva un aumento de la masa muscular (Layman et al. 1980) y, en consecuencia, de la FCSA; 2) la existencia de un fenómeno de hinchazón debido a la posible presencia de edema intramuscular (Yu et al. 2013); y 3) un aumento de la síntesis proteica estimulada por el EEE, como describieron Moore et al. (2005). Aunque no es posible delimitar el alcance de cada uno de estos factores con los experimentos realizados, es probable que todos ellos contribuyan en menor o mayor medida en el aumento observado en la FCSA media. En cualquier caso, estas alteraciones en la capilarización y tamaño de las fibras musculares no fueron encontradas en el grupo AHR, expuesto a HHI y ejercicio aeróbico ligero. Aunque mostraron la misma tendencia en la CD y la

C/F que el grupo PNR durante la primera semana de recuperación (t07), a día 14 se revertió completamente la situación alcanzando o manteniendo valores similares a los del grupo CTRL. Si bien la menor FCSA exhibida por los animales AHR podría explicar los valores superiores en CD y CCA, el incremento en el índice C/F sugiere la formación de nuevos capilares.

Para confirmar la angiogénesis, se analizaron los niveles de VEGF al final del protocolo. Efectivamente, la expresión de VEGF fue significativamente superior en los grupos expuestos a HHI (**II**: Fig. 7), corroborando los hallazgos histológicos. Adicionalmente, en una publicación fruto del mismo proyecto en el que se enmarca esta tesis (Núñez-espinosa et al. 2015), se analizaron mediante citometría de flujo las poblaciones circulantes de células $CD34^+$ (células madre hematopoyéticas) y células $CD34^+/CD45^+$ (células progenitoras endoteliales), las cuales están involucradas en la formación de nuevos vasos sanguíneos y en la reparación del daño muscular (Abedi et al. 2007, Ribeiro et al. 2013). En consonancia con lo expuesto hasta ahora, las ratas expuestas a HHI y ejercicio aeróbico ligero mostraron los niveles más altos de estas poblaciones celulares, sugiriendo una respuesta a nivel sistémico además de a nivel local.

Todos estos resultados indican que la combinación de HHI y ejercicio indujeron una potente respuesta angiogénica, observada a nivel histológico y apoyada por los hallazgos de expresión proteica y de poblaciones celulares en sangre.

5.3.3 Alteraciones mitocondriales

Las alteraciones descritas a nivel capilar y morfométrico en el apartado anterior indican una disfunción de la red vascular tras la realización del protocolo de EEIMD, así como un aumento de la capilarización del músculo sóleo de las ratas sometidas a dos semanas de HHI y ejercicio ligero (AHR). Es de suponer, por lo tanto, una mayor disponibilidad de oxígeno y nutrientes en este grupo. En consecuencia, se decidió analizar la respuesta mitocondrial a estas alteraciones mediante la expresión de diversas proteínas marcadoras de la homeostasis mitocondrial: biogénesis, bioenergética y dinámica mitocondrial, señalización apoptótica y estrés oxidativo.

5.3.3.1 Actividad de la CS

La CS, al ser la primera enzima del Ciclo de Krebs (Essén-Gustavsson y Henriksson 1984), se ha utilizado tradicionalmente como un indicador del contenido mitocondrial (Larsen et al. 2012) y como un marcador clave del metabolismo oxidativo, por lo que su análisis resulta útil como primera aproximación al estado general de las mitocondrias. Sorprendentemente, la actividad de la CS no fue encontrada alterada tras la doble sesión de EEE (**II:** Fig. 8A). Otros autores habían descrito una disfunción inmediata y transitoria en la respiración mitocondrial tras la realización de EEE (Magalhães et al. 2013; Rattray et al. 2013). Esta disparidad podría deberse a que, mientras la CS se encuentra en la matriz mitocondrial, los complejos de la cadena transportadora de electrones, responsables de la respiración mitocondrial, se ubican en la membrana interna, siendo más sensibles a cambios en la permeabilidad de membrana inducida por la liberación excesiva de Ca^{2+}. Leek et al. (2001) reportaron un problema parecido en humanos: a pesar de que el

análisis ultraestructural de biopsias de sujetos sometidos a ejercicio agudo mostraba un gran número de mitocondrias hinchadas y morfológicamente anormales, la actividad de la CS se encontraba aumentada. Ante esta aparente contradicción, Leek y colaboradores. hipotetizaron que, en las mitocondrias hinchadas, la enzima quedaba más expuesta al sustrato durante el ensayo enzimático, dando valores artificialmente elevados, aunque no aportaron pruebas empíricas que justificaran esta explicación. Por último, pero no por ello menos importante, el protocolo de entrenamiento al que fueron sometidos los animales antes del EEIMD pudo conferir un fenotipo protector, retrasando la caída de la actividad de la CS.

De hecho, se observó un descenso de la actividad de la CS en los días 7 y 14. Esta disminución, además de ser un efecto *tardío* del EEIMD, también podría responder a una caída en el aporte de oxígeno y nutrientes, consecuencia de la ya descrita disfunción de la microvasculatura. Similarmente, la actividad de la CS mostró un repunte significativo en el grupo AHR t14, sugiriendo una mejoría en las mitocondrias de este grupo. Para confirmar este punto, se discuten en los siguientes apartados los resultados en la expresión de diferentes proteínas relacionadas con el funcionamiento mitocondrial.

5.3.3.2 Proteínas marcadoras de biogénesis mitocondrial

El análisis de la expresión de PGC-1α (*Peroxisome proliferator-activated receptor Gamma Coactivator 1-a*), TFAM (*Mitochondrial Transcription Factor A*) y TOM20 (*Translocase of the Outer Membrane 20*) sugirió una leve inhibición de la biogénesis mitocondrial (**III:** Fig. 4). PGC-1α, un coactivador transcripcional considerado el principal regulador de la biogénesis mitocondrial (Wu et al. 1999), mostró una disminución en su

expresión 24 horas después del protocolo de EEIMD. Aunque TFAM, factor de transcripción responsable de la replicación del genoma mitocondrial, no fue encontrado alterado, el contenido de TOM20 disminuyó en los días 3 y 14. Dado que TOM20 es necesario para la importación de material y proteínas necesarias para la biosíntesis mitocondrial (Grey et al. 2000), su disminución podría sugerir, efectivamente, una reducción de la biogénesis mitocondrial. Aunque estos resultados van en consonancia con los resultados referentes a la CS (el grupo con mayor expresión de marcadores de biogénesis mitocondrial presentó mayor actividad de CS), sería necesario un análisis ultraestructural por microscopía electrónica de la densidad del volumen mitocondrial para corroborar este resultado.

En cualquier caso, la exposición a HHI, especialmente cuando se combinó con ejercicio aeróbico, indujo un aumento de todos estos marcadores entre los días 7 y 14, indicando una activación de la maquinaria responsable de la biogénesis mitocondrial. Aunque no existen estudios en los que se haya estudiado este fenómeno a nivel de marcadores de biogénesis (PGC-1α, Tfam y TOM20), Zoll et al. (2005) reportó un aumento en el mRNA de PGC-1α en atletas sometidos a dos sesiones de entrenamiento hipóxico durante 6 semanas, sugiriendo que, a diferencia de la hipoxia crónica, la HHI puede inducir un aumento del contenido mitocondrial.

5.3.3.3 Proteínas marcadoras de dinámica mitocondrial y señalización apoptótica

Además de regular su densidad mediante el proceso de biogénesis, las mitocondrias forman un entramado altamente dinámico, capaz de llevar a cabo procesos de fisión y fusión con el objetivo de mantener una red

sana, funcional e interconectada, funcionando como una suerte de control de calidad intracelular (Picca et al. 2016). Así, la fisión permite a las mitocondrias eliminar partes dañadas o poco eficientes. Paralelamente, la fusión permite incorporar a la red nuevas mitocondrias, sanas y funcionales (Suen et al. 2008). Son proteínas marcadoras de fusión mitocondrial Mfn2 (*Mitofusin* 2) y OPA1 (*Optic Atrophy 1*), mientras que Drp1 (*Dynamin-related protein 1*) participa en procesos de fisión. Tal y como se observa en la Fig. 5 (**III**), el EEE perturbó de manera significativa la dinámica mitocondrial, inhibiendo los procesos de fusión y estimulando los de fisión, sugiriendo que parte de la red mitocondrial resultó dañada tras la realización del protocolo de EEIMD. Resulta destacable la persistencia en el tiempo de altos niveles de Drp1 y, consecuentemente, de la fisión mitocondrial, lo cual podría explicar la disminución de TOM20 y CS descrita anteriormente. Es interesante resaltar que el grupo AHR, a pesar de mostrar niveles de Drp1 incluso superiores al grupo PNR, exhibió, adicionalmente, una mayor expresión de las proteínas Mfn2 y OPA-1, sugiriendo un mayor dinamismo y *turnover* mitocondrial. Es decir, la fisión de partes dañadas de la red mitocondrial se vería compensada por la incorporación (fusión) de nuevas mitocondrias, íntegras y funcionales.

La dinámica mitocondrial, además de su impacto lógico en la funcionalidad de la mitocondria, juega también un papel importante en la señalización celular, siendo especialmente relevante su rol en la activación de la apoptosis. No solo media en la liberación del citocromo c, sino que las propias proteínas de fisión (como Drp-1) tienen un rol activo en la activación de la apoptosis (Suen et al. 2008). En consonancia con esto, se observó un aumento significativo de la señalización

apoptótica, representado por el aumento del índice Bax/Bcl-2, el cual expresa el balance entre la señalización pro-apoptótica (Bax) y anti-apoptótica (Bcl-2) (Boise et al. 1995). De acuerdo con lo observado en el análisis histopatológico y con la literatura consultada (Sudo y Kano 2009), este índice muestra un pico de apoptosis a las 72 h, que permaneció relativamente alto hasta el día 14 (**III:** Fig. 6). Aunque no se observaron diferencias significativas, el grupo AHR mostró una tendencia clara a disminuir este índice, sugiriendo que la HHI podría inducir cierto fenotipo protector contra la apoptosis. De hecho, al igual que el porcentaje de fibras anormales (en las que se incluyen fibras en apoptosis) (**II:** Fig. 3), a día 14 el valor de Bax/Bcl-2 fue similar al de las ratas CTRL, sugiriendo una recuperación más rápida.

5.3.3.4 Proteínas marcadoras de estrés oxidativo

También se analizó la expresión de proteínas mitocondriales relacionadas con el estrés oxidativo, especialmente al finalizar el protocolo de recuperación (**III:** Fig. 7). La proteína Sirtuina 3 (Sirt3) es una deacetilasa que en los últimos años ha emergido como una agente importante en la regulación del envejecimiento y de la esperanza de vida, especialmente a través de la modulación que ejerce en enzimas oxidativas y antioxidantes (Wu et al. 2014b). A su vez, Sirt3 es regulada negativamente por el estrés oxidativo (Wu et al. 2014b), por lo que la disminución en su expresión durante las primeras 72 horas indica que el EEE indujo una situación de estrés oxidativo, tal y como ya demostraron estudios previos (Stagos et al. 2015). Siguiendo la dinámica mostrada por otros marcadores, Sirt3 fue regulada positivamente por la HHI en combinación con ejercicio tras una y dos semanas de tratamiento (**III:** Fig. 6A), hecho que no resulta sorprendente teniendo

en cuenta que Sirt3 es un gen diana de PGC-1α (Kong et al. 2010), cuya expresión estuvo elevada a lo largo de la mayor parte del protocolo en el grupo AHR (**III:** Fig. 3A).

Además de Sirt3, también fue analizada la expresión de otro marcador de estrés oxidativo: p66shc. Esta proteína resulta fosforilada en su serina 36 por el daño oxidativo (Migliaccio et al. 1999), por lo que analizar la proporción de p66shc fosforilada respecto a la cantidad total de p66shc (p66shc-Ser36/p66shc) resulta útil para evaluar este tipo de estrés. Así, tal y como muestran las Figuras 7B-D (**III**), los grupos expuestos a HHI exhibieron una menor proporción de p66shc-Ser36 respecto a p66shc total, sugiriendo una mayor resistencia al estrés oxidativo inducido por el EEIMD. Curiosamente, esta reducción fue alcanzada por vías diferentes: mientras que el grupo PHR mostró niveles inferiores de fosforilación, el grupo AHR *tamponó* tal aumento mediante un incremento total en la cantidad de p66shc, lo que sugiere diferentes mecanismos de respuesta. Sería necesario profundizar en este y otros marcadores para elucidar el origen de estas diferencias (por ejemplo, mediante el análisis del balance de la forma reducida y oxidada del glutatión, o de la actividad de enzimas como la catalasa y la superóxido dismutasa).

5.3.3.5 Bioenergética mitocondrial

Por último, aunque no menos importante, se analizó la expresión de diferentes subunidades pertenecientes a los complejos I-V de la cadena transportadora de electrones (ETC, *Electron Transport Chain*) (**III:** Fig. 8A-E) y de la adenina-nucleótido translocasa (ANT) (**III:** Fig. 8F), con el fin de obtener una imagen general del estado de esta cadena fundamental en la generación de ATP. Además de su papel en la

bioenergética celular como proveedor de ADP para la síntesis de ATP (Fiore et al. 1998), ANT forma parte del poro de permeabilidad transitoria mitocondrial (MPTP, *Mitochondrial Permeability Transition Pore*), cuya apertura está íntimamente ligada a la señalización apoptótica (Kinnally et al. 2011). De hecho, reducciones en el contenido de ANT han sido correlacionadas con una mayor susceptibilidad a la apertura del MPTP inducida por Ca^{2+} (Oliveira y Wallace 2006), por lo que el incremento de ANT hallado en los grupos expuestos a HHI no solo sugiere que la ATP sintasa de la ETC tiene a su disponibilidad una mayor cantidad de sustrato, sino que estos animales presentan un fenotipo más resistente a la señalización apoptótica, siendo especialmente evidente en el grupo AHR, el cual también exhibió un menor índice Bax/Bcl-2 (**III:** Fig. 6C).

El análisis del contenido de las diferentes subunidades marcadoras de los complejos I-V de la ETC mostró una tendencia a aumentar en los animales expuestos a HHI, aunque solo resultó significativa en el complejo I (PHR) y IV (PHR y AHR). Tal y como se ha insistido a lo largo de esta memoria, esta divergencia podría deberse a diferencias en la intensidad y la duración de los protocolos de exposición a la hipoxia. En cualquier caso, el grupo PHR mostró una mayor expresión de los complejos I, III y IV, en tanto que los animales AHR solo mostraron una sobreexpresión del IV. El incremento de los complejos I y III podría conducir a un aumento del estrés oxidativo, ya que son los principales productores de ROS de la ECT (Buresh y Berg 2015). Sin embargo, el aumento de la expresión del complejo IV podría contrarrestar este potencial aumento de la producción de ROS, ya que se ha descrito que este complejo puede tener un efecto antioxidante

mediante la reducción, precisamente, de la generación de ROS derivada de la actividad de los complejos I y III (Chen et al. 2003; Parise et al. 2005). En contraposición, los animales AHR exhibieron un incremento de la subunidad marcadora del complejo IV sin la contrapartida del aumento del I y III, sugiriendo una defensa antioxidante neta superior, lo cual reforzaría la sobreexpresión de Sirt3 descrita en la Figura 7A (**III**).

Con todo, resultó sorprendente, al menos inicialmente, no observar un incremento más contundente del contenido de los diferentes complejos, especialmente en el grupo AHR, a tenor de los resultados obtenidos de los marcadores de biogénesis mitocondrial. Esta aparente divergencia podría ser una consecuencia de la complejidad del ensamblaje de las diferentes subunidades de los complejos de la ETC. Así, ha sido descrito un desfase de días/semanas entre el aumento de la expresión de determinadas proteínas relacionadas con la biogénesis y el aumento efectivo del contenido mitocondrial (Hood 2001), lo cual podría explicar los resultados obtenidos.

5.3.4 Relevancia del ejercicio en la exposición a HHI

Tal y como se ha descrito a lo largo de la discusión, los grupos expuestos a HHI (PHR y AHR) arrojaron a menudo resultados dispares, siendo que en muchos casos los animales con recuperación pasiva (PHR) manifestaron valores más próximos al grupo normobárico (PNR) que al grupo con recuperación activa (AHR). El origen de estas diferencias reside, probablemente, en el grado de activación de la vía señalizadora de la hipoxia dependiente de HIF-1, tal y como refleja la expresión de VEGF y GLUT1 (**II:** Fig. 7), genes diana de este factor de transcripción.

Tanto VEGF como GLUT1 poseen elementos de respuesta hipóxica (HRE, *Hypoxic Response Elements*) en sus promotores, a los cuales se une directamente HIF-1, induciendo así su transcripción (Wenger et al. 2005). Consecuentemente, ambos grupos mostraron una mayor expresión de VEGF al finalizar el protocolo. Sin embargo, la expresión de VEGF es modulada por una gran diversidad de factores (Pages y Pouyssegur 2005), por lo que no es un marcador tan específico como GLUT1. En el caso de este último, sí que se observó una expresión diferencial entre las ratas PHR y AHR, indicando que la combinación de HHI y ejercicio aeróbico indujo una activación de la vía dependiente de HIF-1 mucho más robusta y consistente, justificando así el mayor número de respuestas adaptativas y benéficas en este grupo. Esta diferencia en la cascada señalizadora indica que la HHI, por sí sola, no fue capaz de comprometer la homeostasis del oxígeno del músculo sóleo en reposo. Tal y como se ha mencionado anteriormente, la actividad física puede ser un factor necesario para *desafiar* el balance entre aporte y consumo de O_2 y, por lo tanto, activar las vías señalizadoras necesarias para desarrollar respuestas adaptativas.

Parece improbable, además, que el protocolo de ejercicio aeróbico ligero aplicado en combinación con la HHI pudiera, ejecutado aisladamente, inducir una respuesta apreciable. Es importante realzar, en este punto, que todos los animales utilizados en este protocolo fueron entrenados durante varias semanas con dos sesiones diarias de 35 min a 27 $m \cdot min^{-1}$ (1890 m diarios), por lo que la sesión de recuperación, por sí sola (una única sesión diaria de 20 min a 18 $m \cdot min^{-1}$, 360 m diarios) difícilmente podría producir respuestas equivalentes a las observadas en el grupo AHR (para información más detallada de los

diferentes protocolos de ejercicio aeróbico, consultar las Tablas A1, A2 y A3 del Anexo). En este punto es conveniente tener en cuenta que, a pesar de ser posible mantener las adaptaciones fisiológicas al ejercicio físico con protocolos con menor volumen de entrenamiento (Hickson et al. 1982, Madsen et al. 1993), la intensidad continúa siendo un factor clave en la conservación de los beneficios adquiridos (Mujika y Padilla 2000). Es más, en un estudio de Desplanches et al. (1993), en el que un entrenamiento realizado al 50% de la VO_2 máxima en condiciones de hipoxia desencadenó una serie de mejoras metabólicas, el mismo entrenamiento en condiciones normobáricas no indujo ninguna adaptación estructural o funcional, coincidiendo con nuestra hipótesis.

5.3.4.1 Comportamiento hormético de la HHI

Como era de esperar en un fenómeno de hormesis, la HHI mostró un comportamiento acumulativo de dosis-respuesta. La gran mayoría de las diferencias entre el grupo AHR (y, ocasionalmente, PHR) y el PNR fueron encontradas en la parte final del protocolo, concentrándose normalmente en el último punto temporal estudiado (t14). Se resalta, así, la utilidad de la HHI como herramienta pre-condicionante y/o terapéutica, en la que dosis bajas o aisladas apenas producen respuestas fisiológicas duraderas en el organismo, contrastando con la aplicación repetida de dosis de moderada intensidad.

5.3.5 Resumen

La exposición a lo largo de dos semanas a HHI en combinación con ejercicio aeróbico ligero fue capaz de revertir o acelerar la recuperación de la mayoría de las alteraciones inducidas por el EEIMD. Las ratas AHR exhibieron una histopatología más cercana a la encontrada en

animales no sujetos a EEIMD, y las fibras de su músculo sóleo conservaron un tamaño similar al de antes del EEIMD. Más importante aún, este grupo recuperó en 14 días los valores relativos a la capilarización del músculo, no solo debido a la conservación de la FCSA sino también como consecuencia de a un proceso de angiogénesis, tal y como reflejó el incremento en el índice C/F y en la expresión de VEGF. Esta mejora en la capilarización conlleva una mayor disponibilidad de oxígeno y nutrientes en el tejido dañado, acelerando su recuperación. Además, la combinación de HHI y ejercicio aeróbico ligero produjo una fuerte modulación de numerosos procesos mitocondriales, aumentando los marcadores de biogénesis y dinámica mitocondrial, reduciendo la señalización apoptótica y el estrés oxidativo, todo ello sin afectar negativamente la cadena transportadora de electrones. Junto a la atenuación hallada en el descenso de actividad de la CS inducida por el EEIMD, estos cambios sugieren que las ratas AHR poseen una mayor capacidad aeróbica, fundamental en músculos como el sóleo activo durante actividades sostenidas como el mantenimiento de la postura y el ejercicio aeróbico submáximo. La Figura 7 muestra un resumen de los principales resultados.

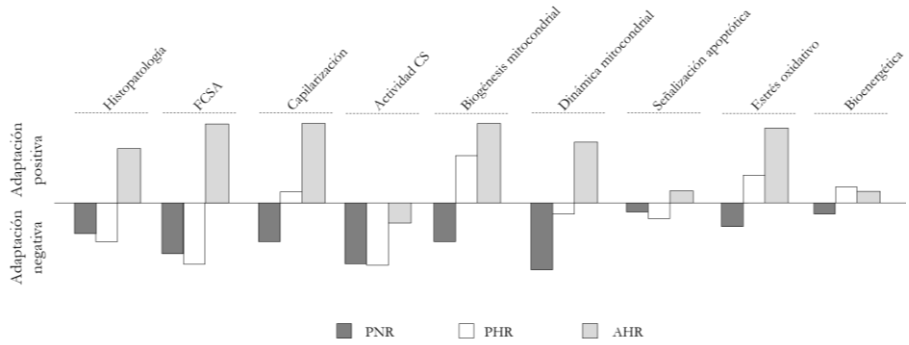

Figura 7. Representación gráfica de las respuestas inducidas por los diferentes protocolos en los principales parámetros y marcadores discutidos a lo largo de la memoria, divididas en benéficas o positivas (parte superior) y perjudiciales o negativas (parte inferior). La altura de las barras representa, de forma aproximada y visual, la magnitud de la alteración. FCSA: área de la sección transversal de la fibra, CS: Citrato Sintasa; PNR: grupo con recuperación normobárica pasiva; PHR: grupo con recuperación hipobárica pasiva; AHR: grupo con recuperación hipobárica activa.

En conclusión, la HHI, acentuada por el ejercicio aeróbico ligero, desencadenó una respuesta benéfica, dosis-dependiente, en la recuperación de las alteraciones inducidas por el EEIMD, sin provocar cambios en la distribución del tipo de fibras. Así, la HHI, ya utilizada en la mejora del rendimiento deportivo, podría potencialmente utilizarse como una herramienta terapéutica para el tratamiento del EEIMD.

6.

Conclusiones

1. La aplicación de dos sesiones de ejercicio excéntrico extenuante induce la aparición de daño muscular en el músculo sóleo de ratas de laboratorio entrenadas, tal y como reflejaron los análisis histopatológicos y la evaluación de los biomarcadores plasmáticos CK-MM y mioglobina.

2. La herramienta metodológica diseñada ha permitido evaluar el daño muscular inducido por ejercicio excéntrico extenuante en sujetos entrenados a partir de la consideración de los siguientes elementos histológicos: morfología de las fibras, infiltrados mononucleares y distensión del tejido conjuntivo.

3. El ejercicio excéntrico extenuante compromete la microvasculatura del músculo sóleo y su capacidad oxidativa. Se observó un incremento del tamaño medio de las fibras, si bien no se pudo concluir que fuera el protocolo de ejercicio excéntrico el responsable de tal hipertrofia. No se observaron diferencias en la distribución de los diferentes tipos de fibras.

4. La aplicación de hipoxia hipobárica intermitente en combinación con ejercicio aeróbico ligero atenúa o revierte la mayoría de las alteraciones inducidas por el daño muscular provocado por el ejercicio excéntrico extenuante.

5. Los individuos sometidos a hipoxia hipobárica intermitente y ejercicio ligero exhibieron una mejora en la capilarización debida a un fenómeno de angiogénesis, reflejado por el incremento de la expresión de VEGF y del índice C/F, atenuándose parcialmente la

reducción en la capacidad oxidativa del músculo inducida por el daño muscular.

6. El protocolo de inducción del daño muscular conlleva un fuerte incremento de proteínas de fisión, sugiriendo la presencia de daño mitocondrial. La aplicación de hipoxia hipobárica intermitente en combinación con ejercicio aeróbico ligero no disminuye la expresión de los marcadores de fisión, pero indujo biogénesis mitocondrial y la sobreexpresión de marcadores de fusión, indicando una red mitocondrial más dinámica y adaptable.

7. La expresión de proteínas mitocondriales de señalización apoptótica y de estrés oxidativo fue menor en los individuos sometidos a hipoxia hipobárica y recuperación activa en comparación con los animales que realizaron una recuperación pasiva normobárica.

8. La exposición a hipoxia hipobárica intermitente resulta insuficiente para activar de manera consistente la vía de señalización dependiente de HIF en el músculo sóleo en reposo.

9. La inclusión de un protocolo de ejercicio aeróbico ligero tras la exposición a hipoxia hipobárica intermitente resultó fundamental para activar de manera consistente la vía de señalización dependiente de HIF.

10. Estos resultados indican que la hipoxia hipobárica intermitente en combinación con ejercicio aeróbico ligero puede ser una potencial herramienta terapéutica para el tratamiento del daño muscular inducido por el ejercicio excéntrico.

7.

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8.

Anexos

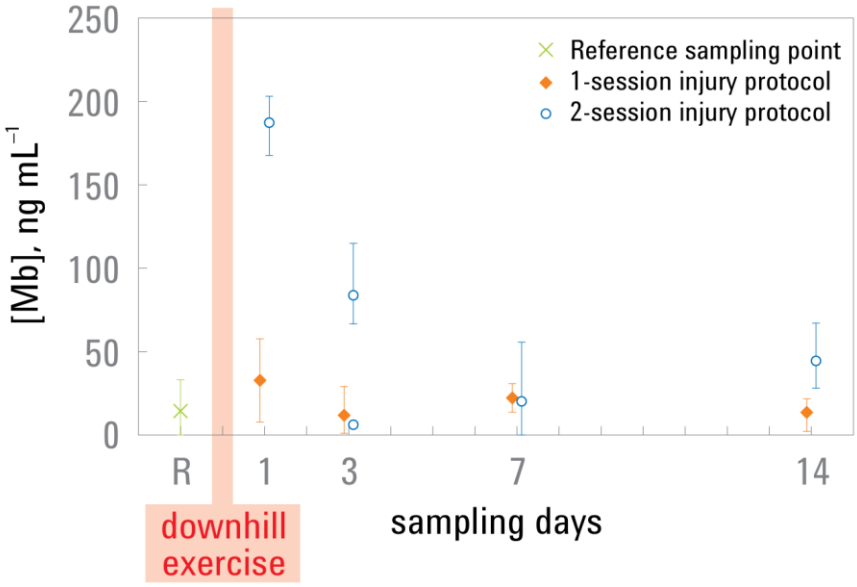

Figura A1. Diferencias en la concentración de mioglobina en plasma tras una (rombos naranjas) y dos (círculos azules) sesiones de carrera continua en pendiente negativa para inducir daño muscular en ratas entrenadas. Una única sesión no alteró significativamente los niveles de mioglobina en plasma, indicando la ausencia de EEIMD. Por lo contrario, la doble aplicación del mismo protocolo indujo un aumento apreciable durante los días 1 y 3. Cuantificación realizada mediante ELISA. Figura publicada en el póster *A method to induce skeletal muscle damage in trained rats* (Ríos-Kristjánsson et al. 2012. Federation of European Physiological Societies Congress, Santiago de Compostela, España, 2012).

Tabla A1. Desarrollo detallado del periodo de adaptación a la carrera continua en tapete rodante.

Exercise Schedule

	phase 0		phase I			phase II			phase III		phase IV		summary				cubicle/channel				
Day	t_0	u_0	$(t_n)_1$	u_1	a_1	$(t_n)_2$	u_2	a_2	t_3	u_3	t_4	u_4	$t_{exerc.}$	t_{total}	I_{min}	I_{max}	Rat's position				
No.	min	cm s^{-1}	min	cm s^{-1}	cm s^{-1}min^{-1}	min	cm s^{-1}	cm s^{-1}min^{-1}	min	cm s^{-1}	min	cm s^{-1}	min	min	mA	mA	**1**	**2**	**3**	**4**	**5**
1A	10.0	0 (5)	0	0	0	0	0	0	0	0	0	0	0	10	0.2	0.6	i	ii	iii	iv	v
2A	10.0	0 (5)	0	0	0	0	0	0	0	0	0	0	0	10	0.2	0.6	v	i	ii	iii	iv
3A	3.5	0 (5)	5.0	10-30	~4	0	0	0	10.0	30	2.0	0	15	20.5	0.2	0.6	iv	v	i	ii	iii
4A	3.0	0 (5)	5.0	10-30	~4	0	0	0	15.0	30	2.0	0	20	25	0.2	0.6	iii	iv	v	i	ii
4B	3.0	0 (5)	5.0	10-30	~4	2.0	30-35	~2	15.0	35	2.0	0	22	27	0.2	0.6	ii	iii	iv	v	i
5A	3.0	0 (5)	5.0	15-30	~3	2.0	30-35	~2	20.0	35	2.0	0	27	32	0.2	0.6	i	ii	iii	iv	v
5B	3.0	0 (5)	5.0	15-30	~3	2.0	30-35	~2	20.0	35	2.0	0	27	32	0.2	0.6	v	i	ii	iii	iv
6A	2.0	0 (5)	5.0	15-30	~3	2.0	30-35	~2	24.0	35	2.0	0	31	35	0.2	0.8	iv	v	i	ii	iii
6B	2.0	0 (5)	5.0	15-30	~3	2.0	30-35	~2	24.0	35	2.0	0	31	35	0.2	0.8	iii	iv	v	i	ii
7A	2.0	0 (5)	5.0	15-30	~3	3.0	30-40	~3	26.0	40	2.0	0	34	38	0.2	0.8	ii	iii	iv	v	i
7B	2.0	0 (5)	5.0	15-30	~3	3.0	30-40	~3	26.0	40	2.0	0	34	38	0.4	0.8	i	ii	iii	iv	v
8A	1.0	0 (5)	5.0	15-30	~3	3.0	30-40	~3	29.0	40	2.0	0	37	40	0.4	0.8	v	i	ii	iii	iv
8B	1.0	0 (5)	5.0	15-30	~3	3.0	30-40	~3	29.0	40	2.0	0	37	40	0.4	0.8	iv	v	i	ii	iii
9A	1.0	0 (5)	5.0	15-35	~4	3.0	30-45	5	32.0	45	2.0	0	40	43	0.4	1.0	iii	iv	v	i	ii
9B	1.0	0 (5)	5.0	15-35	~4	3.0	35-45	5	32.0	45	2.0	0	40	43	0.4	1.0	ii	iii	iv	v	i

La adaptación a la carrera continua se llevó a cabo con dos sesiones diarias durante dos semanas. Cada sesión consistía en cinco fases diferenciadas: 0) en esta fase, el tapete se encuentra desconectado y permite al animal reconocer su nuevo espacio; I) fase de aceleración en la que se alcanza gradualmente una velocidad cercana a la deseada; II) fase en la que se establece la velocidad programada para dicha sesión; III) fase principal del protocolo, en la que transcurre la carrera continua a la velocidad y duración establecidas; y IV) fase de reposo tras la carrera continua, en la que el animal es recompensado positivamente. A pesar de que este conjunto de sesiones se encuadraron bajo la nomenclatura de *aclimatación* o *pre-condicionamiento*, a partir del cuarto día los animales ya realizaban carrera continua a 35 cm·s^{-1} (un 77% de la velocidad establecida durante el protocolo final de entrenamiento).

Tabla A2. Resumen detallado del protocolo de entrenamiento aeróbico.

phase I			phase III		phase IV		summary		current	
$(t_n)_1$	u_1	a_1	t_3	u_3	t_4	u_4	$t_{exerc.}$	t_{total}	I_{min}	I_{max}
min	cm s^{-1}	cm $s^{-1}min^{-1}$	min	cm s^{-1}	min	cm s^{-1}	min	min	mA	mA
5	25-45	4	30	45	1	0	35	36	0.8	1.6

El protocolo de entrenamiento aeróbico en tapete rodante fue aplicado dos veces por día. Tras un breve periodo de *calentamiento* (fase I), en que la velocidad aumentaba gradualmente hasta los 45 cm·s^{-1} (fase III), los animales corrían 30 minutos ininterrumpidamente a dicha velocidad. Debido a que los animales ya se encontraban totalmente adaptados a la carrera continua, la fase II, presente en el protocolo de adaptación, no era necesaria.

Tabla A3. Resumen detallado del protocolo de recuperación aeróbica.

phase 0			phase I				phase III			phase IV		
t_0	u_0	Φ_0	$(t_n)_1$	a_1	u_1	Φ_1	t_3	u_3	Φ_3	t_4	u_4	Φ_4
min	cm s^{-1}	°	min	cm s^{-1} min^{-1}	cm s^{-1}	°	min	cm s^{-1}	°	min	cm s^{-1}	°
1	0 (5)	0	5	~5	10-30	0-(+5)	15	30	+5	1	0 (5)	0

Inmediatamente después de cada sesión de hipoxia hipobárica, los animales asignados al grupo AHR eran emplazados en el tapete rodante para llevar a cabo 20 minutos de carrera continua, tal y como se especifica en esta tabla.