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ACCIÓN DE ALIMENTOS
RICOS EN ÁCIDOS GRASO
SOBRE EL ESTRÉS O

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ACCIÓN DE ALIMENTOS FUNCIONALES RICOS EN ÁCIDOS GRASOS ESENCIALES SOBRE EL ESTRÉS OXIDATIVO

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El qui troba un bon padrí, ja ha corregut mig camí

En memòria de Miquel Pons Ramón

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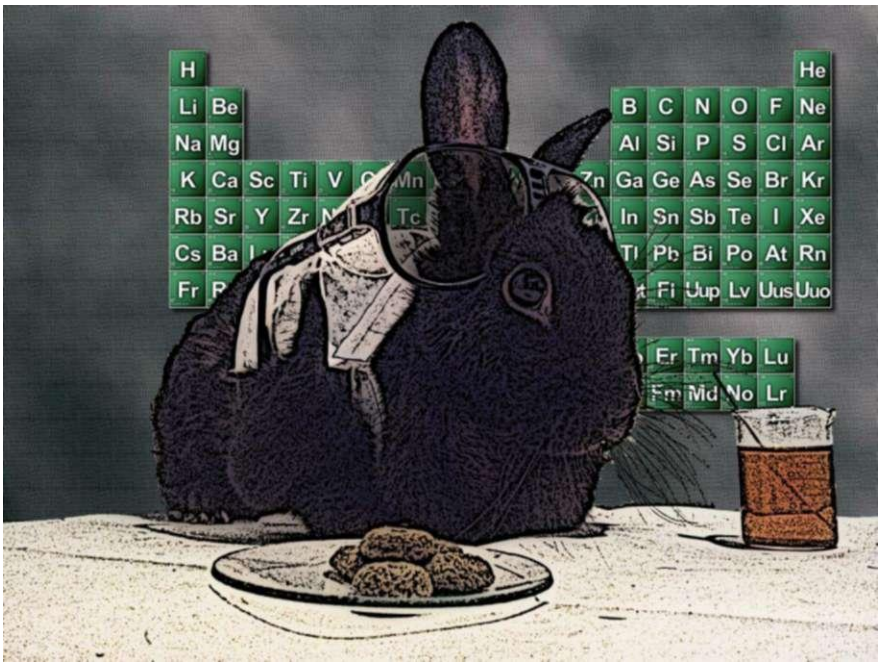

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ÍNDICE / INDEX

ABREVIATURAS / ABBREVIATIONS III

RESUMEN IX

LISTADO DE ARTÍCULOS ORIGINALES / LIST OF ORIGINAL PAPERS. XIII

AGRADECIMIENTOS A LA FINANCIACIÓN RECIBIDA XV

INTRODUCCIÓN 1

ÁCIDOS GRASOS 3

Ácidos grasos esenciales 4

Precursores de los omega-6 y los omega-3 8

Metabolismo de los omega-6 y los omega-3 8

Balance omega-6 i omega-3 11

Necesidades omega-3 13

Alimentos funcionales enriquecidos con ácidos grasos esenciales 15

Omega-3 y expresión de genes 16

EJERCICIO FÍSICO Y ÁCIDOS GRASOS 17

Relación omega-3 y rendimiento deportivo 19

Mecanismos de los omega-3 en los procesos relacionados con el ejercicio físico 20

Modificación de la movilización de los AGs 20

Modificación de la actividad carnitina-aciltransferasa 21

Favoreciendo la actividad oxidativa e induciendo el aumento de las mitocondrias 21

Reduciendo el proceso de inflamación seguido del ejercicio físico 22

Favoreciendo el flujo del ácido láctico 22

EJERCICIO FÍSICO Y ÓXIDO NÍTRICO 23

Suplementos deportivos relacionados con el óxido nítrico 24

EJERCICIO FÍSICO Y RESPUESTA INMUNE DE FASE AGUDA 26

Omega-3, inflamación y enfermedad cardiovascular 27

ESTRÉS OXIDATIVO Y LOS ELEMENTOS QUE PARTICIPAN 29

Especies reactivas de oxígeno 29

Especies reactivas de nitrógeno 31

Defensas antioxidantes 32

Defensas antioxidantes proteicas y enzimáticas 33

Defensas antioxidantes no proteicas 35

Equilibrio redox y estrés oxidativo 37

Formación de radicales libres y estrés oxidativo durante el ejercicio 39

Generación de radicales libres en la contracción muscular y su papel en la señalización celular 41
Mecanismos de formación de radicales libres durante el ejercicio 42
La cadena de transporte electrónico mitocondrial 42
La xantina oxidoreductasa 43
NADPH oxidasa 43
Células sanguíneas, especies reactivas de oxígeno y adaptación al ejercicio .. 44
Eritrocitos 44
Linfocitos 47
Neutrófilos 49
El entrenamiento como antioxidante 51
Omega-3 y estrés oxidativo 53

OBJETIVOS Y PLANTEAMIENTO EXPERIMENTAL 55

OBJETIVOS 57

PLANTEAMIENTO EXPERIMENTAL 62

RESULTADOS Y DISCUSIÓN / RESULTS AND DISCUSSION 69

MANUSCRIPT I 71

MANUSCRIPT II 97

MANUSCRIPT III 127

MANUSCRIPT IV 161

MANUSCRIPT V 201

MANUSCRIPT VI 237

RECAPITULACIÓN 267

Efectos del DHA sobre el daño oxidativo 269
Dinámica del daño oxidativo y nitrosativo asociado a la actividad física aguda y al entrenamiento 271
Efectos del ejercicio sobre la nitración de proteínas 276
Cambios en los marcadores antioxidantes asociados a una actividad física moderada y regular, e intensa 279
La suplementación con omega-3 aumenta su biodisponibilidad afectando al estrés oxidativo y a la función mitocondrial 285

CONCLUSIONES / CONCLUSIONS 289

BIBLIOGRAFIA / BIBLIOGRAPHY 295

ANEXO 335

ABREVIATURAS / ABBREVIATIONS

4-HNE	4-hidroxinonenal / *4-Hydroxynonenal*
AA	Ácido araquidónico / *Arachidonic acid*
AG	Ácido graso / *Fatty acid*
ALA	Ácido alfa-linolénico / *Alpha-linolenic acid*
AP	Fosfatasa alcalina / *Alkaline phosphatase*
AST	Aspartato aminotransferasa / *Aspartate transaminase*
ATP	Adenosina trifosfato / *Adenosine triphosphate*
BHA	Hidroxibutilanisol / Butylated hydroxyanisole
CK	Creatina quinasa / *Creatin kinase*
ConA	Concanavalina A / *Concanavalin A*
CPR	Proteina C reactiva / *C-reactive protein*
DHA	Ácido docosahexaenoico / *Docosahexaenoic acid*
DGLA	Ácido dihomo-gamma-linolénico / *Dihomo-gamma-linolenic acid*
DPA	Ácido docosapentaenoico / *Docosapentaenoic acid*
DTA	Ácido docosatetraenoico / *Docosatetraenoic acid*
EDRF	Factor relajante derivado del endotelio / *Endothelium-derived relaxing factor*
EDTA	Ácido etilendiaminotetraacético / *Ethylenediaminetetraacetic acid*
EPA	Ácido eicosapentaenoico / *Eicosapentaenoic acid*
ETA	Ácido eicosatetraenoico / *Eicosatetraenoic acid*

FOXO	Familia Forkhead / *Forkhead box other*
YGT	gamma glutamil transpeptidasa / *gamma-glutamyl transpeptidasa*
GMPc	Guanosín monofosfato cíclico / *Cyclic guanosine monophosphate*
GLA	Ácido gamma-linolénico / *Gamma-linolenic acid*
GPx	Glutatión peroxidasa / *Glutathione peroxidase*
GRd	Glutatión reductasa / *Glutathione reductase*
GSH	Glutatión / *Glutathione*
GSSG	Glutatión oxidado / *Oxidized glutathione*
HDL	Lipoproteínas de alta densidad / *High-density lipoprotein*
Hb	Hemoglobina / *Hemoglobin*
HNF4A	Factor nuclear 4 alfa de hepatocito / *Hepatocyte nuclear factor 4 alpha*
IFNy	Interferón gamma / *Interferon gamma*
IL	Interleuquina / *Interleukin*
LA	Ácido linoleico / *Linoleic acid*
LCPUFA	Ácido graso de cadena larga polinsaturada / *Long chain polyunsaturated fatty acids*
LDH	Lactato deshidrogenasa / *Lactate dehydrogenase*
LDL	Lipoproteínas de baja densidad / *Low-density lipoprotein*
LOX	Lipooxigenasa / *Lipoxygenase*
LPL	Lipoproteina lipasa / *Lipoprotein lipase*

LPS	Lipopolisacáridos / *Lipopolysaccharide*
LXR	Receptor X hepático / *Liver X receptor*
MAPK	Proteína quinasa activada por mitógeno / *Mitogen-Activated Protein Kinases*
MB	Mioglobina / *Myoglobin*
MCH	Hemoglobina corpuscular media / *Mean corpuscular hemoglobin*
MCHC	Concentración de hemoglobina corpuscular media / *Mean corpuscular hemoglobin concentration*
MCV	Volumen corpuscular mediano / *Mean corpuscular volume*
MDA	Malonildialdehído / *Malondialdehyde*
metHb	Metahemoglobina / *Methemoglobin*
MPO	Mieloperoxidasa / *Myeloperoxidase*
MUFA	Ácido graso monoinsaturado / *Monounsaturated fatty acid*
NEFA	Ácido graso no esterificado / *non esterified fatty acid*
NFKP	Factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas / *Nuclear factor kappa-light-chain-enhancer of activated B cells*
NK	Célula Natural Killer / *Natural killer cell*
NOS	Óxido nítrico sintasa / *Nitric oxide synthase*
NOX	NADPH oxidasa / *NADPH oxidase*
PBMC	Célula mononuclear de sangre periférica / *Peripheral blood mononuclear cell*

PMA Acetato de forbol miristato / *Phorbol myristate acetate*

PLA2 Fosfolipasa A2 / *Phospholipase A2*

PPAR Receptores activados de proliferación de los peroxisomas / *Peroxisome proliferator-activated receptors*

PUFA Ácido graso poliinsaturado / *Poliunsaturated fatty acid*

RDW Ancho de distribución de globulos rojos / *Red blood cell distribution width*

RNS Especies reactivas de nitrógeno / *Reactive nitrogen species*

ROS Especies reactivas de oxígeno / *Reactive oxygen species*

RONS Especies reactivas de oxígeno y de nitrógeno / *Reactive oxygen and nitrogen species*

SDA Ácido estearidónico / *Stearidonic acid*

SFA Ácido graso saturado / *Saturated fatty acid*

SOD Superóxido dismutasa / *Superoxide dismutase*

SREBP Proteína de unión al elemento de respuesta al esterol / *Sterol regulatory element-binding protein*

TCR Receptor de células T / *T-cell receptor*

TGFA Ácido graso de triglicérido / *Triglyceride fatty acid*

THA Ácido tetracosahexaenoico / *Tetracosahexaenoic acid*

TNFa Factor de necrosis tumoral alfa / *Tumor necrosis factor-alpha*

TPA Ácido tetracosapentaenoico / *Tetracosapentaenoic acid*

TSF	Fluoruro de p-toluensulfonilo / *p-toluenesulfonyl fluoride*
TTA	Ácido tetracosatetraenoico / *Tetracosatetraenoic acid*
TLR	Receptor tipo Toll / *Toll-like receptor*
Trx	Tioredoxina / *Thioredoxin*
TrxR	Tioredoxina reductasa / *Thioredoxin reductase*
UCP	Proteína desacopladora / *Uncoupling protein*
XDH	Xantina deshidrogenasa / *Xanthine dehydrogenase*
XO	Xantina oxidasa / *Xanthine oxidase*
VLDL	Lipoproteínas de muy baja densidad / *Very-low-density lipoprotein*

ACCIÓN DE ALIMENTOS FUNCIONALES RICOS EN ÁCIDOS GRASOS ESENCIALES SOBRE EL ESTRÉS OXIDATIVO

Tesis doctoral, Miquel Martore!! Pons, Departament de Biologia Fonamental i Ciències de la Salut, Grup de Recerca en Nutricio Comunitària i Estrés Oxidatiu, Laboratori de Ciències de l'Activitat Física, Universität de les Illes Balears, Palma de Mallorca, España. CIBER de Fisiopatologia de la Obesidad y la Nutrición (CIBERobn CB12/03/30038), Instituto de Salud Carlos III (ISCIII), España.

Resumen

Una suplementación de la dieta con alimentos ricos en omega-3 provoca un aumento de estos ácidos grasos en las membranas celulares, incrementando la capacidad de deformación de los eritrocitos y provocando una mayor capacidad de transporte de oxígeno. Los omega-3 activan la expresión de genes que alteran la utilización energética de los ácidos grasos y de oxígeno a nivel mitocondrial, debido a su interacción con factores de transcripción como los PPARs y NFK0, los cuales están implicados en la regulación de genes de enzimas antioxidantes e inflamatorios. En contraposición, los omega-3 debido a su naturaleza química tienen una marcada susceptibilidad a oxidarse e iniciar procesos de peroxidación lipídica.

Durante el entrenamiento regular y el ejercicio moderado se producen moderadas cantidades de especies reactivas de oxígeno y de nitrógeno (RONS), moléculas altamente reactivas que pueden reaccionar con facilidad con los diferentes componentes moleculares de las células, induciendo daños oxidativo y nitrosativo, alterando su estructura y su función. Sin embargo, estas RONS tienen otro efecto ya que actúan como promotores de la expresión de genes antioxidantes e inducen la activación de las defensas antioxidantes. Las RONS tienen un doble papel, uno que provoca un daño oxidativo y otro que induce una protección frente a altas cantidades de especies reactivas. En este último caso se pone de manifiesto la existencia de efectos horméticos en la producción de bajos niveles de RONS durante el entrenamiento de deportistas. El estado de las defensas antioxidantes previo a la actividad física contribuye a determinar si

ésta genera un desequilibrio oxidativo afectando a la viabilidad y funcionalidad celular.

El objetivo principal de la presente tesis ha sido estudiar la acción de alimentos funcionales ricos en ácidos grasos esenciales sobre la situación de estrés oxidativo inducido por la actividad física aguda y sobre las adaptaciones al estrés oxidativo inducidas por el entrenamiento.

La realización de un entrenamiento deportivo regular, como el entrenamiento futbolístico, mejora la maquinaria antioxidante endógena de diferentes fracciones sanguíneas, plasma, eritrocitos, neutrófilos y células mononucleares de sangre periférica (PBMCs), mediante mecanismos de activación enzimática e incremento proteico de enzimas. Esta mejora de la capacidad antioxidante provocada por el ejercicio regular va acompañada de un daño oxidativo y nitrosativo en las diferentes fracciones sanguíneas, con la excepción de los neutrófilos donde se observa una disminución de los marcadores de daño oxidativo en lípidos y proteínas. Sin embargo, esta mayor capacidad antioxidante inducida por el entrenamiento regular protege a las diferentes fracciones sanguíneas frente a una producción aguda de RONS provocada por un ejercicio intenso. Las PBMCs son las células sanguíneas con mayor daño oxidativo tras un periodo de entrenamiento presentando signos de peroxidación lipídica y nitración de proteínas, probablemente atribuible a una alta producción de RONS en estas células. Esta peor adaptación estaría relacionada con que el entrenamiento regular provoca en las PBMCs un aumento del sistema antioxidante basado en la actividad glutatión peroxidasa, dependiente de la disponibilidad de glutatión, en detrimento del basado en la actividad catalasa, independiente de glutatión, favoreciéndose de esta manera la eliminación de bajas concentraciones de H_2O_2 frente a las altas.

La suplementación de la dieta con una bebida funcional de almendra rica en un ácido graso poliinsaturado omega-3, el docosahexaenoico (DHA), que proporciona una ingesta diaria adicional de 1.14 g diarios de DHA aumenta los niveles plasmáticos y eritrocitarios de este ácido graso. La suplementación con DHA,

durante un periodo de entrenamiento regular, proporciona respecto a los que no han suplementado su dieta, una mayor capacidad antioxidante y un menor daño nitrosativo tras un ejercicio agudo en eritrocitos, una menor tasa de producción de ROS en las PBMCs y una disminución del tiempo para generar especies reactivas de oxígeno durante la denominada explosión oxidativa en neutrófilos activados después del ejercicio.

LISTADO DE ARTÍCULOS ORIGINALES / LIST OF ORIGINAL PAPERS

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I. Cordova A, Martorell M, Sureda A, Tur JA, Pons A. **Plasma cytokine inflammatory markers in elite cyclists throughout a competition.**

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V. Capó X, Martorell M, Sureda A, Llompart I, Tur JA, Pons A. **Diet supplementation with DHA enriched food during training season in soccer players enhances the mitochondrial antioxidant capabilities in PBMCs.**

VI. Bescós R, Ferrer-Roca V, Galilea PA, Roig A, Drobnic F, Sureda A, Martorell M, Cordova A, Tur JA, Pons A (2012) **Sodium nitrate supplementation does not enhance performance of endurance athletes.** Medicine & Science in Sports & Exercise 44 (12):2400-9.

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INTRODUCCIÓN

ÁCIDOS GRASOS

Un ácido graso (AG) se define como una biomolécula formada por una cadena hidrocarbonada lineal, de diferente longitud o número de átomos de carbono, con un grupo carboxilo en un extremo. Los AGs se pueden clasificar según el número de insaturaciones presentes en la cadena hidrocarbonada en saturados (SFA, *saturated fatty acid),* monoinsaturados (MUFA, *monounsaturated fatty acid)* y poliinsaturados (PUFA, *poliunsaturated fatty acid).* Normalmente, el doble enlace de carbono de los MUFAs se encuentra entre el átomo de carbono 9 y 10, empezando a contar por el carbono del metilo terminal (omega-9). Estos omega-9 también son conocidos como derivados del ácido oleico (C18:1n9). Cuando existe más de un doble enlace en los AGs naturales las insaturaciones no se hallan nunca conjugadas (-CH=CH-CH=CH-) sino que se encuentran separadas por un grupo metileno (-CH=CH-CH_2-CH=CH-). Según la posición de cada doble enlace podemos diferenciar dos tipos de PUFAs, aquellos en los cuales el primer doble enlace se halla a 6 átomos de carbono del metilo terminal, doble enlace entre el carbono 6 y 7, los omega-6 (como el ácido linoleico, LA, C18:2n6, y derivados) y aquellos en los cuales el primer enlace doble se encuentra a 3 átomos de carbono del metilo terminal, doble enlace entre carbono 3 y 4, los omega-3 (como el ácido a-linolénico, ALA, C18:3n3, y derivados). En menor presencia están los omega-7, o derivados del ácido palmitoleico (C16:1n7).

En consecuencia, las características que diferencian los distintos AGs son el número de átomos de carbonos de la molécula, el número de dobles enlaces de la cadena hidrocarbonada y la posición y configuración de dichas insaturaciones. Se suelen clasificar como AGs de cadena corta cuando estos poseen menos de 8 átomos de carbono, de cadena media cuando poseen de 8 a 12 y de cadena larga cuando poseen más de 12. La mayoría de AGs de los alimentos son de cadena larga (entre 12 y 22 átomos de carbono), aunque hay alimentos como la leche o el aceite de coco que contienen AGs de cadena corta.

Los SFA pueden ser sintetizados por nuestro organismo y presentan algunas características que los hacen poco recomendables al sobrepasar una proporción determinada en la dieta. Los AG insaturados presentan características físicas, químicas y biológicas diferentes a los SFA. Los SFA cuando tienen menos de 10 átomos de carbono son líquidos a temperatura ambiente, pero al ir aumentando la longitud de la cadena su punto de fusión aumenta, de manera que a partir de 12 carbonos son sólidos insolubles en agua. Los lípidos ricos en SFA se llaman grasas, sólidos a temperatura ambiente, mientras que a los lípidos líquidos a temperatura ambiente se les llaman aceites.

En la dieta humana los lípidos aportan alrededor del 34% de la energía (Vidal, 2007). A los lípidos se les atribuyen multitud de funciones, entre ellas, una función de reserva energética, una función estructural al formar parte de la bicapa lipídica de las membranas, una función biocatalizadora, una función transportadora (como el transporte de vitaminas liposolubles) (Kazantzis and Stahl, 2012), funciones señalizadores (mensajeros intracelulares y extracelulares), algunos son hormonas (hormones esteroideas, por ejemplo) y otros son vitaminas (vitaminas A, D y E). Algunos lípidos son esenciales de manera que su presencia en el organismo depende del aporte dietético. Los lípidos, comparados con las grandes estructuras poliméricas de los hidratos de carbono, son pequeñas moléculas insolubles en agua y constituyen un grupo heterogéneo de compuestos.

Ácidos grasos esenciales

Los AG se pueden dividir en esenciales y no esenciales, entendiendo como nutrientes esenciales aquellos nutrientes necesarios para el desarrollo y función corporal que nuestro organismo no pueden sintetizar por lo que deben ser aportados por la dieta. Sin aporte externo a la dieta los mamíferos podemos sintetizar los AGs omega-9, podemos sintetizar el ácido oleico, introducir dobles enlaces en dirección al extremo carboxilo y alargar la cadena

hidrocarbonada. Las plantas pueden introducir dobles enlaces tanto en dirección al extremo metilo como al carboxilo (Belitz and Grosch, 1997). Para fabricar los AG de las series omega-6 y omega-3 necesitamos precursores provenientes de la dieta que ya posean el doble enlace en el carbono omega-6 y omega-3, respectivamente. Los AGs de cadena más larga y más insaturados (LCPUFAs, *long chain poiyunsaturated fatty acids)* son sintetizados mediante sucesivas desaturaciones y elongaciones. A partir del LA, omega-6 esencial, se forma el ácido y-linolénico (GLA, 18:3n6) y por alargamiento de la cadena el ácido araquidónico (AA, C20:4n6). A partir del ALA, omega-3 esencial, se pueden sintetizar ácidos eicosapentaenoico (EPA, C20:5n3) y docosahexanoico (DHA, C22:6n3). En la tabla 1 se recogen los principales AGs esenciales y no esenciales.

Las principales funciones de los PUFAs, tanto de esenciales como de no esenciales, se centran en dos áreas fundamentales para el organismo, el crecimiento y la capacidad funcional. Además, los PUFAs forman parte de todas las membranas celulares, condicionando la estructura de éstas y modulando la actividad de enzimas, receptores celulares, la transmisión de impulsos nerviosos, el comportamiento de los leucocitos, la actividad de trombocitos... El control y regulación de la composición de los fosfolípidos de membrana aún no está del todo comprendido (Tepsic, et al., 2009), sin embargo se sabe que el perfil de los AGs de la dieta influencia las funciones de la membrana (Baylin and Campos, 2006, Egert, et al., 2012, McMurchie, et al., 1984). Al mismo tiempo, los PUFAs tienen un papel importante en las interacciones entre lípidos y proteínas en las sinapsis de las membranas celulares, afectando a la conformación del receptor, a los canales iónicos, enzimas y a los movimientos dentro y fuera de la célula (Salem, et al., 1989, Salem, et al., 1988).

Los AGs omega-3 y omega-6 de cadena larga son precursoras, mediante procesos de oxidación, de eicosanoides, como las prostaglandinas y los leucotrienos, que participan en eventos de inflamación y en la respuesta inmune. Los eicosanoides modulan reacciones secretoras del músculo liso (contracción y relajación) y están involucrados con los sistemas cardiovascular, renal y pulmonar

y en la actividad protectora de las células sanguíneas (plaquetas, monocitos, macrófagos y neutrófilos).

Los omega-3 absorbidos de la dieta pueden tener diferentes dianas como su conversión a otros omega-3, su incorporación a las membranas como fosfolípidos, su oxidación o su almacenamiento (Browning, et al., 2012). Están implicados en la maduración y crecimiento cerebral y retiniano, intervienen en procesos de inflamación, coagulación, presión arterial, reproducción, metabolismo de lípidos e hidratos de carbono (Weber, 1999). Las membranas neuronales son ricas en DHA y AA, los cuales constituyen un 25% del contenido en fosfolípidos (Mahadik, et al., 1989). Por tanto, aunque el DHA sea un componente menor en los lípidos plasmáticos, está presente en todos los órganos y es el mayor componente de los fosfolípidos del cerebro y retina (Neuringer, et al., 1988). El ALA se encuentra en cloroplastos de las hojas verdes y en algunos vegetales (aceite de linaza, aceite de soja, nueces, etc.). El EPA y el DHA se encuentran en el pescado y en el aceite de pescado, principalmente en el pescado azul (Lee, et al., 1986).

Los omega-6 tienen funciones parecidas a los omega-3, aunque parece que tienen un efecto potenciador de los procesos inflamatorios y de la arteriosclerosis cuando la dieta es rica en éstos. Las plantas son la principal fuente de omega-6, muchas semillas (girasol, maíz, soja y algodón) son ricas en LA.

Tabla 1. Nomenclatura de ácidos grasos esenciales i no esenciales

	Nombre común y nombre sistemático	Abreviatura	Fórmula
Familia omega-3	Ácido a-linolénico Cis-9,12,15-octadecatrienoico (ALA)	18:3n3	C18H30O2
	Ácido estearidónico Cis-6,9,12,15-octadecatetraenoico (SDA)	18:4n3	C18H28O2
	Ácido timnodónico Cis-5,8,11,14,17-eicosapentaenoico (EPA)	20:5n3	C20H30O2
	Ácido clupanodónico Cis-7,10,13,16,19-docosapentaenoico (DPA)	22:5n3	C22H34O2
	Ácido cervónico Cis-4,7,10,13,16,19-docosahexaenoico (DHA)	22:6n3	C22H32O2
Familia omega-6	Ácido linoleico Cis-9,12-octadecadienoico (LA)	18:2n6	C18H32O2
	Ácido y-linolénico Cis-6,9,12-octadecatrienoico (GLA)	18:3n6	C18H30O2
	Ácido dihomo-y-linolénico Cis-8,11,14-eicosatrienoico (DGLA)	20:3n6	C20H34O2
	Ácido araquidónico Cis-5,8,11,14-eicosatetraenoico (AA)	20:4n6	C20H32O2
	Ácido adrénico Cis-7,10,13,16-docosatetraenoico (DTA)	22:4n6	C22H36O2
	Ácido osmond Cis-4,7,10,13,16-docosapentaenoico (DPA)	22:5n6	C22H34O2
Familia omega-9	Ácido oleico Cis-9-octadecenoico	18:1n9	C18H34O2
	Ácido eicosenoico Cis-11-eicosenoico	20:1n9	C20H38O2
	Ácido eicosatrienoico Cis-5,8,11-eicosatrienoico	20:3n9	C20H34O2
	Ácido erúcico Cis-1-docosenoico	22:1n9	C22H42O2
	Ácido nervónico Cis-15-tetracosenoico	24:1n9	C24H46O2

Precursores de los omega-6 y los omega-3

Los miembros más simples de los AG omega-6 y omega-3 son el LA y el ALA, respectivamente (Das, 2006). Aunque estos dos AGs sean esenciales para nuestra supervivencia y la del resto de mamíferos carecemos de las enzimas A12- y A15-desaturasas, las cuales son responsables de la inserción de dobles enlaces entre los carbonos 6-7 y 3-4, empezando por el metilo terminal. Estas enzimas sí se expresan en plantas, pero al tener tejidos pobres en grasa no son una fuente cuantitativamente importante de AGs esenciales. En cambio, los aceites y margarinas vegetales, al ser extractos lipofílicos, nos aportan cantidades importantes de LA y ALA.

Metabolismo de los omega-6 y los omega-3

Los ácidos grasos omega-3 y omega-6 después de ser asimilados a partir de la dieta son transportados en el plasma sanguíneo de manera esterificada (como componentes de lipoproteínas) o no esterificada (en forma libre o unidos a proteína) (Fielding, 2011). El LA (18:2n6) y el ALA (18:3n3) son metabolizados en el hígado a través de vías de desaturación y elongación dando lugar a los diferentes ácidos grasos correspondientes de las familias omega-6 y omega-3, respectivamente. La enzima A6-desaturasa convierte el LA a GLA insertando un doble enlace, y posteriormente éste es elongado dos unidades de carbono, mediante una elongasa, a ácido dihomo-y-linolénico (DGLA, C20:3n6), el cual a su vez es el sustrato de la enzima A5-desaturasa dando como producto el ácido araquidónico (AA, C20:4n6). Estos mismos enzimas que intervienen en el metabolismo de los omega-6 también pueden metabolizar el ALA, dando lugar a toda la familia de omega-3. Siguiendo la misma ruta enzimática, ALA se convierte en ácido estearidónico (SDA, C18:4n3), éste en ácido eicosatetraenoico (ETA, C20:4n3) y éste en EPA. El EPA se puede metabolizar a DHA en cuatro etapas. Primero el EPA se elonga con dos átomos de carbono para dar lugar al ácido docosapentaenoico (DPA, C22:5n3). Después actúa otra elongasa para añadir otros dos átomos de carbono y obtener el ácido tetracosapentaenoico (TPA,

C24:5n3), sobre el cuál actúa la A6-desaturasa dando lugar al ácido tetracosahexaenoico (THA, C24:6n3) el cual finalmente pierde dos átomos de carbono mediante B-oxidación originando el DHA.

En la figura 1 se observa que el AA se puede metabolizar de forma paralela por las mismas enzimas, produciendo los ácidos docosatetraenoico (DTA, C22:4n6), tetracosatetraenoico (TTA, C24:4n6), tetracosapentaenoico (TPA, C24:5n6) y docosapentaenoico (DPA, C22:5n6), lo que implica que LA y ALA compiten constantemente por las mismas enzimas (Arterburn, et al., 2006, Das, 2006).

Aunque la conversión de LA y ALA a AA, EPA y DHA sea posible, en realidad es un proceso poco eficiente, sobretodo en personas adultas, de manera que lo más habitual es incorporar estos AGs esencia a través de la dieta (Neff, et al., 2011, Swanson, et al., 2012).

Se estima que los adultos consumen entre 50 y 300 mg al día de AA. Este omega-6 se encuentra principalmente en aceites de semillas (soja, girasol, maíz, etc.) y también junto con proteína animal, fundamentalmente carnes rojas y vísceras (Sears, 2002). Los omega-3, como el ALA, lo obtenemos de plantas silvestres, caracoles, nueces, higos, huevos y aceite de oliva (Arterburn, et al., 2006, Villegas, 2007). Por otra parte, la principal fuente de omega-3, EPA y DHA, es el pescado. El pescado magro, como el bacalao, acumula la grasa en el hígado en forma de triglicéridos y el pescado graso, como el atún, el salmón y la sardina, acumula los triglicéridos en su carne. El aceite de pescado tiene un alto contenido en EPA y DHA, y los aceites extraídos de distintos tipos de pescado contienen diferente perfil de omega-3 debido a sus hábitos alimentarios y características metabólicas de cada pez. Principalmente, los peces obtienen los PUFAs omega-3 de las plantas marinas como las algas, las cuales sí poseen las enzimas necesarias para producir omega-3 que no tenemos los mamíferos.

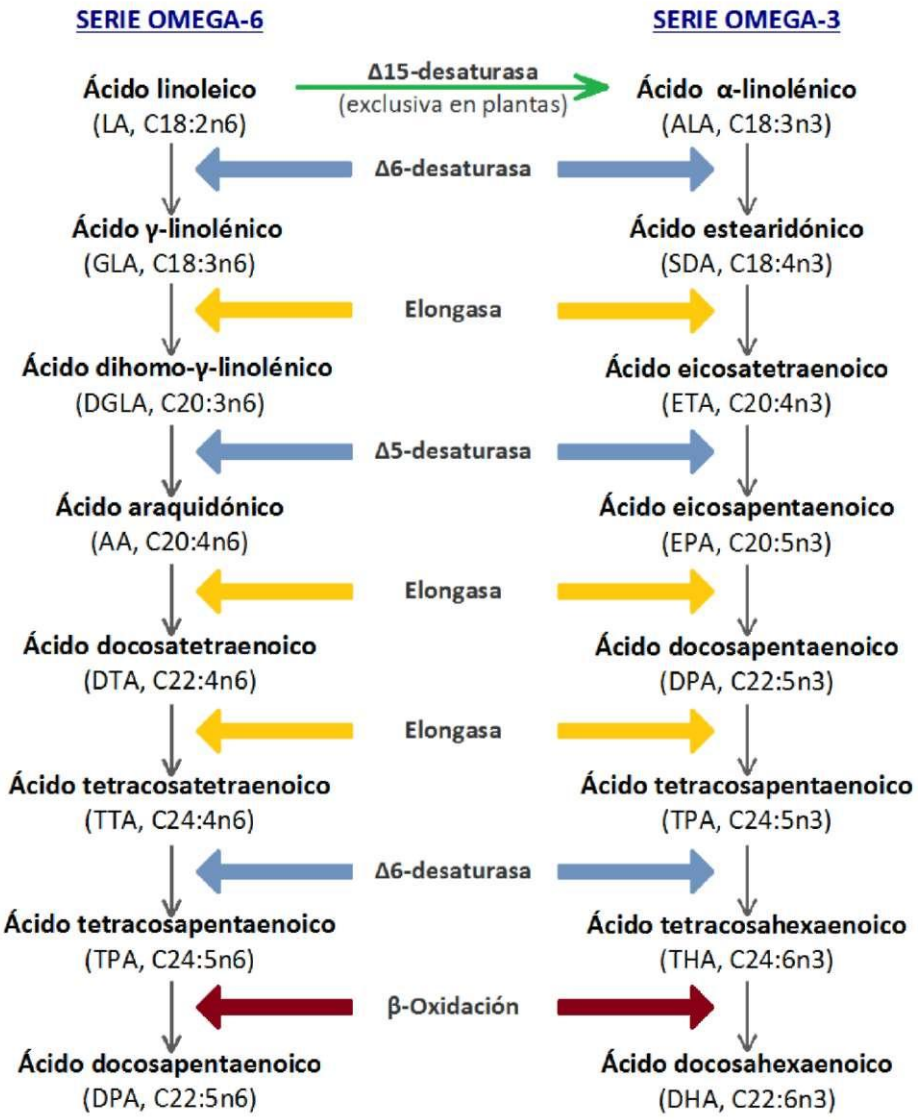

Figura 1. Metabolismo de los ácidos grasos omega-6 y omega-3.

Balance omega-6 i omega-3

El balance entre los omega-6 y los omega-3 es importante ya que compiten por las mismas enzimas. El balance altera el perfil de los eicosanoides, afectando de esta manera algunas funciones como la agregación plaquetaria, la actividad trombogénica, el tono vascular y procesos antiinflamatorios, antiinfecciosos e inmunoprotectores. Además, DHA, EPA y ALA compiten con AA para la posición sn-2 de los fosfolípidos de membrana (Arterburn, et al., 2006). Las fosfolipasas A2, enzimas con actividad hidrolasa y lipasa, son responsables de la movilización de los PUFAs liberados de la posición sn-2 de los fosfolípidos, de manera que la acción de esta enzima puede inducir cambios en la composición de las membranas, activar cascadas inflamatorias y generar vías de señalamiento celular (transducción de señales) (Alfonso and García, 2009).

El principal problema entre los omega-6 y los omega-3 es el desequilibrio dietético, a favor de los omega-6, que se da en muchas zonas del planeta. Algunas poblaciones tienen un equilibrio entre estos dos tipos de AGs, como la dieta cretense (auténtica dieta mediterránea) que tiene una relación omega-6/omega-3 de 1.5:1 o como la dieta japonesa que la tiene de 4:1. En contra posición, en Estados unidos existe una relación omega-6/omega-3 de 16:1 y en el Reino Unido y Europa del norte una relación de 15:1 (Villegas, 2007).

La obtención de los diferentes PUFAs a través de la dieta puede variar aunque dos individuos se alimenten de la misma manera, ya que la relación omega-6/omega-3 de muchos alimentos provenientes de animales puede variar según la diferente ingesta de pasto o pienso. Por ejemplo, la carne de vacas alimentadas con pastos tiene una razón omega-6/omega-3 de 2.5:1 y las alimentadas con grano de 20:1 (Villegas, 2007).

Un consumo mayor de omega-6 respecto a los omega-3 puede disparar la producción de prostaglandinas y eicosanoides de carácter proinflamatorio, aumentando el riesgo de padecer enfermedades inflamatorias crónicas. De tal manera, se les llama eicosanoides "malos" a los derivados omega-6 del AA, y eicosanoides "buenos" a

los derivados de omega-3, como el EPA que frena la producción de AA (Sears, 2002). Para este autor, los eicosanoides derivados de los omega-3 causan dilatación de los vasos sanguíneos, reducen el dolor y la proliferación celular anormal, mejoran el sistema inmune y la función cerebral. En cambio, los eicosanoides derivados de los omega-6 tienen los efectos contrarios como coágulos, constricción, aumento de dolor, entre otros. Por tanto, se incentiva una dieta rica más en omega-3 y que disminuya la presencia de omega-6, para llegar a un equilibrio de estas familias de AGs propio de la alimentación originaria de nuestra especie, más ligada al mar y en la cual se consumían más nutrientes naturales ricos omega-3.

Los AGs de la dieta se incorporan en muchas partes del cuerpo incluyendo las membranas celulares (Lazzarin, et al., 2009). La composición lipídica de las distintas células presenta un patrón característico y suele sufrir pocas variaciones (Leidl, et al., 2008). El DHA es el omega-3 más abundante en las membranas y está presente en todos los órganos, y de manera abundante en el cerebro y retina. Por la parte de los omega-6, el AA también es abundante en la mayoría de los tejidos pero con una distribución diferente a la de DHA (Figura 2).

Una suplementación con omega-3, combinando DHA y EPA, reducirá por competición la concentración de AA (los AA de los fosfolípidos del plasma, por ejemplo) y evitará los efectos perjudiciales de un exceso de este omega-6 (Arterburn, et al., 2006).

Para tener una relación omega-6/omega-3 adecuada, se ha propuesto incluso regresar a una dieta Paleolítica (Sears, 2002) o solución Paleolítica (Villegas, 2007), no obstante se han propuesto soluciones más viables como incorporar los omega-3 a los alimentos, aumentar el consumo de alimentos ricos en omega-3 y evitar el consumo de grasas saturadas y grasas trans, además de ingerir proporciones equilibradas del resto de nutrientes (Esteve, 2012) y favorecer una dieta Mediterránea (Estruch, et al., 2013, García-Calzón, et al., 2013, Martínez-Lapiscina, et al., 2013, Martínez-Lapiscina, et al., 2013, Medina-Remón, et al., 2012) .

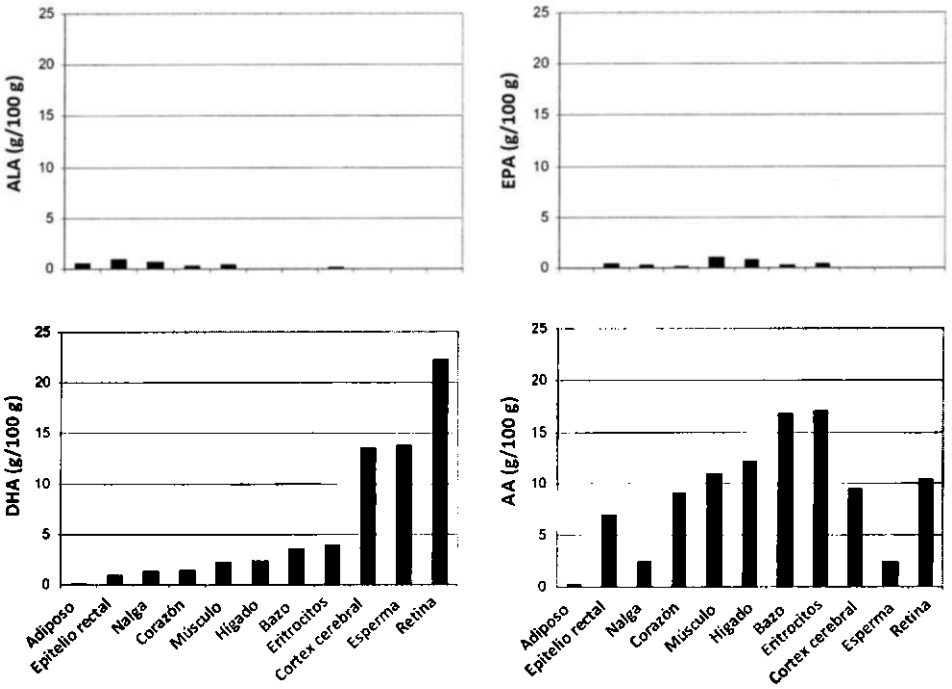

Figura 2. Concentraciones de ácidos grasos (g/100 g de ácidos grasos totales) en los tejidos de los adultos de los Estados Unidos, Canadá, Australia, o Europa. Adaptado de Arterburn et al. (2006).

Necesidades omega-3

No existe un consenso sobre las necesidades nutricionales de omega-3 en humanos ni si un elevado consumo proporciona efectos beneficiosos para la salud. Los estudios de suplementación de la dieta con ácidos grasos esenciales son escasos aunque están en aumento, con resultados muchas veces dispares. El establecimiento del estado nutricional basal en relación con las demandas de ácidos grasos esenciales y su respuesta a la actividad física es imprescindible para poder estudiar los efectos de la suplementación de la dieta con PUFAs sobre sus implicaciones funcionales y sobre el estrés oxidativo.

La determinación de las concentraciones de varios AGs esenciales omega-3 en plasma, suero o membranas eritrocitarias se ha utilizado como marcadores útiles de la necesidad de los AGs esenciales. Las membranas eritrocitarias son un buen indicador de una suplementación con omega-3 ya que reflejan su incorporación en otros tipos celulares (Giannl, et al., 2012, Shohet and Nathan, 1970), aunque también se ha evidenciado su incorporación a las células cerebrales (Babin, et al., 1993) o en las células periféricas mononucleares (Toft, et al., 2000). Sin embargo no se ha establecido ningún criterio que evidencie la cobertura de las necesidades de omega-3 a partir de la composición lipídica de las membranas celulares. Un marcador que se ha utilizado ampliamente para la detección de deficiencias nutricionales de los AGs esenciales es el ácido meádico (ácido 5,8,11-eicosatrienoico, 20:3n9), un ácido graso omega-9 que se sintetiza a partir de oleico en condiciones de déficit de LA omega-3 con el que compite para su elongación y desaturación (Goodgame, et al., 1978, Siguel, et al., 1987). También se ha utilizado la relación plasmática entre los AG trienoicos y tetranoicos (entre 0.2 y 0.4) (Goodgame, et al., 1978). La utilización de cuestionarios de frecuencia de consumo de alimentos han permitido establecer las ingestas diarias de AGs esenciales en diferentes grupos de población (Martínez-González, et al., 2012, Milá-Villarroel, et al., 2011, Ritter¬ Gooder, et al., 2006, Salas, et al., 2013, Sanz París, et al., 2012). Hay estudios que reflejan poblaciones con un déficit en el consumo de AGs omega-3 (McNamara, 2010, McNamara, et al., 2013, Neuringer, et al., 1984), aunque existen controversias en relación a las necesidades diarias de los AG esenciales. Un metaanálisis sobre la ingesta de omega-3 durante un periodo mínimo de 6 meses realizado a partir de 48 ensayos controlados aleatorios (36,913 participantes) y 41 estudios de cohorte concluye que no se puede documentar objetivamente un efecto beneficioso del consumo de omega-3 sobre la mortalidad relacionada con eventos cardiovasculares o cáncer (Hooper, et al., 2006). Aun así, los omega-3 LCPUFAs, incluyendo el DHA, son AGs consumibles con un amplio abanico de beneficios saludables (Su, et al., 2008, Swanson, et al., 2012, Tur, et al., 2012). Hay estudios de suplementación con ácidos grasos insaturados que incrementan la ingesta con dosis muy altas (Bloomer, et al., 2009,

Egert, et al., 2012, Mehra, et al., 2006, Peoples, et al., 2008, Toft, et al., 2000) mientras que en otros las dosis que se suplementan son moderadas (Barceló-Coblijn, et al., 2008, Din, et al., 2008), habiéndose observado efectos funcionales de estos suplementos en muchos de estos estudios (Swanson, et al., 2012, Tur, et al., 2012). En estos momentos no se han observado efectos tóxicos de la suplementación de la dieta con AGs insaturados (Tur, et al., 2012), pero se ha apuntado que debido a la mayor posibilidad de oxidación de los ácidos grasos insaturados se pueden producir problemas de oxidación de las membranas celulares y de hemólisis, aunque los omega-3 parecen preservar la integridad de la membrana (Mabile, et al., 2001). Se sugiere que la ingesta de AGs esenciales presente una relación omega-6/omega-3 de 1/50, siendo mayoritario el DHA aunque también deben estar presentes el ALA y el EPA.

Alimentos funcionales enriquecidos con ácidos grasos esenciales

La necesidad de aumentar el consumo de AGs omega-3 en relación con los AGs saturados y los omega-6 ha propiciado el diseño de un número elevado de alimentos funcionales enriquecidos en este tipo de AGs. En el mercado podemos encontrar una amplia variedad de estos productos, como leches, yogures, flanes, leches en polvo para recién nacidos y lactantes, mayonesas, bebidas de soja, zumos, huevos, aceites, margarinas, mantequillas de cacahuete, galletas, panes, aceites, aceitunas, etc.

Los frutos secos son un buen alimento para proveer de AGs esenciales y permiten ser una buena base para el diseño de alimentos funcionales que aporten las cantidades adecuadas de AGs esenciales. La relación entre el consumo de frutos secos y su acción sobre el perfil lípido está bien establecida, tanto por el tipo de AGs que contiene, como por los antioxidantes y fitoesteroles que posee (Sabaté, et al., 2003). La almendra, junto con las nueces, es uno de los frutos secos que presenta mayor contenido de AGs esenciales. Se ha utilizado la almendra como base para la obtención de una bebida que se enriquece con ácidos grasos esenciales, básicamente con DHA de origen marino. Una de las principales limitaciones del enriquecimiento de alimentos con ácidos grasos esenciales es el sabor

característico que presentan los ácidos grasos que pueden producir rechazo del alimento funcional. Normalmente no se pueden enriquecer alimentos con omega-3 a valores superiores de 0,1-0,2% de ácidos grasos insaturados procedentes de fuentes marinas. En el procedimiento de elaboración de la bebida se evitan los procesos de peroxidación de los PUFAS, además de evitar la pérdida de vitamina E (de la cual la almendra contiene niveles altos). La bebida se diseña como isotónica y contiene una cantidad importante de carbohidratos y proteínas (especialmente ricas en arginina) y un contenido graso que facilita la asimilación de la vitamina E.

Omega-3 y expresión de genes

Los omega-3 influyen una mejora en el perfil lipídico, específicamente en las fracciones de triglicéridos y colesterol circulantes, y un incremento de la fluidez de las membranas (Connor, 2000). Estos efectos son similares a los que se atribuyen a la práctica regular de ejercicio aeróbico (Brilla and Landerholm, 1990). El efecto sobre la oxidación de las grasas inducida por los omega-3 se puede relacionar con la activación de los receptores nucleares PPAR-a (receptores activados de proliferación de los peroxisomas) del músculo esquelético por acción de EPA y DHA, dando lugar a una disminución de la acumulación de triglicéridos en los miotúbulos (Huffman, et al., 2004). Por otra parte, los PUFA no solo afectan a la expresión de genes relacionados con la síntesis de triglicéridos y con su oxidación, sino que también provocan cambios en la composición de las membranas, en los niveles intracelulares de calcio (Billman, 2013) y en la producción de eicosanoides (Duda, 2012).

Los PUFA y sus metabolitos pueden actuar a nivel nuclear afectando la transcripción de una variedad de genes en conjunción con factores de transcripción y receptores nucleares. Algunos de estos factores son el PPAR, el HNF4A (factor nuclear 4 alfa de hepatocito), el LXR (receptor X hepático), el SREBP (proteína de unión al elemento de respuesta al esterol) y el NFKB (factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas). Además, algunos de estos factores están relacionados con la expresión de otros genes no necesariamente relacionados con el metabolismo lipídico. Un ejemplo

es el caso del factor nuclear NFK0, el cual está relacionado con la expresión de genes antioxidantes y de la síntesis del óxido nítrico (Ji, et al., 2007). Al mismo tiempo el factor PPAR está relacionado con una disminución del estrés oxidativo. Las vías activadas por las PPARs reducen el estrés oxidativo mediante la expresión de la proteína desacopladora mitocondrial (UCP) (reduciendo la producción de especies reactivas de oxígeno), optimizando la actividad FOXO (subgrupo de la familia Forkhead) mediante una mejora de la sensibilidad a la insulina y suprimiendo NFK0 a nivel transcripcional. Por tanto, los PUFA pueden modular la acción de los PPAR y de NFK0, interaccionando así sobre la expresión de genes relacionados con el metabolismo lipídico y el estrés oxidativo. En conclusión, mediante la vía de PPAR como de NFK0 es posible poner de manifiesto que la suplementación de la dieta con PUFAs puede provocar cambios importantes en la expresión y actividades de enzimas antioxidantes, afectando el equilibrio oxidativo celular.

EJERCICIO FÍSICO Y ÁCIDOS GRASOS

Hay que diferenciar los conceptos de actividad física, ejercicio y deporte. Se define como actividad física cualquier movimiento corporal producido por los músculos esqueléticos que consuma energía. Ejercicio se denomina a una actividad física que al ser planificada, estructurada y repetida mantiene o mejora las funciones del organismo. El deporte es un ejercicio físico que se realiza bajo unas reglas y de forma competitiva.

La práctica regular de una actividad física es beneficiosa para la salud i el bienestar de las personas de todas las edades, casi todos los individuos pueden beneficiarse de la actividad física regular (Prasad and Das, 2009). En roedores se ha demostrado la práctica usual de ejercicio físico aumenta la vida media y la vida máxima (Domenéch, 2007). Además, reduce el riesgo de sufrir enfermedades coronarias, diabetes mellitus tipo 2, obesidad, hipertensión arterial (Domenéch, 2007), osteoporosis (Bérard, et al., 1997) y distintos tipos

de cáncer (Friedenreich, et al., 1998, Hoffman-Goetz, 1998, McTiernan, et al., 1999). En contraposición, las actividades físicas de intensidad elevada se asocian a un mayor riesgo cardiovascular, de lesión músculo-esquelética y de estrés oxidativo (Fisher-Wellman and Bloomer, 2009, Knez, et al., 2007, Nikolaidis, et al., 2012).

Los AGs plasmáticos provenientes de la lipólisis de los triglicéridos almacenados en el músculo son un combustible importante para las células musculares durante la realización del ejercicio físico (Jeppesen and Kiens, 2012). Los AG cumplen funciones esenciales durante el ejercicio físico y es necesario consumir grasas para asegurar el aporte de AGs esenciales y vitaminas liposolubles. El metabolismo lipídico está condicionado por varios factores (Culebras, 2012, Esteve, 2012):

- Tipo, duración e intensidad del ejercicio. La entrada de AGs de cadena larga en la mitocondria se inhibe al realizar un ejercicio de alta intensidad y, a la vez, inhibe la actividad de la acetil-CoA carboxilasa durante el ejercicio.

- Reservas de glucógeno. A menor reserva de glucógeno mayor es la oxidación de los AG.

- Estado físico del individuo. Los atletas entrenados mediante trabajos de larga duración y alta intensidad tienen la capacidad de oxidar más AG durante el ejercicio, haciéndose más notorio a intensidades de trabajo muy elevadas. Parece ser que el entrenamiento produce una mayor oxidación de las grasas, debido a una mayor facilitación de la entrada de los AGs a la mitocondria, entre otros (González-Gross, et al., 2001).

- Composición de la dieta ingerida los días previos a la realización del ejercicio. Los AGs son una fuente importante de energía para la contracción del músculo esquelético, particularmente en ejercicios de intensidad leve o moderada, de larga duración, i en estado de ayuno. Los AGs libres plasmáticos transportados desde el tejido adiposo de reserva y los triglicéridos contenidos dentro de las fibras musculares

esqueléticas son las principales fuentes de estos AGs. La contribución relativa de cada fuente de AGs depende del tipo, la intensidad y la duración del ejercicio y del estado de entrenamiento. La oxidación de los AGs libres plasmáticos está directamente relacionada con la tasa de lipolisis del tejido adiposo (Martin, 1996). Una dieta alta en grasas incrementa la oxidación de éstas (Schrauwen, et al., 2000).

En la realización de un ejercicio moderado el tejido adiposo derivado de los AGs no esterificados constituyen la mayor fuente de combustible del músculo, y la importancia de estos AGs incrementa cuando el ejercicio es prolongado (Frayn, 2010). Por otra parte, la realización de un ejercicio muy intenso, condición donde los carbohidratos son la principal fuente de energía, provoca un alto incremento de catecolaminas en plasma que puede provocar una inhibición de la lipolisis del tejido adiposo implicando mecanismos a-adrenérgicos (Frayn, 2010).

Relación omega-3 y rendimiento deportivo

La realización de actividad física modifica el metabolismo de los PUFA (Benatti, et al., 2004) e induce cambios en la composición de los AG de los fosfolípidos de las membranas musculares (Helge, et al., 2001). Además, la actividad física parece disminuir la relación relativa de los omega-3/omega-6 (Helge, et al., 2001). Siendo esto así, se considera una hipótesis aceptable que los deportistas entrenados a un alto nivel de rendimiento podrían necesitar ingestas superiores de omega-3 para satisfacer sus necesidades. La suplementación con 4 g diarios de aceite de pescado durante 10 semanas aumenta, respecto a los controles, los umbrales anaeróbicos ventilatorios de sujetos sedentarios sometidos a un plan de entrenamiento aeróbico (Brilla and Landerholm, 1990). La mejora de los umbrales ventilatorios se puede explicar por la incorporación de los omega-3 en las membranas de los eritrocitos, aumentado su capacidad de deformación y viscosidad (Conquer, et al., 2000, Smith, et al., 2011) y su transporte de oxígeno. Por otro lado, con una suplementación mayor, 5.2 g

diarios, y de misma duración no se ha observado esta mejora sobre los umbrales ventilatorios en sujetos deportistas bien entrenados, pero sí que se han observado disminuciones de los triglicéridos plasmáticos e incrementos de los ácidos omega-3 (EPA y DHA) (Raastad, et al., 1997).

Otros estudios evidenciaron que la suplementación de la dieta con aceite de pescado mejora significativamente la resistencia a la fatiga (Segura, 1988), tanto en animales de experimentación (Escrich, et al., 1994, Escrich, et al., 2001) como en humanos (García-Closas, et al., 1993, Javierre, et al., 2005, Navarro, et al., 1988, Navarro and Segura, 1988). Además, se observó que con este tipo de suplementación los individuos participantes producían menor cantidad de ácido láctico realizando el mismo volumen de trabajo, lo que hace pensar que la suplementación con omega-3 mejora la capacidad oxidativa de los músculos y estos usan mayor cantidad de AGs. En ratas se ha evidenciado que el efecto del consumo de aceites de pescado sobre el daño oxidativo en proteínas y actividades antioxidantes depende de las variaciones del contenido en EPA/DHA (Méndez, et al., 2012).

Mecanismos de los omega-3 en los procesos relacionados con el ejercicio físico

Los omega-3 pueden participar en diferentes procesos relacionados con la actividad del músculo esquelético e influir sobre su capacidad de trabajo y resistencia, tanto en individuos que practican regularmente alguna especialidad deportiva, como en los individuos que no realizan un ejercicio asiduo.

Modificación de la movilización de los AGs

La triacilglicerol lipasa es una enzima tisular dependiente de hormonas la cual condiciona la cantidad de AGs liberados por unidad de tiempo a partir de los triglicéridos de reserva. Estudios realizados por Awad y colaboradores (Awad, 1986, Awad and Chattopadhyay, 1986, Awad and Chattopadhyay, 1986, Awad and Zepp, 1979) indican que la ingesta de PUFAs produce una respuesta lipolítica más intensa

(cuando ésta es inducida por estimulación con noradrenalina) en comparación a una ingesta rica en SFA. La triacilglicerol lipasa forma parte de un complejo lipoprotéico en el cual los fosfolípidos representan aproximadamente el 50% de la totalidad de sus componentes. Por tanto, variaciones en la composición de los AG de los fosfolípidos pueden variar la actividad de la lipasa del adipocito. Esta hipótesis fue refutada por Awad y colaboradores (1979 y 1986) comprobando que la actividad específica de dicho sistema enzimático era mayor con una alimentación con aceite de cártamo (rica en PUFAs) frente a la alimentación con grasa de origen animal.

Modificación de la actividad carnitina-aciltransferasa

Para que la fibra muscular obtenga energía de los AGs es necesario que estos entren en la matriz mitocondrial. Para pasar del citoplasma al interior de la mitocondria los AG tienen que sufrir una activación (acilación) y posterior translocación.

La carnitina-aciltransferasa I transfiere los grupos acilos (RCO-) a una molécula de carnitina, que facilita la difusión a través de la membrana mitocondrial utilizando la translocasaacil-carnitina/carnitina. En la matriz mitocondrial, la carnitina-aciltransferasa II, transfiere nuevamente el grupo acilo a una molécula de CoA-SH y se separa de la carnitina.

Diferentes estudios han puesto de manifiesto que la actividad de este sistema enzimático, encargado de transportar los AGs al interior de las mitocondrias para su posterior B-oxidación, es mayor en animales (ratas) alimentados con aceite de pescado que en aquéllos con una dieta convencional (Demoz, et al., 1994, Fr0yland, et al., 1996, Jacques, et al., 1995, Madsen, et al., 1999).

Favoreciendo la actividad oxidativa e induciendo el aumento de las mitocondrias

El entrenamiento favorece el aumento del tamaño y el número de mitocondrias presentes en la célula muscular, aumentando la capacidad oxidativa para poder obtener mayor cantidad de adenosina trifosfato (ATP) por unidad de tiempo a partir de los AGs (Vidal, 2007).

Los omega-3 inducen la formación de mitocondrias de mayor tamaño y con mayor capacidad oxidativa (Christiansen, et al., 1981).

Reduciendo el proceso de inflamación seguido del ejercicio físico

La suplementación con aceite de pescado modifica la proporción de los PUFAs en el plasma y en las membranas celulares, con un incremento de DHA, lo cual podría dar lugar a una respuesta inflamatoria más moderada y discreta, en relación a la que se produce con una dieta rica en omega-6 (precursores del ácido araquidónico) (Srivastava, 1985). Parece ser que la frecuencia de los impulsos nerviosos, de carácter nociceptivo, que pasarían el filtro del asta posterior de la médula espinal sería menor en una dieta rica en omega-3. Además, esto conllevaría una menor actividad de los sistemas centrales de control del dolor, lo que permitiría realizar un ejercicio durante más tiempo y se retardaría la aparición de la fatiga. Se sugiere que una ingesta de aproximadamente 1-2 g/día de EPA y DHA (relación 2:1), puede ser beneficiosa contra la inflamación inducida por el ejercicio y para la salud en general del atleta (Mickleborough, 2013).

Favoreciendo el flujo del ácido láctico

El ácido láctico es generado y consumido en diferentes situaciones metabólicas y por distintos tipos celulares. La movilización del ácido láctico y del anión lactato entre los distintos órganos y tejidos depende del flujo entre los compartimientos intra- y extracelular. El flujo depende de diferentes factores, como la superficie de intercambio, la velocidad de difusión entre tejidos, el gradiente de concentración de lactato y de los protones a través de la membrana celular, la permeabilidad de la membrana a los mencionados iones, etc. (Roth, 1991). Por tanto, una dieta rica en omega-3 podría modificar las características del sarcolema, facilitando el flujo de lactato y protones desde la célula muscular al espacio intersticial, y acelerando el proceso de recuperación muscular (Esteve, 2012).

EJERCICIO FÍSICO Y ÓXIDO NÍTRICO

El óxido nítrico o monóxido de nitrógeno (NO) es una pequeña especie radicalaria gaseosa inicialmente caracterizada como factor relajante derivado del endotelio (EDRF). El ejercicio físico puede estimular la síntesis de NO de las células endoteliales por dos mecanismos, uno químico y uno físico (Bescós, 2011).

El estímulo químico se origina mediante la interacción de los agonistas endógenos y exógenos con receptores específicos presentes en las células endoteliales, como acetilcolina (neurotransmisor), trifosfato de adenosina (ATP) y bradiquinina (péptido vasodilatador). El ejercicio físico estimula la liberación de éstas moléculas, por ejemplo, la unión neuromuscular de los nervios motores, que sintetizan, almacenan y liberan acetilcolina, es una fuente fisiológica de estos compuestos durante el ejercicio (Campbell, et al., 2006). Además, los glóbulos rojos pueden liberar ATP (Campbell, et al., 2006) y la actividad muscular aumenta las concentraciones intersticiales de bradiquinina durante el ejercicio (Buford and Koch, 2004).

La estimulación física se lleva a cabo por la presión que realiza la sangre sobre las paredes de la arteria, llamada tensión de cizallamiento. El mecanismo por el cual la tensión de cizallamiento favorece la formación de NO todavía no está claro. Las células endoteliales tienen mecanoreceptores, los cuales pueden activar directamente proteínas G, canales iónicos y enzimas proteína quinasa y fosfatasa, promoviendo la formación de segundos mensajeros como el guanosín monofosfato cíclico (GMPc). Estudios en seres humanos y animales han demostrado que la tensión de cizallamiento producida por el ejercicio físico es un estimulante de la liberación de factores vasorelajantes producidos por el endotelio vascular, como el NO, y aumenta la expresión de la óxido nítrico sintasa (NOS) endotelial y neuronal (Stevens, et al., 2000). Por esta razón se asocia a los efectos beneficiosos del ejercicio regular sobre las enfermedades cardiovasculares una mayor producción de agentes vasodilatadores derivados del endotelio.

La magnitud del beneficio del ejercicio físico depende de la intensidad o del volumen trabajo. Si bien el entrenamiento a corto plazo aumenta rápidamente la bioactividad de NO, si éste se mantiene, hay una adaptación mediante cambios estructurales NO-dependientes que conducen a la remodelación arterial para una normalización de la tensión de cizallamiento (Colombani, et al., 1999). Se tiene que realizar un ejercicio de intensidad moderada-alta para afectar a la función endotelial en sujetos asintomáticos sanos (Abel, et al., 2005). Estas evidencias plantean la posibilidad de un que exista un umbral, de volumen e intensidad de trabajo, para el entrenamiento físico y los mecanismos asociados a la producción de NO.

Suplementos deportivos relacionados con el óxido nítrico

Para la suplementación deportiva se ha sugerido que los compuestos dietéticos relacionados con NO, como la L-arginina y el nitrato inorgánico, son ayudas ergogénicas. Para ello existe la base de los efectos potenciales sobre la regulación del flujo sanguíneo y sobre la respiración mitocondrial del NO durante el ejercicio físico (Carter, et al., 2000, Lundberg, et al., 1994). Se sugiere que el aumento de flujo sanguíneo provocado por la ingesta de donantes de NO, como L-arginina y nitrato, puede mejorar el suministro de sangre y sustratos a los tejidos activados (Hauk and Hosey, 2006). Esta respuesta fisiológica puede mejorar la capacidad de trabajo muscular y el rendimiento deportivo. El nitrito es un marcador sensible de la síntesis de NO vía las NOS, pero además proporciona una fuente independiente de la actividad NOS para la generación de NO (Bescós, et al., 2012). El nitrito puede generarse a partir de nitrato por las bacterias anaeróbicas presentes en la cavidad bucal, entrando rápidamente en sangre donde puede ser transformado en NO por acción de enzimas como la xantina oxidasa (XO) (Bescós, et al., 2012, Shiva, 2013). La concentración de nitrito se relaciona con la capacidad de ejercicio en sujetos entrenados moderados (Totzeck, et al., 2012) y el ejercicio de resistencia estimula la NOS del músculo esquelético (Linden, et al., 2011). Estos estudios involucran al NO con la

prolongación de la capacidad de duración de una actividad física. Un aumento en la carga de trabajo general estimula la tensión en los tejidos musculares ejercitados, dando como resultado una hipertrofia muscular (Figura 3). Estos efectos se otorgan a suplementos que presuponen una estimulación de la síntesis de NO, aunque en muchos casos están compuestos por una gran variedad de ingredientes, los cuales, sin ser estimulantes de la síntesis de NO, ya se ha demostrado que tienen una cierta capacidad ergogénica (creatinina, carbohidratos, aminoácidos, etc.) (Eto, et al., 1994, Wu, et al., 2009, Wu and Morris, 1998). Por esta razón, los estudios que pretendan evidenciar efectos ergogénicos de los precursores de NO deben realizarse con los componentes clave para la generación de NO y evitar la interferencia de otros componentes dietéticos.

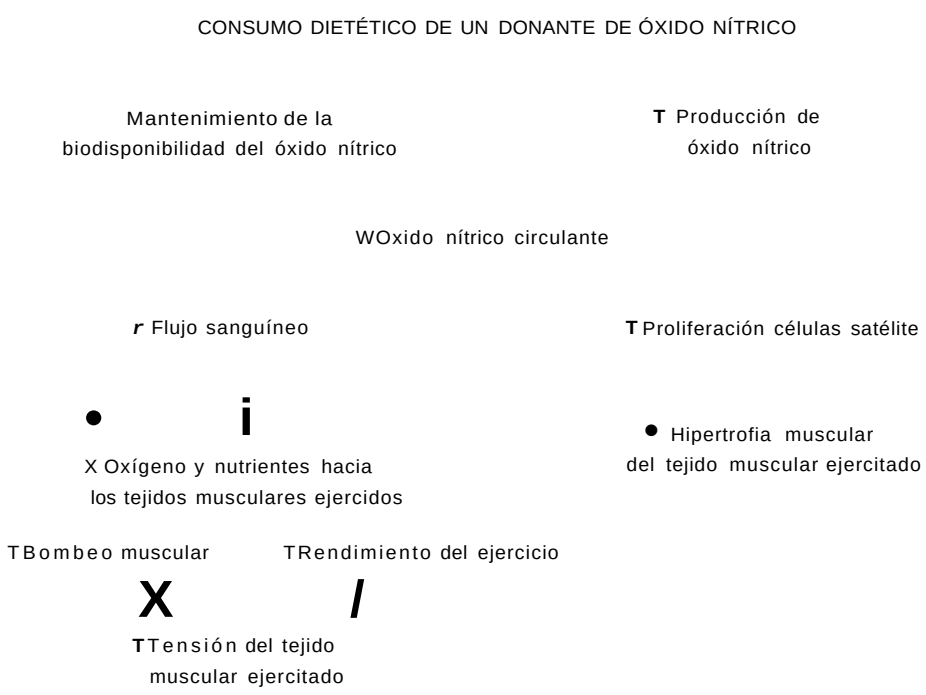

Figura 3. Modelo teórico propuesto para los beneficios dietéticos de los suplementos de óxido nítrico, adaptado de Bescós (2011).

EJERCICIO FÍSICO Y RESPUESTA INMUNE DE FASE AGUDA

La realización de una sesión de ejercicio físico intenso conlleva una secuencia de eventos similares a los que se producen en una respuesta de fase aguda inducida por una infección, pero de una magnitud menor (Bailey, et al., 2011, Ferrer, 2009). Esta respuesta se da en un ejercicio agudo normal (Petersen and Pedersen, 2005, Walsh, et al., 2011) y en un ejercicio intenso (Febbraio, 2007, Petersen and Pedersen, 2005, Suzuki, et al., 2006). Tras el ejercicio se activa el sistema del complemento a través de la denominada vía alternativa, la cual puede ser estimulada por fragmentos de tejido dañado (Cannon, et al., 1994, Dufaux and Order, 1989, Gelfand, et al., 1983). La leucocitosis (neutrofilia principalmente) es otra de las características típicas de la respuesta inmunitaria al ejercicio. Este fenómeno ya se describió hace más de 100 años tras una maratón (Blake and Larrabee, 1903) y posteriormente se comprobó en ejercicios mucho más cortos (Martin, 1932, Steel, et al., 1974). La neutrofilia asociada al ejercicio parece ser debida al daño muscular y la consecuente activación del complemento. A parte de incrementar su número de células en circulación, los neutrófilos también se activan durante el ejercicio. Esta activación se ha determinado *in vivo* a través de las concentraciones plasmáticas elevadas de enzimas almacenados normalmente en los gránulos del neutrófilo, como la elastasa (Kokot, et al., 1988), la lactoferrina (Taylor, et al., 1987) y la mieloperoxidasa (MPO) (Pincemail, et al., 1990); e *in vitro* por una mayor producción de especies reactivas de oxígeno (ROS) por parte de neutrófilos aislados tras el ejercicio (Cannon, et al., 1990). Los neutrófilos se infiltran rápidamente en el tejido muscular dañado, y esta infiltración es proporcional al grado de daño en las bandas z de las fibras musculares (Fielding, et al., 1993). Tras el ejercicio los monocitos también migran a las regiones dañadas del músculo, pero mucho más tarde que los neutrófilos, al igual que en la respuesta de fase aguda a la infección. Está bien establecido que la reparación muscular después de una lesión aguda incluye una fase proinflamatoria inicial durante la cual el músculo libera citoquinas y

en el tejido dañado se infiltran células inmunes que coordinan la eliminación de las células musculares muertas (Pillon, et al., 2013).

La producción de citoquinas, especialmente las interleuquinas 1 y 6 (IL1 e IL6), también se ve modulada por la práctica de ejercicio. La presencia simultánea de niveles aumentados de IL6 y TNFa (factor de necrosis tumoral) son indicativos de un proceso de inflamación (Walsh, et al., 2011). Diferentes ensayos tanto *in vitro* como *in vivo* han puesto de manifiesto incrementos tanto en la expresión como en la secreción de IL1 e IL6 asociados al ejercicio (Cannon, et al., 1989, Haahr, et al., 1991, Sprenger, et al., 1992). Entre los factores postulados como inductores de esta producción de citoquinas se encuentran lipopolisacáridos producidos por el tracto gastrointestinal, la fagocitosis de fragmentos de tejido y los peróxidos lipídicos producidos por las ROS (Cannon and Blumberg, 2000).

Omega-3, inflamación y enfermedad cardiovascular

Los esquimales que vivían en Groenlandia y tenían una dieta rica en PUFAs, especialmente DHA y EPA, provenientes de los aceites y grasa de animales marinos, presentaban menor incidencia de infarto y coronariopatía (10 veces menos) que los esquimales que habían emigrado a Dinamarca y habían adoptado la dieta rica en proteína y grasa animal propia de los daneses (Bang and Dyerberg, 1972). A partir de este tipo de estudios se pudo demostrar que los omega-3 podían prevenir el desarrollo de arteriosclerosis y explicar así la baja mortalidad por enfermedad cardiovascular de los esquimales alimentados de animales marinos (pescado, focas, ballenas...) ricos en DHA y EPA. Además, este grupo de población presenta un perfil lipídico con valores plasmáticos bajos de colesterol, triglicéridos y ciertas lipoproteínas, lo que da lugar a un menor riesgo de padecer una enfermedad cardiovascular.

Una dieta rica en omega-3 incrementa los niveles sanguíneos de estos AGs (Egert, et al., 2012). Los omega-3 han demostrado disminuir en el plasma sanguíneo la concentración de lipoproteínas de baja densidad (LDL), aumentar la concentración de las lipoproteínas

de alta densidad (HDL) y reducir la presión sanguínea (Egert, et al., 2009, Okuda, et al., 2005, Weber and Raederstorff, 2000).

La cantidad de AGs de la dieta y su tipología condicionan el ritmo de síntesis y metabolización de las distintas lipoproteínas. Los SFAs tienden a incrementar los niveles de colesterol, LDL y triglicéridos, mientras que los PUFAs omega-3 favorecen la reducción de estos. Se ha demostrado que una ingesta rica en EPA y DHA, mediante el consumo de aceite de pescado, va seguida de una reducción significativa de los niveles de triglicéridos plasmáticos (Weber, 1999, Weber and Raederstorff, 2000).

Actualmente se acepta la hipótesis de que los omega-3 reducen los niveles de triglicéridos y de lipoproteínas de muy baja densidad (VLDL), inhibiendo la síntesis de triglicéridos a nivel hepático (Azra, 2005, Weber and Raederstorff, 2000).

Otros estudios han investigado la relación existente entre el consumo de PUFAs y enfermedad cardiovascular, hiperlipidemia, hipertensión arterial, problemas inflamatorios y autoinmunes, cáncer y diabetes (Banning, 2005, Carley and Severson, 2005, Harrison and Abhyankar, 2005, Heird and Lapillonne, 2005, Judé, et al., 2006, Lee and Austen, 1986, Mozaffarian, 2005, Verbeke, et al., 2005). Se ha observado que los AGs AA, DGLA, EPA y DHA son precursores de la síntesis de eicosanoides (prostaglandinas, tromboxanos y leucotrienos), moléculas involucradas en el funcionamiento del Sistema Nervioso Central (Heird and Lapillonne, 2005). El DHA es un omega-3 con propiedades antiinflamatorias (Tur, et al., 2012). Además, los omega-3 han demostrado tener una influencia beneficiosa en enfermedades inflamatorias como la artritis reumatoide, mediante la modulación de la expresión génica, suprimiendo la producción de varias citoquinas y aumentando la producción de eicosanoides antiinflamatorios (Berbert, et al., 2005, Oh, 2005).

ESTRÉS OXIDATIVO Y LOS ELEMENTOS QUE PARTICIPAN

Especies reactivas de oxígeno

El oxígeno molecular (O_2), esencial para la vida, puede ser convertido mediante una gran variedad de procesos celulares implicados en una multitud de procesos fisiológicos (función inmune, envejecimiento, comienzo y progresión de patologías, etc.) a ROS (Ferrer, 2009, Fisher-Wellman and Bloomer, 2009). Las ROS son altamente reactivas y entre ellas destacan el peróxido de hidrogeno (H_2O_2), el anión superóxido (O_2^-) y el radical hidroxilo libre (OH-). Las ROS pueden ser radicales libres, formados en reacciones hemolíticas, heterolíticas o redox, o no radicalarias (átomos o moléculas sin electrones desapareados). Se define como radical a una molécula con un electrón desapareado muy reactivo en un orbital exterior, que puede iniciar reacciones en cadena por eliminación de un electrón para completar su propio orbital. Paralelamente a los ROS nos encontramos con las especies reactivas de nitrógeno (RNS).

El O_2^- se produce como intermediario en un gran número de reacciones bioquímicas, y se forma cuanto el O2 capta un electrón (Halliwell, 1995). Tiene una vida media relativamente larga, por lo que puede difundir por la célula y reaccionar con múltiples dianas. Aunque es una especie impermeable a las membranas celulares se ha descrito que se puede protonar a pH fisiológico en las proximidades de las membranas, dando lugar al radical hidroperoxilo (HO2-) y facilitando el paso a través de membranas (Salvador, et al., 2001). Las principales fuentes intracelulares de $O2^-$ son la cadena respiratoria, las reacciones catalizadas por ciertas oxidasas como la XO (cataliza la oxidación de xantina e hipoxantina en el metabolismo de las purinas) (Wassmann, et al., 2004) y la activación de células del sistema inmune como los neutrófilos y macrófagos. Estas células inmunológicas expresan la enzima NADPH oxidasa (NOX), la cual cataliza la generación de $O2^-$ a partir de O2 y NADPH (Fuchs, 1992). La presencia de NOX se ha generalizado a multitud de tipos celulares, incluyendo las células de

endotelio vascular. Los productos de la NOX, O_2^- y H_2O_2 extracelular, son considerados moléculas mensajeras (Paletta-Silva, et al., 2013).

El H2O2 es un agente citotóxico, pero no tiene un potencial oxidante muy elevado, por lo que no puede oxidar el ADN o lípidos directamente, pero sí que puede inactivar ciertos enzimas (Halliwell, 2007). El H2O2 se forma espontáneamente por dismutación del O2- o de manera catalizada por la enzima superóxido dismutasa (SOD).

O2- + O2- + 2H+ H2O2 + O2

Además, el H2O2 se puede generar durante reacciones enzimáticas como las catalizadas por las enzimas urato oxidasa, glucosa oxidasa y aminoácido oxidasa (Fridovich 1986). Al ser un compuesto reactivo, el H_2O_2 puede generar otros ROS como el OH- a través de reacciones catalizadas por metales como la reacción de Fenton:

O2- + Fe3+ Fe2+ + O2

H_2O_2 + Fe2+ Fe3+ + OH- + OH-

El OH- tiene un elevado potencial oxidante lo que la convierte en una especie altamente reactiva y por tanto sólo reacciona con las moléculas cercanas a su lugar de formación. Son consideradas las ROS más dañinas y dado a su alta reactividad sólo se ha confirmado su existencia en organismos vivos detectando sus productos de reacción (Alessio, et al., 2000).

Otra ROS es el oxígeno singlete ($^1O_2$), una especie no radicalaria que se puede formar a nivel celular a partir de la dismutación del O2- en medio acuoso. Al igual que el radical OH-, el ^{1}O2 tiene una vida media muy corta, pero a diferencia del anterior puede difundir a través de la membrana. El ^{1}O2 puede peroxidar directamente ácidos grasos insaturados dando lugar a los correspondientes hidroperóxidos de estos ácidos grasos.

El hipoclorito (HOCl) se forma a partir de H_2O_2 y Cl^-, reacción que puede ser catalizada por la enzima MPO, la cual está principalmente presente en los neutrófilos:

$$H_2O_2 + Cl^- \quad HOCl + OH^-$$

El HOCl puede dañar diferentes biomoléculas a partir de la oxidación de grupos tioles, lípidos, etc. Puede atravesar membranas y provocar la rotura y agregación de proteínas.

Los hidroperóxidos de los ácidos grasos insaturados son precursores de radicales libres como el radical peroxilo y el radical alcóxido. Esta reacción está catalizada por la presencia de metales de transición como el hierro y el cobre. Estos radicales libres pueden capturar un hidrógeno a partir de AGs insaturados, los cuales se transforman a su vez en radicales libres, en este caso el radical está centrado en el carbono del AG. Los radicales peroxilo orgánicos (ROO-) se formar por la adición de O_2 a un radical de carbono, generando un ciclo de producción de radicales libres a partir de ácidos grasos insaturados. El ataque de un radical a un compuesto hidrocarbonado supone la pérdida de un átomo de hidrógeno provocando la formación de un radical de carbono, lo que es inestable y reacciona rápidamente con una molécula de O_2 formando ROO-. A partir de ROO- se pueden generar otros radicales como el radical alcoxilo (RO-).

Especies reactivas de nitrógeno

El óxido nítrico gaseoso es la RNS más abundante en los sistemas biológicos. Se sintetiza a partir de L-arginina por la familia de isoenzimas de la NOS. Hasta el momento se han descrito tres isoformas de NOS, dos de ellas constitutivas (eNOS y nNOS, endotelial y neuronal, respectivamente) y una inducible (iNOS). La iNOS, presente en muchas células del sistema inmune, produce grandes cantidades de NO cuando es activada para actuar contra agentes infecciosos (Sureda, et al., 2005). El NO, además de este efecto antimicrobiano, participa en diversos procesos fisiológicos tales como

la regulación del flujo sanguíneo, inhibición de la agregación plaquetaria, posee actividad antiinflamatoria y actúa como neurotransmisor (Coleman, 2001, Liaudet, et al., 2000, Wiesinger, 2001).

El NO puede reaccionar con $O2^{\cdot}$ y formar el anión peroxinitrito ($ONOO^{\cdot}$):

$$O2^{\cdot} + NO \quad ONOO^{\cdot}$$

Esta reacción ocurre tres veces más rápido que la dismutación de $O2^{\cdot}$ a $H2O2$, por lo que es la reacción predominante cuando coexisten ambas especies reactivas (Zalba, et al., 2003). El $ONOO^{\cdot}$ es un fuerte agente oxidante, capaz de dañar el ADN y provocar la nitración de proteínas (Pacher, et al., 2007).

Defensas antioxidantes

Para evitar un exceso de ROS y RNS (RONS) nuestro organismo está dotado de una maquinaria antioxidante, la cual puede actuar reduciendo la producción de RONS o aumentando su destoxificación (Fisher-Wellman and Bloomer, 2009, Ji, 2008, Nikolaidis, et al., 2012, Sachdev and Davies, 2008). Pueden actuar controlando las concentraciones locales de O2, quelando iones metálicos, inactivando sustancias con carácter prooxidante, eliminando las especies activadas de oxígeno o transformándolas en moléculas menos activas, previniendo la formación de radicales o favoreciendo la transformación de las formas más reactivas en formas menos peligrosas, reparando el daño oxidativo e incrementando la eliminación de moléculas dañadas. Los antioxidantes se encuentran dentro y fuera de la célula. Entre estas defensas antioxidantes encontramos las enzimas antioxidantes y los antioxidantes de bajo peso molecular, como el glutatión reducido (GSH) o las vitaminas C y E.

Defensas antioxidantes proteicas y enzimáticas

Las principales enzimas antioxidantes son la catalasa, la glutatión peroxidasa (GPx) y la SOD, pero también existen otros enzimas que complementan la función antioxidante como son la glutatión reductasa (GRd) y la tioredoxina reductasa (TrxR), y proteínas antioxidantes como las UCPs que controlan la producción mitocondrial de ROS (Carrera-Quintanar, et al., 2012, Ferrer, et al., 2010, Sluse, et al., 2006, Sureda, et al., 2013, Wang, et al., 2013).

La SOD es la primera línea de defensa frente a $O2^{\cdot}$ dismutandolo a H2O2 (menos reactivo que $O2^{\cdot}$) y oxígeno. En mamíferos se han descrito 3 isoformas, todas ellas con un metal de transición en su centro activo que permite la rotura catalítica de $O2^{\cdot}$ (Culotta, et al., 2006). La isoforma Cu/Zn-SOD (SOD1) requiere cobre-zinc como cofactor y se localiza en el citoplasma y en el espacio intermembrana de la mitocondria. La isoforma Mn-SOD (SOD2) requiere manganeso como cofactor y se localiza en la matriz mitocondrial. Finalmente, la ecSOD (SOD3) también requiere cobre-zinc como cofactor, pero se encuentra en el espacio extracelular.

La GPx es una enzima tetramérica, posee un residuo de selenio-cisteína en cada una de sus subunidades, y cataliza la reducción de H2O2 e hidroperóxidos (ROOH) a H2O y alcohol (ROH), respectivamente, usando GSH como donador electrónico, el cual es oxidado a disulfuro de glutatión (GSSG), también llamado glutatión oxidado (Halliwell, 2007, Matés, et al., 1999). Por el momento se han descrito 5 isoformas de GPx (Brigelius-Flohé, 2006), las cuales presentan diferente especificidad de sustrato (diferentes hidroperóxidos) y localización celular (citosólica, mitocondrial, etc.) (Brigelius-Flohé, 1999). Esta variedad en la especificidad de sustrato y localización optimiza la función de GPx como defensa antioxidante, que puede reducir tanto el H2O2 como ROOH orgánicos complejos resultado de la oxidación de lípidos de membrana, proteínas y ácidos nucleicos (Ji, 1999). Al necesitar un aporte continuo de GSH para una óptima función de la GPx, la célula dispone de un mecanismo de regeneración de GSH a partir de GSSH. Para ello cuenta con la GRd,

una flavoenzima que se encarga de la reducción de GSSH a GSH utilizando NADPH como cofactor (Meister and Anderson, 1983).

La catalasa es un homotetrámero ampliamente distribuido por toda la célula (Kirkman and Gaetani, 2007, Matés, et al., 1999). También cataliza la descomposición de H_2O_2 a H_2O como la GPx, pero sin la necesidad de otro sustrato. En su centro activo presenta hierro como cofactor, el cual forma parte de un grupo hemo y necesita de NADPH para mantener su adecuado estado de oxidación (Kirkman and Gaetani, 1984, Korth, et al., 2012). A pesar de compartir sustrato con la GPx, la catalasa posee una menor afinidad por el H2O2 a bajas concentraciones (GPx K_m = 1 u.M; catalasa K_m = 1 mM) (Siess, 1985).

El sistema de la tioredoxina (Trx) está compuesto por la Trx y la TrxR. La Trx es una proteína de 12 kDa altamente conservada que se encuentra en el citoplasma (Trx1) y en la mitocondria (Trx2) (Berndt, Lilling 2007). Mantiene las proteínas en su estado reducido a través de su actividad disulfuro reductasa, pero también contribuye en otras funciones como la reducción de factores de transcripción y el control de la apoptosis. Una vez oxidada la Trx es reciclada a su forma reducida por TrxR, la cual utiliza los electrones provenientes de la oxidación del NADPH. Además de regenerar los grupos disulfuro de la Trx, la TrxR reduce otros grupos disulfuro de otras proteínas oxidadas (Wang, et al., 2013). La TrxR también actúa como enzima antioxidante a través de la reducción directa de hidroperóxidos y del reciclaje de la vitamina C (Arnér and Holmgren, 2000).

La mitocondria es una fuente importante y continua de ROS, principalmente a través de la cadena respiratoria, por lo que cuenta con toda una serie de proteínas con características antioxidantes. Entre estas podemos encontrar las proteínas desacoplantes (UCPs), presentes en la membrana interna de las mitocondrias (Figueira, et al., 2013). Diferentes isoformas (UCP1 - UCP5) se encuentran en diferentes tejidos y células. Las UCPs están ligadas a la composición de las membranas, por lo que los AGs libres se consideran reguladores de las UCPs (Sluse, et al., 2006). Además los AGs en forma aniónica son activadores de las UCPs (Figueira, et al., 2013).

Otros sistemas antioxidantes consisten en proteínas que transportan y secuestran posibles agentes prooxidantes como el hierro (transferrina, ferritina), el cobre (ceruloplasmina) o el grupo hemo (haptoglobina). El reciclaje de proteínas, la síntesis de nuevas proteínas funcionales, la degradación de proteínas modificadas, además de los procesos de reparación del ADN también pueden considerarse como parte del sistema de defensa antioxidante (Ferrer, 2009, Hellsten Y In: Sen CK, 2000).

Defensas antioxidantes no proteicas

Las defensas antioxidantes no proteicas comprenden un amplio y heterogéneo abanico de moléculas orgánicas de bajo peso molecular que ayudan al mantenimiento del equilibrio redox intracelular y extracelular mediante su acción destoxificadora de especies reactivas o de sus productos. Se pueden clasificar en antioxidantes endógenos (glutatión, ubiquinona, bilirrubina, etc.), antioxidantes dietéticos (vitaminas E y C, carotenoides, compuestos fenólicos, fitoestrógenos, etc.) y productos finales del metabolismo excretados (ácido úrico, bilirrubina, hipoxantina, etc.).

La vitamina E (mayoritariamente en la forma a-tocoferol, aunque existen al menos 8 isómeros estructurales del tocoferol) (Packer, 1991) es un antioxidante liposoluble localizado en membranas y entornos lipofílicos, lo que le permite actuar eficientemente sobre las especies reactivas originadas en la membrana mitocondrial interna y otras membranas. Su función es la de reaccionar con ROO- deteniendo las reacciones de autooxidación. Tras esta reacción se forma el radical de tocoferol que puede ser reciclado por otros antioxidantes como la vitamina C o el GSH (Ji, 1995).

La vitamina C (ácido ascórbico) es una vitamina antioxidante hidrosoluble que se localiza principalmente en el citoplasma y en los fluidos extracelulares. Reacciona directamente con el $O2^{\cdot}$ y el OH- en la fase acuosa del plasma previniendo la aparición de daño oxidativo en las membranas de las células sanguíneas (Beyer, 1994, Carr and Frei, 1999). Además reduce la forma radicalaria del tocoferol a su

forma activa, dando lugar a la forma oxidada de la vitamina C (ácido dihidrolipoico) que puede ser reducida por GSH o por el ciclo redox del ácido dihidrolipoico (Halliwell, 2007).

Los carotenoides, como el 0-caroteno, tienen una estructura formada por largas cadenas de dobles enlaces conjugados y actúan a nivel de membranas y lipoproteínas eliminando $O2^{\cdot}$, ROO- y $^{1}O2$ (Young and Lowe, 2001). Debido a su localización celular en membranas y a su potencial destoxificador, los caroteroides tienen un papel importante contra la peroxidación lipídica (Krinsky, 1998).

Otras sustancias antioxidantes ingeridas con los alimentos son los compuestos fenólicos como la antocianidina, las isoflavonas o los taninos, que pueden capturar radicales libres o disminuir su producción, además de actuar como quelantes de metales (Rice-Evans and Miller, 1996); o el ácido lipoico, capaz de reaccionar con especies reactivas de oxígeno así como con otros antioxidantes como las vitaminas E y C o el glutatión, regenerándolos a partir de sus formas oxidadas (Packer, et al., 1995). Los fitoestrógenos que se encuentran ampliamente distribuidos ampliamente en el reino vegetal también poseen propiedades antioxidantes (Mestre-Alfaro, et al., 2011, Seidel, et al., 2000).

Entre los antioxidantes no enzimáticos endógenos, el GSH es el más abundante en las células, participa en la reducción de ROO- por la GPx, en la neutralización de OH- y $^{1}O2$ y en la reducción de semihidroascorbato y radicales tocoferol (Ji, 1999). El GSH se regenera a partir de la reducción de su forma oxidada GSSH mediante la acción de GRd (Meister and Anderson, 1983).

Algunos productos finales del metabolismo como el ácido úrico, que deriva del metabolismo de las purinas, o la bilirrubina, que deriva del catabolismo del grupo hemo, también poseen propiedades antioxidantes. El ácido úrico neutraliza ROO-, OH- y $^{1}O2$, quelata iones metálicos como el hierro y el cobre previniendo la formación de radicales OH- por la reacción de Fenton (Powers and Jackson, 2008). La bilirrubina posee un fuerte potencial antioxidante frente ROO- y H_2O_2 (Baranano, et al., 2002).

Equilibrio redox y estrés oxidativo

Cuando la tasa de generación de RONS se encuentra neutralizada por una similar tasa de eliminación por mecanismos antioxidantes decimos que nos encontramos en equilibrio redox. Si las RONS se producen a una mayor velocidad que su eliminación se produce un desequilibrio oxidativo y se pueden dañar estructuras celulares mediante oxidación de macromoléculas. Este desequilibrio en la homeóstasis de las RONS es conocido como estrés oxidativo. Después de un ejercicio agudo e intenso la capacidad antioxidante puede no ser suficiente para restaurar el balance redox oxidante-antioxidante, dando como resultado una situación de estrés oxidativo y una mínima adaptación (Ji, 2008).

Los PUFAs presentes en las membranas son especialmente susceptibles de ser atacados por las RONS, especialmente por el radical OH-, a través de reacciones de peroxidación lipídica. Estas reacciones consisten en la extracción de un electrón al ácido graso y su reacción con oxígeno dando lugar a la formación de ROO-. Cuando un AG con dobles enlaces conjugados es atacado por radicales, éstos enlaces pueden reaccionar fácilmente con O2 produciendo ROO-, que al capturar un nuevo electrón pueden finalmente transformarse en hidroperóxidos lipídicos. Estos hidroperóxidos tienen una vida media relativamente corta y se descomponen hasta formar aldehídos de cadena corta como 4-hidroxinonenal (4-HNE) y malondialdehído (MDA), que pueden unirse a proteínas, carbohidratos, otros lípidos y ADN, alterando su estructura y función (Halliwell, 2007). Los lípidos de membrana peroxidados son más hidrofílicos, lo que puede dar lugar a una entrada en exceso de agua y conllevar a una inflamación celular, la cual, a su vez, puede promover una mayor producción de $O2^{\cdot}$ haciendo una alimentación positiva que provoque las reacciones de peroxidación (Alessio, et al., 2000).

Las proteínas, al tener en su compleja estructura un elevado número de grupos funcionales oxidables, son susceptibles de reaccionar con RONS, principalmente con H2O2, OH- y NO (en forma de peroxinitrito). La oxidación puede ser catalizada por metales formando grupos carbonilo en las cadenas laterales (Stadtman, 1992,

Stadtman, et al., 1993), siendo la prolina, arginina, lisina e histidina los aminoácidos más susceptibles a esta transformación. Estos grupos carbonilo son grupos aldehídos y cetonas, los cuales también se pueden formar en la proteína a través de la reacción con carbohidratos y lípidos. Por otra parte, metionina, histidina, triptófano y cisteína tienen tendencia a ser atacados por ROO- y RO¬ en ausencia de metales. Este proceso oxidativo puede conllevar a una modificación estructural ligada a un cambio funcional o hasta la degradación oxidativa de la proteína. La determinación de los grupos carbonilo presentes suele ser un indicador de la oxidación de proteínas y un biomarcador de estrés oxidativo (Dalle-Donne, et al., 2003).

Las RONS también pueden reaccionar con el ADN, tanto es así que el ADN oxidado es bastante abundante en células humanas y se va acumulando con la edad (Ames, et al., 1995, Shigenaga and Ames, 1991). Los daños sobre el ADN oxidado son la modificación de las bases o los nucleótidos, la aparición de roturas en una cadena y la aparición de entrecruzamientos ADN-proteína.

Las células y tejidos poseen mecanismos para reestablecer el estado redox tras un período de producción excesiva de RONS. Por ejemplo, la producción de NO está sujeta a una regulación negativa de retroalimentación, ya que el propio NO inhibe la NOS. Otro mecanismo para la homeostasis redox es la inducción por RONS de cascadas de señalización que conducen a la expresión de enzimas antioxidantes. Las RONS pueden actuar a través de diversas vías de transducción de señales, interaccionando con elementos señalizadores tales como calcio, tirosina quinasas y fosfatasas, serina/treonina quinasas y fosfatasas. La interacción de las especies reactivas con estas proteínas quinasas y fosfatasas resulta de vital importancia en los procesos reguladores ya que las cascadas de fosforilación están implicadas en numerosos mecanismos de transmisión de señales extracelulares desde la membrana plasmática hasta el núcleo. Entre las principales vías de regulación en las que participan las RONS encontramos la activación de factores de transcripción (NFKB, AP-1, p53), la activación de las vías de las MAPK y del PI_3K/Akt, etc (Ferrer, 2009, Ji, 2007). Si el incremento de RONS es

relativamente pequeño, la maquinaria antioxidante suele ser suficiente para volver al equilibrio redox original. Sin embargo, bajo condiciones de producción de RONS incrementadas de forma prolongada la respuesta antioxidante fisiológica puede no ser suficiente para volver a una condición de homeostasis original (Ji, 2008). En estos casos, el sistema puede volver a alcanzar con el tiempo un nuevo equilibrio, el cual irá asociado a concentraciones de RONS más elevadas y a diferentes patrones de expresión génica.

Formación de radicales libres y estrés oxidativo durante el ejercicio

El ejercicio regular de intensidad moderada tiene una gran cantidad de efectos beneficios, pero el ejercicio agotador puede generar una situación de estrés oxidativo, sobretodo en sujetos no entrenados, que puede provocar daños estructurales a las células musculares y reacciones inflamatorias en el músculo. Algunos de estos efectos son consecuencia de una producción elevada de RONS provocada por la contracción muscular. En 1954 ya se detectaron radicales libres en el músculo esquelético mediante resonancia paramagnética electrónica (COMMONER, et al., 1954), aumentando el interés sobre ROS y su papel en la función muscular y el daño asociado al ejercicio. Actualmente se conoce que un ejercicio intenso y prolongado produce condiciones de estrés oxidativo desfavorables para el deportista que pueden llegar a desencadenar un proceso patológico (Bloomer, et al., 2009, Peternelj and Coombes, 2011).

Aunque las fuentes de RONS durante el ejercicio no están del todo caracterizadas no cabe duda que el exceso de RONS provoca oxidación en biomoléculas y estrés oxidativo durante la práctica de una actividad física intensa o prolongada. Estudios tempranos ya detectaron niveles elevados de pentano oxidado después de una sesión de ejercicio prolongado (Dillard, et al., 1978). Desde entonces, numerosos estudios han puesto de manifiesto incrementos en los marcadores de peroxidación lipídica relacionados con la intensidad del ejercicio (Alessio, 1993, Davies, et al., 1982, Ji, 1995, Ji, et al., 1992). Se ha evidenciado que la realización de un ejercicio agudo como un partido de fútbol, una etapa ciclista y la realización de un

duatlón o triatlón incrementa los marcadores oxidativos de lípidos (Ascensao, et al., 2008, Fisher, et al., 2011, Medina, et al., 2012). También se han descrito daños en proteínas, como la formación de grupos carbonilo, después de una maratón (Radák, et al., 2003), una etapa ciclista (Sentürk, et al., 2005), una sesión de ejercicio de intensidad submáxima (al 70% de la VO_2max) (Bloomer, et al., 2007, Morillas-Ruiz, et al., 2005) y una sesión de ejercicio de intensidad máxima (Gochman, et al., 2007). También se ha descrito daño oxidativo en el ADN producido por una actividad física intensa (Almar, et al., 2002, Hartmann, et al., 1994, Mastaloudis, et al., 2004, Niess, et al., 1996, Radák, et al., 2000, Tsakiris, et al., 2006).

Para hacer frente a los procesos oxidativos del ejercicio las células adaptan sus defensas antioxidantes. Los niveles de vitamina E en los tejidos suele mantenerse constante tras una sesión de ejercicio, lo que sugiere que los niveles fisiológicos de esta vitamina son adecuados para proteger contra la producción de especies reactivas asociada al ejercicio (Gohil, et al., 1987, Tiidus, et al., 1993). Durante el ejercicio, elevadas cantidades de GSH son oxidadas a GSSG, en músculo y corazón, para neutralizar las especies reactivas y productos de oxidación. Sin embargo, el ratio GSH:GSSG no se suele alterar significativamente debido a los mecanismos enzimáticos de regeneración de GSH y a la importación de GSH desde el plasma (Ji and Fu, 1992, Leeuwenburgh and Ji, 1995, Lew, et al., 1985). Los enzimas antioxidantes pueden activarse selectivamente en respuesta a la situación de estrés oxidativo provocada por el ejercicio. Por ejemplo, se ha descrito que un episodio agudo de actividad física incrementa la actividad SOD en hígado, músculo esquelético y eritrocitos (Ji, 1999, Lawler, et al., 1993, Shin, et al., 2008, Tauler, et al., 2005), aunque la actividad SOD se regula diferente en las distintas isoformas del enzima (Cu/Zn-SOD y Mn-SOD) y de los diferentes tipos de ejercicio (Ji, 1999). En cambio, se ha descrito que la actividad GPx frente al ejercicio varía en función del tipo de músculo esquelético, aumentando (Leeuwenburgh and Ji, 1996) o no variando tras realizar ejercicio (Brady, et al., 1979).

Generación de radicales libres en la contracción muscular y su papel en la señalización celular

Las RONS son producidas continuamente en individuos sanos y parece ser que tienen un papel en la modulación del desarrollo, metabolismo, control del flujo sanguíneo y contracción muscular. Las contracciones musculares en un ejercicio agudo incrementan la producción de RONS aumentado los marcadores de estrés oxidativo en sangre (Newham, et al., 1983, Raastad, et al., 2010), los cuales regresan a sus niveles basales después de 2-3 días (Close, et al., 2004, Nikolaidis, et al., 2007, Theodorou, et al., 2011, Theodorou, et al., 2010). La formación en reposo de $O2^{\cdot}$ en el músculo esquelético es pequeña, pero aumenta durante la contracción muscular. Al $O2^{\cdot}$ y al $H2O2$ se les atribuye una función en la señalización, lo que no ocurre con OH- ya que con su alta reactividad y baja vida media es prácticamente indetectable en condiciones basales. Éste aumenta en un ejercicio agotador y se relaciona con la fatiga muscular (Reid, 2001). El NO también es generado continuamente en el músculo esquelético en reposo y aumenta sus niveles con la contracción muscular, cataliza una variedad de señales que regulan procesos intracelulares e interacciones entre células.

La activación de estas rutas podría estar relacionada con la activación de las defensas antioxidantes. Cada vez hay más evidencias que indican que unos elevados niveles de ROS pueden producir daño en los componentes celulares, mientras que unos niveles moderados-bajos tienen un papel regulador (Dróge, 2002, Ferrer, et al., 2010, Powers, et al., 2010, Powers and Jackson, 2008). Se ha descrito que tras una sesión de ejercicio se activa simultáneamente el factor de transcripción NFKP y la expresión génica de la Mn-SOD en músculo esquelético de rata (Hollander, et al., 1999, Ji, et al., 2004), la activación de la vía de las MAPK (proteínas quinasas activadas por mitógenos; p38, ERK1 y ERK2), activación del NFK0 y aumento de la expresión de genes como la SOD y la iNOS en músculo de rata (Gomez-Cabrera, et al., 2005), y la activación de la subunidad p50 del NFKP en linfocitos humanos (Gomez-Cabrera, et al., 2006). El hecho de que el alopurinol, inhibidor de la producción de ROS por parte de

la XO (Stofan, et al., 2000, Viña, et al., 2000), inhibiera estas respuestas adaptativas apunta a las ROS como principales efectoras.

Mecanismos de formación de radicales libres durante el ejercicio

No existe una única fuente de RONS durante el ejercicio, las principales son la cadena de transporte electrónico mitocondrial, la XO y la NOX presente en los macrófagos.

La cadena de transporte electrónico mitocondrial

El papel de la cadena respiratoria como fuente de ROS durante el ejercicio es objeto de debate (Jackson, et al., 2007). Durante el ejercicio físico el músculo puede aumentar su consumo de oxigeno más de 100 veces (Tonkonogi and Sahlin, 2002) y el del organismo entero 20 veces (Meydani, et al., 1993). El oxígeno utilizado por la célula se consume en un 90% en la cadena de transporte electrónico, actuando como aceptor de electrones en el proceso de respiración mitocondrial (Chance, et al., 1979, Shigenaga, et al., 1994). De esta fracción, un 2-5% se reduce formando $O2^{\cdot}$, el cual puede reducirse a $H2O2$ y ser liberado por la mitocondria (Boveris and Chance, 1973). Para prevenir el posible daño del radical superóxido la mitocondria presenta la enzima SOD. También se ha evidenciado la presencia de OH- en el músculo esquelético ejercitado (Barclay and Hansel, 1991). No obstante, la formación de radicales libres por parte de la mitocondria cuando está en estado 3 (consume oxígeno activamente, hay ADP) es insignificante (Chance, et al., 1979, Papa, et al., 1997). En estado 3 la mitocondria produce grandes cantidades de ATP, con el correspondiente incremento de flujo electrónico, y la proporción de oxígeno que es convertido a radicales libres cae hasta aproximadamente el 0.25%, lo que supone un orden de magnitud menos que en condiciones basales (Gomez-Cabrera, et al., 2008, St-Pierre, et al., 2002). En esta menor producción de ROS podrían estar implicadas las proteínas desacoplantes UCP-2 y UCP-3, las cuales actuarían protegiendo a la mitocondria frente a la producción de ROS y la aparición de daño celular (Brand and Esteves, 2005).

Además, la fosfolipasa A2 (PLA2), presente en la mitocondria y en el sarcolema, se relaciona como una fuente de $O2^{\cdot}$ en el músculo esquelético y como reguladora mitocondrial de la producción de radicales libres (Li, et al., 1999, Nethery, et al., 1999, Supinski, 1998).

También se propone un mecanismo para explicar un posible aumento de la producción mitocondrial de ROS en el cuál las elevadas temperaturas que se alcanzan en el interior del músculo (41-45°C), durante el ejercicio, provocarían una desestabilización de la ubisemiquinona, induciendo un descenso en la transferencia electrónica a lo largo de la cadena a la vez que la transferencia directa del electrón al oxígeno aumentaría (Sachdev and Davies, 2008, Salo, et al., 1991).

La xantina oxidoreductasa

La xantina oxidoreductasa se puede encontrar como dos isoformas con actividad diferente, la XO y la xantina deshidrogenasa (XDH). En condiciones fisiológicas normales predomina la actividad XDH (produce NADH), mientras que en condiciones temporales de hipoxia o isquemia predomina la actividad XO (produce $O_2^{\cdot}$ y H_2O_2).

Parece ser que la XO tiene un papel importante en la generación de ROS durante el ejercicio. Inicialmente la XO fue identificada como una fuente potencial de radicales libres en el citosol de la célula muscular (Laughlin, et al., 1991), aunque en el músculo esquelético se localiza principalmente en el endotelio vascular (Linder, et al., 1999). Administrando un inhibidor de XO se previno la formación de GSSG, creatina quinasa (CK), aspartato aminotransferasa (AST) y MDA tras la realización de una sesión de ejercicio en humanos (Heunks, et al., 1999, Viña, et al., 2000). Además, administrando inhibidores de XO disminuye la liberación de $O2^{\cdot}$ en el espacio vascular en los músculos durante la contracción (Stofan, et al., 2000) y parcialmente inhibe la fatiga (Barclay and Hansel, 1991).

NADPH oxidasa

En un ejercicio exhaustivo aumenta la producción de especies reactivas por parte de neutrófilos y otras células fagocitarias (que

ejercerían una respuesta inmune en respuesta a una actividad física intensa o de larga duración). Los neutrófilos migran hacia los tejidos dañados para fagocitar las células dañadas, liberando O_2^- y lisozimas (Petrone, et al., 1980). La NOX es la enzima responsable de la denominada explosión oxidativa *(oxidative burst)* de los neutrófilos en respuesta a una infección o su estimulación. Diversos estímulos, como estímulos exógenos como el zymosan opsonizado, los lipopolisacáridos (LPS) o el miristato de acetato (PMA), interaccionan con diversos receptores tipo Toll (TLRs) propiciando el acoplamiento de las diferentes subunidades de la NOX en la membrana vacuolar, generando a partir de este momento una producción elevada de O_2^-, precursor de otras especies reactivas con que se enriquece la vacuola fagocitaria como el HOCl y el H_2O_2 (Knight, 2000, Lee, et al., 2003, Segal, 2005). También se han descrito diferentes isoformas de la NOX presentes en la membrana plasmática de multitud de tipos celulares, dotándola de importancia en el papel señalizador de las ROS que generan (Han, et al., 2013), siendo diana de tratamientos para combatir diferentes patologías como diabetes (Di Marco, et al., 2013), cáncer (Ushio-Fukai and Nakamura, 2008), desarrollo cerebral (Coyoy, et al., 2013), etc.

Células sanguíneas, especies reactivas de oxígeno y adaptación al ejercicio

Eritrocitos

Los eritrocitos son células anucleadas y representan el tipo celular más abundante y uno de los más especializados del cuerpo humano. Su principal función es transportar O2 a los diferentes tejidos. Se forman en la médula ósea y al salir al torrente sanguíneo pierden el núcleo, los ribosomas y las mitocondrias, por lo que pierden su capacidad de división, síntesis proteica y capacidad oxidativa mitocondrial (Volpe, 1993). Más del 95% de la proteína citoplasmática del eritrocito es hemoglobina (Hb), la cual posee un grupo hemo que permite la unión reversible al O2. La membrana contiene un 40% de lípidos, incluye fosfolípidos y colesterol en un ratio 1.2:1, y aproximadamente la mitad de los AGs presentan

insaturaciones. El combustible del eritrocito es la glucosa, la cual se metaboliza a través de glucólisis anaeróbica. Los eritrocitos tienen una vida media de 120 días (Ganz and Nemeth, 2012).

La elevada proporción de AGs insaturados en la membrana, el ambiente rico en O2 y los niveles abundantes de hierro del grupo hemo de la Hb hacen de los eritrocitos un tipo celular especialmente susceptible a sufrir daño oxidativo (Sureda, et al., 2009). Al no tener mitocondrias, los eritrocitos no pueden producir ROS mitocondriales, pero sí que producen continuamente especies reactivas debido al alto contenido en O2 y a su elevado contenido en grupo hemo (Baynes and Domoniczak, 2005). La Hb se puede oxidar produciendo $O2^{\cdot}$ y metahemoglobina (metHb), la cual no posee capacidad de unión al O2. La metHb puede ser reducida a Hb por el sistema NADH-citocromo b5-metHb reductasa, y en menor medida, por la metHb reductasa NADPH-dependiente (Kennett, et al., 2005).

El $O2^{\cdot}$ generado es transformado casi inmediatamente a una especie más estable, el H2O2. Esta especie puede provocar oxidaciones a la Hb, actuando como señales para la proteólisis (Giulivi and Davies, 2001). Además, un incremento de H2O2 se asocia a un incremento de metHb y de la peroxidación lipídica. A la vez, el H2O2 puede reaccionar con $O_2^{\cdot}$ y formar OH-, molécula altamente reactiva. A pesar de que el estrés oxidativo eritrocitario puede dañar la propia célula, la liberación de grandes cantidades de ROS a la circulación también puede dañar otros componentes de la circulación (Johnson, et al., 2005).

La incapacidad de los eritrocitos para reparar los componentes dañados por ROS mediante resíntesis (Clemens and Waller, 1987) puede causar una disminución en el número de eritrocitos en sangre (Banfi, et al., 2007). Se ha observado una disminución de Hb y hematocrito durante periodos de entrenamiento prolongados, con sesiones de actividad física intensa, en diferentes modalidades deportivas como el ciclismo, natación y correr, pero con menor evidencia en la práctica de fútbol y rugby (Banfi, et al., 2011).

Para contrarrestar la producción de ROS los eritrocitos contienen los enzimas SOD, catalasa, GPx, GRd y NADH-metHb reductasa (Knez, et al., 2007, Sureda, et al., 2005, Tauler, et al., 1999), como también el sistema GSSG/GSH y otros antioxidantes de bajo peso molecular como la vitamina C y E (Tauler, et al., 2003). Al no tener mitocondrias, la isoforma mayoritaria de la SOD es la Cu/Zn-citoplasmática. La presencia elevada de Cu/Zn-SOD permite la eliminación de $O2^{\cdot}$, previniendo la formación de metHb y peroxinitrito, de manera que la actividad SOD también regula la biodisponibilidad de NO (Gunduz, et al., 2004). El H2O2 producida por la SOD se puede destoxificar por la GPx y por la catalasa. La GPx es la primera barrera contra el H2O2 a bajas concentraciones, en cambio, a niveles incrementados la catalasa adquiere un papel predominante. Junto con estos sistemas antioxidantes también se ha descrito la función de proteasas que degradan selectivamente proteínas oxidadas, como la serina proteasa (Fujino, et al., 1998).

Además, los eritrocitos poseen antioxidantes no enzimáticos, como las vitaminas C y E, carotenoides y GSH. El GSH es el antioxidante mayoritario en eritrocitos, el cual protege a proteínas importantes como la espectrina, cuya oxidación puede provocar rigidez en las membranas, y mantiene los grupos sulfidrilo (-SH) de la Hb y otros enzimas en su estado reducido (Cimen, 2008). Los eritrocitos transportan glutatión por el torrente sanguíneo aportando un sistema importante de destoxificación en la circulación (Awasthi, et al., 2001, Dumaswala, et al., 2001).

Durante el ejercicio los músculos necesitan un mayor aporte de oxígeno, por lo que los eritrocitos deben transportar mayores cantidades de O2, lo que les hace más susceptibles de padecer estrés oxidativo. Después de un ejercicio se ha observado un incremento de la fluidez de la membrana eritrocitarias (Kamada, et al., 1993), así como una variación de la composición lipídica (Tepsic, et al., 2009) e incrementos en los marcadores de daño oxidativo (Sureda, et al., 2005, Tauler, et al., 2008).

Aunque los eritrocitos son células que no tienen capacidad de síntesis proteica son capaces de adaptar las defensas antioxidantes en

respuesta al estrés oxidativo asociado al ejercicio mediante mecanismos de regulación post-traduccional. Tras un duathlon las actividades catalasa y GPx aumentaron en eritrocitos (Tauler, et al., 2003, Tauler, et al., 1999). Tras una etapa ciclista se observó también un incremento de la actividad catalasa y GRd, pero también un descenso en la GPx (Aguiló, et al., 2005). Sin embargo, la activación de la maquinaria antioxidante puede no ser suficiente para contrarrestar el estrés oxidativo, puesto que en ciclistas se ha descrito un aumento de daño oxidativo en lípidos y proteínas (Muñoz Marín, et al., 2010, Sureda, et al., 2005).

Linfocitos

Los linfocitos constituyen entre el 20 y el 40% de los leucocitos totales y su función principal es reconocer antígenos específicos y mediar la respuesta inmunitaria específica o adquirida. Se pueden dividir en 3 poblaciones celulares en base a su función y composición de membrana: linfocitos B, linfocitos T y células natural killer (NK).

Algunos estudios se han centrado en la producción de ROS por parte de los linfocitos, y la importancia de éstas en la activación del linfocito frente a estímulos inmunitarios. Estimulado a los linfocitos con agentes mitogénicos como PMA o concanavalina A (ConA), así como las señales que estimulan los receptores de las células T (TCR), se produce un incremento en la generación de ROS por parte de estas células (Hume, et al., 1981, Williams and Kwon, 2004). Entre las ROS producidas tras la estimulación de los TCR se encuentran el H2O2 y el $O2^{\cdot}$, que parecen ser producidos por vías independientes (Devadas, et al., 2002). El metabolismo del AA (Los, et al., 1995), la mitocondria (Schulze-Osthoff, et al., 1993) y la NOX (Jackson, et al., 2004) parecen ser algunas de las principales fuentes de ROS del linfocito tras la activación del TCR. Como ocurre en otros tipos celulares, las ROS producidas tras la estimulación de receptores de membrana parecen estar implicados en procesos e señalización celular. Entre las dianas de las ROS generadas a este nivel se encuentran las tirosina fosfatasas (Williams and Kwon, 2004). Tras la activación de TCR empieza una

cascada de fosforilaciones sucesivas reguladas por proteínas tirosina quinasas y fofatasas que acaban formando el complejo de señalización, a partir del cual se activan diferentes vías de señalización, como MAPK o NFKß. En los linfocitos, las vías de las MAPK están involucradas en la regulación del ciclo celular, mientras que el NFKß participa en la regulación de la expresión de genes inflamatorios (Li and Verma, 2002).

Los linfocitos muestran una respuesta al ejercicio opuesta a la de los neutrófilos, el número de linfocitos aumenta durante la realización del ejercicio para luego caer drásticamente hasta niveles inferiores a los iniciales (McCarthy and Dale, 1988). Las actividades de lo enzimas antioxidantes tienden a aumentar tras un ejercicio intenso (Tauler, et al., 2003), pero esta respuesta es dependiente de la duración e intensidad del mismo. Así, tras una prueba de esfuerzo máximo de corta duración el número de linfocitos aumenta y las actividades catalasa y GPx disminuyen, mientras que tras una prueba de esfuerzo submáximo de mayor duración no se observa linfocitosis y la actividad GPx aumenta (Tauler, et al., 2004). Estos resultados apuntan a una posible inactivación temprana de los enzimas antioxidantes en respuesta a un incremento de los niveles de ROS, pero que luego es compensada incrementando los niveles o la actividad de estos enzimas si el estímulo persiste. En línea con este modelo, tras la realización de una etapa ciclista de montaña se observa la activación de catalasa, GPx y GRd y SOD, aunque esta activación de la maquinaria antioxidante no es suficiente para evitar la aparición de daño oxidativo (Sureda, et al., 2005, Tauler, et al., 2006). La SOD parece ser uno de los enzimas implicados en la adaptación al estrés oxidativo, tal como parece ser en el músculo esquelético. Tras una etapa ciclista sin tramos de montaña (con menor intensidad del ejercicio que en una etapa de montaña) se detecta únicamente la potenciación de la actividad SOD pero no del resto de enzimas antioxidantes, potenciación debida, en parte, a la activación de la expresión génica tanto de Cu/Zn-SOD y Mn-SOD (Cases, et al., 2006). Estos resultados apuntan a una respuesta adaptativa al ejercicio por parte del linfocito similar a la observada en

otros tejidos como el músculo esquelético, aunque no se puede descartar otros mecanismos reguladores.

Neutrófilos

La función principal de los neutrófilos en la respuesta inmune innata es llevada a cabo a través de toda una serie de respuestas rápidas y coordinadas, las cuales culminan con la fagocitosis y la eliminación de los patógenos (Nathan, 2002). Para ello, los neutrófilos contienen un diverso arsenal antimicrobiano, el cual consta de agentes oxidantes, proteinasas y péptidos antimicrobianos, y también producen cantidades importantes de RONS, como O_2^- y NO, a través de sistemas generadores de oxidantes, como puede ser la NOX y la NOS (Fialkow, et al., 2007). Durante la fagocitosis el neutrófilo libera en el interior del fagosoma los compuestos contenidos en diferentes gránulos (proteasas, fosfolipasas, glucosidasas y lisozimas) y RONS generados en la membrana del propio fagosoma, compartimentalizando así tanto al patógeno como a los productos citotóxicos, y facilitando la eliminación del patógeno. A pesar de esta estructuración celular, que se da en condiciones patológicas, los compuestos citotóxicos pueden ser liberados al espacio extracelular e inducir daño en los tejidos circundantes.

Las principales fuentes de RONS en el neutrófilos son la NOX, la cadena de transporte electrónico, el metabolismo de AA y las iNOS. La NOX de los neutrófilos es un complejo enzimático unido a su membrana, que al activarse genera grandes cantidades de ROS (Babior, et al., 2002), lo que se conoce como la explosión oxidativa. Esta enzima está latente en el neutrófilo inactivo, pero se activa rápidamente por toda una serie de mediadores solubles (péptidos quimioatrayentes y quimioquinas) y determinados estímulos (bacterias y complejos inmunes) que interactúan con los receptores de membrana. Su activación provoca la producción masiva de O_2^-, el cual se puede transformar rápidamente en otras ROS como H_2O_2, OH^- y HOCl, especies que serán liberadas en los fagosomas para oxidar proteínas y lípidos bacterianos. La aportación de la cadena de

transporte a la producción de ROS tiene poca importancia debido a que los neutrófilos poseen pocas mitocondrias. El metabolismo del AA por las cicloxigenasas y lipoxigenasas también genera ROS. La prostaglandina H sintasa, clave en la síntesis de prostaglandinas, prostaciclinas y tromboxanos, posee actividad cicloxigenasa e hidroperoxidasa, siendo esta última la responsable de la liberación de $O_2^{\cdot}$. La 5-lipoxigenasa, responsable de la síntesis de leucotrienos, también genera $O_2^{\cdot}$ (Fialkow, et al., 2007). Los neutrófilos poseen iNOS y pueden formar NO, el cual puede reaccionar con ROS como el $O2^{\cdot}$ dando lugar a peroxinitrito, un compuesto potencialmente citotóxico pero que también funciona en la modificación fisiológica por nitración de moléculas de señalización.

Para hacer frente a esta elevada producción de RONS los neutrófilos poseen un elaborado sistema de defensas antioxidantes, enzimáticos (catalasa, GPx, SOD, GRd, TrxR) como no enzimáticos (vitaminas C y E, glutatión). Además contienen cantidades importantes de ascorbato para preservar la integridad celular y la del tejido circundante a través de la neutralización de los productos bactericidas producidos durante la explosión oxidativa (Anderson, et al., 1987).

El principal efecto del ejercicio agudo sobre los neutrófilos es un incremento de su número de células circulantes (Ascensao, et al., 2008, Ferrer, et al., 2007, Ferrer, et al., 2009, Sureda, et al., 2009, Sureda, et al., 2006, Tsubakihara, et al., 2012). Esta movilización de los neutrófilos tras la realización de un ejercicio agudo es similar a la que se produce en un proceso infeccioso (Gravina, et al., 2012, Suda, et al., 2012). La neutrofilia se asocia con la intensidad y duración del ejercicio, pero también con la temperatura central del cuerpo que se alcanza durante el ejercicio (Mestre-Alfaro, et al., 2012).

Los neutrófilos participan activamente en la respuesta de fase aguda al ejercicio, activándose y produciendo grandes cantidades de especies reactivas en la explosión oxidativa. Se ha comprobado que tras un ejercicio intenso este incremento en el potencial oxidativo del neutrófilo va acompañado de un descenso en la actividad de los enzimas antioxidantes catalasa, SOD y GPx, así como los niveles de

glutatión total y del ratio GSSH:GSSG, y un incremento en los niveles intracelulares de ascorbato (Tauler, et al., 2002, Tauler, et al., 2003). Tras una etapa ciclista de montaña se observó una marcada neutrofilia así como una disminución de las actividades catalasa y GPx en neutrófilos, aunque estas menores actividades enzimáticas no iban acompañadas de la aparición de daño oxidativo en proteínas (Sureda, et al., 2005). Parece ser que el neutrófilo podría actuar aportando sus defensas antioxidantes al plasma sanguíneo para protegerlo así de la posible aparición de estrés oxidativo y, a pesar de quedar en cierta medida indefenso, el neutrófilo parece resistente a padecer una situación de estrés oxidativo. Se ha evidenciado el aumento de enzimas antioxidantes en el plasma en respuesta al ejercicio extremo, y estos enzimas, como la catalasa, pueden proceder de una secreción de los neutrófilos (Sureda, et al., 2007).

La suplementación con una bebida de almendras y enriquecida con vitamina E y C parece atenuar los daños oxidativos del ejercicio y aumentar las defensas antioxidantes en neutrófilos (Sureda, et al., 2013).

El entrenamiento como antioxidante

Si bien un episodio puntual de ejercicio aérobico y anaeróbico pueden generar daño oxidativo y conllevar una situación de estrés oxidativo (Ferrer, et al., 2009), la práctica regular de una actividad física moderada potencia las defensas antioxidantes y aporta una mayor resistencia a la aparición de estrés oxidativo (Tian, et al., 2010). Una explicación para el efecto positivo de la práctica regular de un ejercicio moderado es el fenómeno conocido como hormesis (Fisher-Wellman and Bloomer, 2009). La presencia de pequeños estímulos, como bajas concentraciones de RONS y antioxidantes de bajo peso molecular, podría inducir la expresión de enzimas antioxidantes y otros mecanismos de defensa (Calabrese and Baldwin, 2003, Carrera-Quintanar, et al., 2012, Ji, et al., 2006, Mestre-Alfaro, et al., 2011, Radak, et al., 2005, Tauler, et al., 1999). El ejercicio regular provoca la presencia continuada de estimulantes fisiológicos oxidantes, lo que provoca una adaptación frente al estrés oxidativo inducido por el

ejercicio, dando como resultado una mayor protección antioxidante (Fisher-Wellman and Bloomer, 2009, Radak, et al., 2008, Radak, et al., 2008), mediante diferentes mecanismos como un incremento de las actividades enzimáticas y de sus niveles proteicos (Ferrer, et al., 2010, Knez, et al., 2007, Pikosky, et al., 2006).

Animales entrenados muestran menores niveles de daño oxidativo muscular tras un ejercicio extenuante o tras la inducción de estrés oxidativo en comparación con animales no entrenados (Alessio and Goldfarb, 1988, Radák, et al., 2002, Radák, et al., 2000, Salminen and Vihko, 1983). Además, enzimas involucrados en la reparación del ADN también responden de forma positiva al entrenamiento, lo que implica una mayor resistencia a la aparición de lesiones en el ADN (Radák, et al., 2003, Radák, et al., 2002).

El entrenamiento en deportistas es un factor que influye en el nivel de daño oxidativo en los diferentes compartimentos sanguíneos, generalmente el daño oxidativo es mayor después de episodios sucesivos de ejercicio a lo largo de varias semanas, con un aumento de la peroxidación lipídica de las membranas eritrocitarias (Carrera-Quintanar, et al., 2012, Miyazaki, et al., 2001, Shin, et al., 2008). Sin embargo, no se ha observado este aumento de peroxidación lipídica en las membranas eritrocitarias de ratas entrenadas (Marsh, et al., 2006) o en linfocitos de futbolistas tras un periodo de 3 meses de entrenamiento (Ferrer, et al., 2009). Las actividades antioxidantes de los eritrocitos incrementan como resultado del entrenamiento, en concordancia a una protección antioxidante preventiva (Fielding and Meydani, 1997, Metin, et al., 2003, Shin, et al., 2008).

Individuos entrenados suelen poseer mayores actividades enzimáticas que los individuos no entrenados. En el músculo esquelético la actividad SOD se ha visto incrementada en respuesta a diferentes tipos de entrenamiento, pero también hay estudios que no detectaron estos cambios (Ji, 1999). Las diferencias observadas podrían ser debidas a las diferentes isoformas del enzima estudiadas y métodos de detección, a los diferentes modelos de entrenamiento y a los diferentes tipos de fibras estudiadas.

Omega-3 y estrés oxidativo

Los PUFAs, debido a sus dobles enlaces de carbono no conjugados, son susceptibles de oxidarse con mayor facilidad que los SFAs, dando lugar a hidroperóxidos, los cuales pueden iniciar reacciones en cadena, mediadas por radicales libres, potenciando la propia peroxidación lipídica. De hecho, se ha evidenciado que altas ingestas de DHA provocan daño oxidativo en las membranas de los eritrocitos, hepatocitos y riñón y en el ADN de la médula ósea (Song and Miyazawa, 2001). La oxidación de los PUFA puede potenciarse mediante la actividad lipooxigenasa (LOX), enzima presente en eritrocitos (Flamand, et al., 2006, Rádmark and Samuelsson, 2009). Por tanto, la suplementación de la dieta con PUFAs puede provocar un incremento del potencial prooxidante, comprometer el equilibrio oxidativo e inducir estrés oxidativo. Por otra parte, se ha evidenciado la existencia de correlaciones entre las defensas antioxidantes endógenas de los linfocitos y el daño oxidativo que se produce en eritrocitos inducido por una actividad física intensa (Sureda, et al., 2005). Así, un incremento de la fracción lipídica insaturada puede aumentar la capacidad de producir derivados oxidantes, pero, paradójicamente, una dieta rica en omega-3 parece favorecer el mantenimiento del estado antioxidante y reducir los marcadores de daño oxidativo (Bloomer, et al., 2009, Tayyebi-Khosroshahi, et al., 2010, Tur, et al., 2012). Sin embargo, se necesitan de más estudios para conocer las adaptaciones que se producen en las defensas antioxidantes endógenas frente a una ingesta elevada de PUFAs y su interacción con una actividad física intensa. Para mantener reducidos los PUFA suplementados en la dieta se recomienda que estos vayan acompañados con vitamina E, además de evitar posibles estados carenciales de esta vitamina liposoluble. La interacción entre la suplementación de la dieta con PUFA y antioxidantes está falta de estudios, y no se conocen los efectos sobre el daño oxidativo ni sobre el estado de las defensas antioxidantes endógenas. *A priori* se podría encontrar tanto efectos potenciadores como efectos inhibidores de las defensas antioxidantes endógenas y de los factores prooxidantes a tenor de lo descrito en relación de los efectos de los PUFA sobre la acción de PPAR y de NFK0.

OBJETIVOS Y PLANTEAMIENTO EXPERIMENTAL

OBJETIVOS

Los omega-3 son nutrientes esenciales con carácter antiinflamatorio, precursores de multitud de mediadores celulares con funciones relevantes en la regulación del proceso de inflamación aguda y crónica (Swanson, et al., 2012, Tur, et al., 2012). A la ingesta crónica de omega-3 se le atribuyen efectos adaptativos en la obtención de energía para la contracción muscular que afectan al rendimiento deportivo (Jeppesen and Kiens, 2012), incrementando la VO2max de los deportistas sin que se vea afectada la respuesta inflamatoria asociada a la actividad física. La mejora en el rendimiento deportivo está asociada a la incorporación de los omega-3 de la dieta a las membranas eritrocitarias, incrementado la capacidad de deformar los eritrocitos y aumentando así su accesibilidad a los tejidos periféricos y, por ello la capacidad de transporte de O2. Sin embargo, los omega-3 también afectan a la expresión génica mitocondrial de enzimas clave del catabolismo de los ácidos grasos incrementando la capacidad metabólica de obtención de energía a partir de los AGs a nivel muscular. En contraposición a estos efectos beneficiosos que afectan al rendimiento deportivo, los omega-3 tienen una marcada susceptibilidad a oxidarse e iniciar procesos de peroxidación lipídica aumentando el daño oxidativo (Richard, et al., 2008) que, dada su ubicación en la estructura de las membranas celulares, puede verse afectada en gran manera la consistencia de membranas celulares y favorecerse la lisis celular. El balance oxidativo celular también puede verse afectado por la ingesta de omega-3 dado que su capacidad de activar la expresión de genes que alteran la utilización energética de los ácidos grasos a nivel mitocondrial se atribuye a su interacción con factores de transcripción como los PPARs y NFKB (Huffman, et al., 2004). Estos mismos factores de transcripción participan de la regulación de la expresión de genes de enzimas antioxidantes e inflamatorios, por lo que los omega-3 podrían interaccionar con la expresión de estos genes antioxidantes. Por tanto, los omega-3 pueden tener una acción sobre el metabolismo mitocondrial que se pondría de manifiesto tanto en la potenciación metabólica del uso energético de los AGs y del O2 para

mantener la contracción muscular, pudiendo incrementar la producción de ROS, como en un incremento de la capacidad antioxidante celular. El balance de producción - eliminación de especies reactivas de oxígeno puede verse afectado por el consumo crónico de suplementos de omega-3, especialmente en situaciones de actividad física intensa. Hemos de tener en cuenta que, adicionalmente al incremento del potencial pro-oxidante que comporta el consumo de ácidos grasos poliinsaturados, durante la realización de una actividad física intensa también aumenta el consumo de O_2, las demandas energéticas, y la producción de RONS (Fisher-Wellman and Bloomer, 2009) con lo que el carácter pro¬ oxidante de los omega-3 podría exacerbar la situación de estrés oxidativo asociado al ejercicio agudo. Es preciso valorar como afecta una suplementación crónica con omega-3 al balance oxidativo en la realización de una actividad física aguda e intensa.

El consumo crónico de suplementos de omega-3 en deportistas no puede desligarse del hecho de que los deportistas entrenan de forma regular. La realización de una actividad física moderada y regular presenta diferencias respecto a la realización de una actividad física aguda e intensa. La práctica regular de una actividad física es beneficiosa para la salud y el bienestar (Bérard, et al., 1997, Domenéch, 2007, Friedenreich, et al., 1998, Hoffman-Goetz, 1998, McTiernan, et al., 1999). Por otra parte, la actividad física de intensidad elevada se asocia a un mayor riesgo cardiovascular, de lesión músculo-esquelética y de estrés oxidativo (Fisher-Wellman and Bloomer, 2009, Knez, et al., 2007, Nikolaidis, et al., 2012). Los deportistas entrenados presentan una alta capacidad antioxidante para hacer frente a la elevada generación de ROS inducida por una actividad física intensa. Aparentemente, en el entrenamiento regular se produce proceso de hormesis (Nikolaidis, et al., 2012, Radak, et al., 2008). La generación de dosis bajas de ROS provoca una adaptación de la capacidad antioxidante que permite hacer frente a la producción de dosis más altas de ROS cuando se practica un ejercicio agudo de alta intensidad. Los omega-3 como pro-oxidantes o como inductores de genes, pueden tener efectos que interaccionen con los procesos de adaptación que se dan durante un periodo prolongado de

entrenamiento, sobre el daño oxidativo que se va generando y sobre la capacidad antioxidante.

Los diferentes compartimentos sanguíneos, el plasma, eritrocitos, neutrófilos y células mononucleares periféricas (PBMCs, *peripheral blood mononuclear cells)* tienen diferentes maneras de producir y eliminar las RONS, representando diferentes modelos de estudio del estrés oxidativo. El plasma recoge la producción tisular de RONS (peróxido de hidrógeno, actividad NADPH oxidasa), tiene capacidad propia de producción de RONS (xantina oxidasa) y también recoge moléculas dañadas de diferentes tejidos durante el ejercicio, principalmente el tejido muscular que es el que se encuentra más activo durante el ejercicio. Las RONS pueden dañar los propios componentes plasmáticos, por ello el plasma presenta un conjunto de defensas antioxidantes enzimáticas (catalasa, SOD) que pueden actuar eliminando las ROS sin necesidad de cosustratos, y defensas no enzimáticas (otras moléculas antioxidantes como vitaminas antioxidantes, el ácido úrico, proteínas que mantienen ligados los metales pro-oxidantes) que permiten mantener el equilibrio oxidativo plasmático. Los eritrocitos son células especializadas en el transporte de O2, lo que conlleva un alto riesgo de daño oxidativo a los componentes celulares, necesitando de potentes mecanismos antioxidantes que permitan una correcta funcionalidad celular, entre ellos el sistema antioxidante GSSG/GSH y el sistema SOD/catalasa. Los eritrocitos maduros son células sin núcleo, sin expresión génica ni síntesis de proteínas, por lo que inicialmente deben presentar un bagaje con todo el contenido enzimático suficiente para hacer frente a una situación de estrés oxidativo. Las PBMCs representan un tipo de células en las cuales la producción de ROS mitocondrial es importante, los mecanismos antioxidantes y de regulación mitocondrial de producción de RONS operan para mantener la situación de equilibrio oxidativo. Entre ellas se encuentran los linfocitos, los cuales son muy sensibles frente a una situación de estrés oxidativo asociada a la actividad física intensa que puede incluso derivar en situaciones de linfopenia. Los neutrófilos son células inmunológicas con una producción de ROS lisosomal importante, aunque también se ha descrito una producción

mitocondrial. Al tener núcleo, las células que constituyen el acervo PBMC y los neutrófilos, pueden responder de una manera adaptativa a la producción de RONS.

Los efectos horméticos de las RONS también pueden ponerse de manifiesto con la suplementación de precursores de NO. El NO es una especie reactiva de nitrógeno que tiene importantes funciones reguladoras en diferentes niveles, cuya síntesis y metabolismo está muy regulada. Se han descrito diferentes moléculas como la arginina, la citrulina y el nitrato, cuya suplementación dietética produce una cierta potenciación de la producción de NO, con efectos diversos sobre el rendimiento deportivo y la situación de estrés oxidativo. La suplementación con arginina o citrulina se dirige hacia el incremento de la concentración del substrato de la óxido nítrico sintentasa (NOS), enzima responsable de la síntesis de NO. Sin embargo, la suplementación con nitrato es una vía alternativa a la NOS que necesita del concurso de las bacterias anaerobias de la cavidad bucal para su transformación primero en nitrito, que es asimilado, y, éste, por acción de enzimas como la XO u otras oxidasas, transformarse en NO. El mecanismo parece ser operativo de manera que se detecta una disminución significativa de la presión sanguínea a las pocas horas de ingerir nitrato. Parece ser que la ingesta de nitrato también mejora la eficiencia energética del deportista consiguiendo realizar el mismo trabajo con menores consumos de oxígeno (Bescós, et al., 2011). Como en el caso de los omega-3, el nitrato, como precursor de NO, tendría un carácter prooxidante pudiendo provocar un daño nitrosativo, principalmente debido a su reacción previa con $O_2^{\cdot}$ que genera NOO-, altamente reactivo. Así, el potencial prooxidante del nitrato podría incrementarse tras la realización de una actividad física intensa dado que también se genera $O2^{\cdot}$, aunque se debe tener en cuenta las defensas antioxidantes propias del deportista. Es necesaria una valoración del equilibrio oxidativo tras el ejercicio y del daño oxidativo derivada de la suplementación aguda con nitrato para mejorar el rendimiento deportivo.

El **objetivo general** de esta tesis es estudiar la acción de alimentos funcionales ricos en ácidos grasos esenciales sobre la situación de estrés oxidativo inducido por la actividad física aguda y sobre las adaptaciones al estrés oxidativo inducidas por el entrenamiento.

Este objetivo general se divide a su vez en los siguientes objetivos específicos:

1. Estudiar el efecto de una actividad física regular de entrenamiento deportivo y de la suplementación de la dieta con ácido docosahexaenoico (DHA) sobre el daño oxidativo en plasma y en células con diferentes sistemas de equilibrio oxidativo. Valorar si la suplementación con DHA conlleva un daño oxidativo asociado potencialmente perjudicial para la salud.
2. Evaluar los procesos de adaptación antioxidante inducidos por la realización de una actividad física regular de entrenamiento deportivo y de la suplementación de la dieta con DHA en plasma y en células con diferentes sistemas de equilibrio oxidativo. Valorar si la suplementación crónica con DHA afecta al proceso de adaptación que se produce con el entrenamiento deportivo.
3. Estudiar el efecto de una sesión de ejercicio físico agudo e intenso y la suplementación de la dieta con DHA sobre el daño oxidativo y las defensas antioxidantes en sujetos entrenados.
4. Estudiar el efecto de la repetición de sesiones intensas de ejercicio físico sobre la acumulación de daño oxidativo y de marcadores de inflamación en sujetos entrenados.
5. Valorar si una suplementación con nitrato mejora el rendimiento deportivo en deportistas entrenados y si conlleva un daño oxidativo.

PLANTEAMIENTO EXPERIMENTAL

Para llevar a cabo los objetivos de la presente tesis se han diseñado tres experimentos con sujetos voluntarios. Se cumple con los requisitos indicados en la Declaración de Helsinki para experimentación humana y cuyos protocolos fueron previamente aprobados por el Comité Ético de Investigación Clínica de las Illes Balears (CEIC-Illes Balears, número de expediente IB 994PI).

Todos los participantes de los diferentes experimentos han accedido voluntariamente a su participación tras haber sido debidamente informados del objetivo del estudio, de los posibles riesgos y de firmar la hoja de consentimiento informado. Los participantes fueron varones, no fumadores, con escaso o nulo consumo de alcohol y seguían una dieta equilibrada, planificada por los respectivos servicios dietéticos de los clubs deportivos.

El planteamiento experimental para llevar a cabo el estudio de los efectos del entrenamiento, del ejercicio intenso agudo y repetitivo, y de los suplementos dietéticos esta detallado a continuación.

Experimento 1. Efectos de una actividad física repetitiva sobre marcadores de oxidación e inflamación en ciclistas de élite.

Los sujetos participantes en este experimento fueron ciclistas de la Vuelta al Bidasoa del 2008. Una semana antes de la prueba física los 8 participantes en el estudio completaron un cuestionario médico, un examen cardiopulmonar y electrográfico y un control biológico (hematológico y bioquímico) de acuerdo con la Unión Internacional de Ciclismo. La prueba deportiva consistió en 4 etapas ciclistas realizadas en 4 días consecutivos. La realización completa de la prueba implicó un recorrido de 460 km con 9 puertos de montaña.

Los tres primeros días de competición se obtuvieron 2 muestras sanguíneas, la primera en estado basal antes de la prueba y

la segunda 3 horas después de la prueba. En el cuarto día solo se obtuvo la muestra en estado basal. Las muestras de sangre obtenidas se fraccionaron para la obtención de plasma y suero.

Este experimento se recoge en el Manuscrito I. En suero se han determinado una serie parámetros bioquímicos como alanina aminotransferasa (ALT), AST, cortisol, CK, creatinina, fosfatasa alcalina (AP), lactato deshidrogenasa (LDH), mioglobina (MB) y proteínas totales. En plasma se ha determinado un marcador de daño en lípidos (MDA), niveles de nitrito y marcadores de inflamación como interferón gamma (IFNy), IL1B, IL2, IL6 y TNFa.

Experimento 2. Efectos de una suplementación crónica con DHA sobre la respuesta del balance oxidativo al entrenamiento deportivo de futbolistas y al ejercicio agudo e intenso.

Se diseña un estudio a doble ciego en el cual se realiza una intervención nutricional que empieza al inicio de una temporada deportiva futbolística. La duración de la intervención es de 8 semanas y consiste en el consumo de una bebida funcional rica en DHA o una bebida placebo.

Los sujetos participantes en el estudio son 22 futbolistas del Reial Club Deportiu Mallorca en los inicios de la temporada 2011¬ 2012. Se distribuyen aleatoriamente en un grupo placebo (11) o en un grupo experimental (11). Durante el desarrollo del estudio tan sólo pudieron completar con todos los requisitos 15 deportistas, 6 en el grupo placebo y 9 en el grupo experimental. Las causas de las bajas fueron en todos los casos ajenas al estudio, ya que dejaron de entrenar en el equipo del RCD Mallorca B por lesión o por entrenar con la primera plantilla del RCD Mallorca.

Al inicio del estudio se realiza una valoración nutricional y antropométrica de los participantes para comprobar que los participantes de ambos grupos experimentales presenten unas dietas y características antropométricas similares. Durante la intervención

nutricional los participantes consumen un litro de una bebida (placebo o experimental) cinco veces a la semana. Las ingestas se realizan los días de entrenamiento, al inicio. Las dos bebidas utilizadas como vehículo tienen una base de almendras, son de composición similar, con la diferencia de que la experimental está enriquecida con DHA. El enriquecimiento se realiza mediante triglicéridos ricos en DHA, con baja presencia de ALA y EPA. Tanto la bebida placebo como la experimental contienen un nivel alto de antioxidantes (vitamina E) para protegerlas de la oxidación antes de su consumo y equilibrar la mayor ingesta de ácidos grasos poliinsaturados (pro-oxidantes) que implica la intervención nutricional. La recomendación está establecida en 0.6 mg equivalentes de tocoferol para 1 g de PUFAs (FAO, 1995). Al final de las 8 semanas de suplementación los sujetos realizan una sesión de entrenamiento convencional, con una actividad física intensa, de 2 horas de duración, incluyendo la realización de un test de capacidad máxima (el test de *Leger Boucher),* para determinar indirectamente la VO2max, y de ejercicios tácticos que simulan situaciones propias de competiciones futbolísticas, que requieren una alta intensidad de actividad física. En las bebidas vehículo se han determinado los ácidos grasos presentes y la cantidad de vitamina E.

En total se practican tres extracciones de sangre, una en condiciones basales al inicio del estudio y otras dos al final del estudio, una en condiciones basales y otra tras 2 horas de acabar el ejercicio agudo e intenso que conlleva la sesión de entrenamiento convencional. Se fraccionan las muestras sanguíneas para obtener plasma, suero, eritrocitos, PBMCs y neutrófilos.

Los datos de este experimento se recogen en los Manuscritos II, III, IV y V.

En las muestras sanguíneas obtenidas se analizan los siguientes parámetros hematológicos y bioquímicos: Hb, hematocrito, volumen corpuscular mediano (MCV), hemoglobina corpuscular media (MCH), concentración de hemoglobina corpuscular media (MCHC), ancho de distribución de glóbulos rojos (RDW). Además de se ha hecho el recuento de eritrocitos, leucocitos, linfocitos, monocitos, eosinófilos, basófilos, PBMCs y neutrófilos.

En suero se determinan los niveles de nitrito y nitrato, y parámetros clínicos de inflamación, daño celular y del metabolismo proteico, lipídico y glucídico: ácido úrico, albumina, ALT, AST, bilirrubina, colesterol HDL, colesterol LDL, colesterol total, creatinina, gamma-glutamil transpeptidasa (YGT), glucosa, LDH, proteína C reactiva (CPR), proteínas totales, triglicéridos y urea.

En plasma se determinan los ácidos grasos no esterificados (NEFAs) y los ácidos grasos de triglicéridos (TGFAs). Se ha puesto a punto una metodología cuantitativa, segura y rápida, para determinar una amplia variedad de NEFAs y TGFAs plasmáticos, la cual se recoge en el Anexo I. Además se determinan en plasma marcadores de daño oxidativo en lípidos (MDA), de daño oxidativo (índice de carbonilos) y daño nitrosativo (índice de nitrotirosinas) en proteínas. Como marcadores antioxidantes plasmáticos se determinan actividades de enzimas antioxidantes (catalasa y SOD) y niveles de vitamina E.

Con los eritrocitos aislados se determinan los ácidos grasos individuales y marcadores lipídicos y proteicos de daño oxidativo y nitrosativo (MDA, carbonilos y nitrotirosina). Como marcadores antioxidantes se determinan actividades enzimáticas antioxidantes (catalasa, GPx, GRd y SOD) y niveles de proteínas antioxidantes (catalasa, GPx, GRd y Cu/Zn-SOD).

Con las PBMCs aisladas se determinan marcadores de daño oxidativo y nitrosativo en lípidos (MDA), proteínas (carbonilos y nitrotirosina) y ADN *(comet assay).* Como marcadores antioxidantes se determinan actividades de enzimas antioxidantes (catalasa, GPx y GRd), niveles de proteínas antioxidantes (catalasa, GPx, GRd, Cu/Zn-SOD, Mn-SOD, TrxRI, UCP2 y UCP3) y de iNOS y expresión de genes antioxidantes (catalasa, GPx, Cu/Zn-SOD, Mn-SOD y UCP3). Además se determinan niveles de nitrato y la capacidad de producción de ROS de las PBMCs mediante su activación con LPS o PMA.

Con los neutrófilos aislados se determinan marcadores lipídicos y proteicos de daño oxidativo y nitrosativo (MDA, carbonilos y nitrotirosina). Además se han determinado actividades enzimáticas (catalasa, GPx, GRd y MPO), niveles proteicos (catalasa, GPx, GRd,

Mn-SOD, TrxR1, UCP2 y UCP3) y expresión de genes (catalasa, GPx, GRd, MPO y Mn-SOD). También se determinan niveles de nitrato y la capacidad de producción de ROS de los neutrófilos tras su activación con PMA o zymosan.

Experimento 3. Efectos de una suplementación con nitrato de sodio sobre el rendimiento deportivo y el daño nitrosativo.

Es un experimento a doble ciego con 13 sujetos en el cual los mismos sujetos toman el suplemento experimental ($NaNO_3$) o el placebo (NaCl). Se realizan dos pruebas físicas de la misma intensidad, una habiendo tomado la suplementación de nitrato y la otra habiendo tomado la suplementación placebo.

Los participantes en el estudio fueron ciclistas y triatletas pertenecientes al Centro de Alto Rendimiento de Sant Cugat. Los participantes realizaron 3 ingestas en 3 días consecutivos, antes de desayunar, de nitrato o cloruro de sodio disueltos en agua. En el tercer día los sujetos realizaron una prueba física tres horas después de la suplementación. Los participantes siguieron una dieta baja en nitratos los 2 días antes de la prueba física. Se dejaron 4 días de separación para que los participantes tomaran la otra correspondiente suplementación.

La prueba física se realiza en una bicicleta ergométrica en la cual deben recorrer la máxima distancia posible en 40 minutos. Durante la prueba se miden las principales variables respiratorias. Semanas antes del estudio los sujetos se familiarizan con la bicicleta ergométrica, el analizador de gases y el procedimiento a seguir. Se toman 2 muestras sanguíneas para la obtención de plasma, la primera después de la suplementación con nitrato o placebo y antes de la prueba física, y la segunda tras 3 minutos de realizar la prueba física. Además se toman 2 muestras de orina, la primera después del periodo de suplementación antes de la prueba física y la segunda al cabo de una hora tras realizar la prueba física.

En plasma se determina el daño nitrosativo en proteínas (índice de nitrotirosina) y los niveles de endotelina-1 (vasoconstrictor). Tanto en plasma como en orina se han determinado los niveles de nitrito y nitrato.

Este experimento se recoge en el Manuscrito VI.

RESULTADOS Y DISCUSIÓN / RESULTS AND DISCUSSION

MANUSCRIPT I

Plasma cytokine inflammatory markers in elite cyclists throughout a competition

PLASMA CYTOKINE INFLAMMATORY MARKERS IN ELITE CYCLISTS THROUGHOUT A COMPETITION

ABSTRACT

Purpose: to determine the changes in the basal and post-exercise plasma markers of muscular damage and cytokines in response to a three consecutive-day cycling competition.

Methods: blood samples were obtained before each stage in basal conditions and up to 3 h after the race and on the morning of the fourth day. Markers of muscular damage were determined in serum by standardized clinical methods. Oxidative damage markers and nitrite were determined in plasma by spectrophotometric methods and citokyne levels were determined by inmunoassays.

Results: creatine kinase activity, lactate dehydrogenase activity, myoglobin and malondialdehyde, creatinine and nitrite levels followed a sawtooth-type representation throughout the competition, with post-race values higher than respective basal ones. On each consecutive day, the elevated post-race levels decreased and returned to basal levels. MDA showed an accumulative pattern, evidenced in the post-race values of the third stage which were significantly higher with respect to the values of the first stage. Cortisol levels were significantly influenced by an interaction between the exercise and the stage factors, with higher values on the fourth day. Plasma cytokine levels were only determined before the first stage and post-race, after the third stage. The exercise increased TNFa, IL6, IL2 and IFNy levels, whereas IL1B was unchanged.

Conclusion: cyclist stages induced oxidative and cellular muscle damage which is recovered to basal values by the next morning. Repetitive stages during the cycling competition accumulated plasma oxidative damage markers and pro-inflammatory cytokines, probably as a result of local inflammatory responses.

Keywords: cytokines; exercise; inflammation; oxidative stress

INTRODUCTION

The finding that exercise promotes oxidative stress in humans was first reported over 30 years ago [1]. Since this early report, growing evidence indicates that although high levels of reactive oxygen species (ROS) production can damage cellular components, low-to-moderate levels of cellular oxidants play important regulatory roles [2-4] and even up-regulate antioxidant defenses. Chronic exercise can up-regulate antioxidant defenses, particularly in tissues directly involved in exercise [5]. However, muscle-damaging exercise or even non-muscle-damaging exercise alters the blood redox homeostasis, causing oxidative and nitrosative damage [6, 7]. Eccentric contractions increase the production of ROS and induced oxidative stress in the blood, peaking in most cases 2-3 days after exercise and returning towards baseline afterwards [8-11]. In races lasting multiple days, a portion of the race must be performed while the body is affected by physiological perturbations usually only observed during recovery [12], such as tissue damage [13], elevations in serum concentrations of inflammatory markers [14] and oxidative damage [15]. Scarce information concerning oxidative damage and its repair in races lasting multiple days is available [12]. The rise in ROS production does not have to be considered detrimental, since it represents stimuli for an up-regulation in endogenous antioxidant defenses [1, 16]. ROS released from muscle, endothelial, and immune cells are necessary to stimulate adaptation to intense physical exercise. This provides adaptive protection from ROS during subsequent training sessions as well as during non-exercise-related conditions [17].

It is well established that contracting skeletal muscle *per se* is the main source of interleukin-6 (IL6) in the circulation in response to exercise [18, 19]. There is a consensus regarding the anti-inflammatory effects of regular acute exercise [20, 21] and even intense exercise induces an anti-inflammatory response [14, 21, 22]. On the other hand, pro-inflammatory cytokines, tumor necrosis factor-alpha (TNFa) and interleukin-1 (IL1) increase in plasma during and after eccentric prolonged exercises such as a marathon [12, 23]. It

has been hypothesized that the exercise-induced increases in TNFa and IL6 are a consequence of an immune response to local damage in the working muscles [24, 25]: TNFa initiates the breakdown of damaged muscle tissue, and IL6 stimulates the proliferation and differentiation of satellite cells [3]. Thus, both cytokines play a very important role in muscle reconstruction after strenuous exercise and in the development of tolerance to ROS-induced muscle damage [26]. However, the pro-inflammatory cytokine response is minimal whereas the anti-inflammatory cytokines are induced after the Western Australia Ironman triathlon race despite evidence of muscle damage and an immune acute phase response [14]. Adaptation to regular exercise also includes changes in the immune and energy status related to cytokine synthesis such as TNFa and IL6 [27-29]. Little information is available on the effects of repetitive exercise over races lasting multiple days on plasma inflammatory markers [30].

Since exercise has been shown to alter the levels of cytokines and oxidative markers, it provokes an anti-inflammatory response, we attempted to evaluate the changes in basal and post-exercise plasma markers of ROS activity and inflammatory mediators including IL6 and TNFa, over a cycling competition lasting four days. The aim of this study is observe the effect of repetitive stages of intense exercise, such as multi-stage cycling race, on the oxidative damage and if the elevated post-race oxidative markers returns to basal conditions the next-day race or if exist an accumulative pattern which can provoke a pro-inflammatory situation.

MATERIALS AND METHODS

Human volunteers

Eight male well trained semiprofessional cyclists participated in this study (Table 1). Cyclists were participating in the "Vuelta al Bidasoa 2008" which is characterized in Figure 1. All participants were informed about the research protocol and volunteered to participate in the study. The study was designed in compliance with the recommendations for clinical research of the Declaration of Helsinki

of the World Medical Association and was approved by the local ethics committee. Furthermore, all participants signed a written informed consent. All participants completed a medical questionnaire and a cardiopulmonary and electrocardiographic examination and biological control according to International Cycling Union (UCI) mandatory (hematological and biochemistry) parameters one week prior to the onset of the study. None of the participants smoked, drank alcohol, or were taking medications known to alter the hormonal response. Concomitant pathology was discarded by clinical rapport and medical examination. All participants followed a similar diet supervised by the medical doctor of the team.

The cycling competition involved the completion of 460 km, with nine mountain passes, over four consecutive days. The part of the cycling competition followed in this study corresponds to the first three stages: 351 km long, with six mountain passes. Each cycling stage began at 14:00 - 15:00 h. The longest stages were the first and second stages whereas the mountain passes with the greatest degree of difficulty were in the third stage (Figure 1). The classification of the mountain passes was established by the race organizers as follows: first, second and third category. With these parameters, the first stage had two category 3 passes; the second stage had two passes: category 2 and 3; and the third stage had two category 1 passes. The evaluation was carried out over 4 daily consecutive stages, before and at the end of the race. The last stage was a short stage and we only analyzed the moment before the competition because the cyclists had to leave the competition zone immediately after finishing the race. The time for completing the stages by the participants in the study was significantly the same for the first and the second, and shorter for the third and there were no differences between the average speeds.

Experimental procedure

Blood samples were obtained from the antecubital vein from all participants participating in the study before each stage in basal conditions and 3 h after the race. Gel vacutainers were used for biochemical determinations and heparinized vacutainers were used to

obtain plasma. Serum and plasma were obtained after centrifugation of the blood samples at 1,000 x g and stored (-80°C). All biochemical parameters were corrected according to the observed hemodilution, calculated on the basis of the changes in plasma protein levels.

Biochemical analysis

Serum activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), creatine phosphokinase (CK), myoglobin (MB), lactate dehydrogenase (LDH), creatinine and total proteins (TP) were measured by spectrophotometry in an autoanalyzer (Hitachi 917, Japan). Serum levels of cortisol were determined using a radioimmunoassay kit (Diagnostic Products Corporation, USA).

Malondialdehyde determination

MDA as a marker of lipid peroxidation was analyzed in plasma using the colorimetric assay kit Bioxytech LPO-586 (Oxis Research, Portland, OR, USA) following the manufacturer's instructions. Absorbance was measured at 586 nm.

Nitrite determination

Nitrite levels were determined in plasma by the acidic Griess reaction [31] using a spectrophotometric method [32]. Plasma samples were deproteinized with cold sulfosalicylic acid 6% at room temperature (30 min). Samples were centrifuged for 15 min at 10,000 x g at 4°C, and supernatants were recovered. A hundred microliters of samples or nitrite standard solutions were added to 50 ul of sulfanilamide (2% w/v) in 5% HCl, and 50 ul of N-(1-naphthyl)-ethylenediamine (0.1% w/v) in water was later added. Absorbance at 540 nm was measured after an incubation of 30 min.

Cytokine determination

Plasma samples were used for cytokine determination (interleukin-1-beta [IL1|3], interleukin-6 [IL6], interleukin-2 [IL2], interferon-gamma [IFNy] and tumor necrosis factor-alpha [TNFa]), using a commercially available enzyme immunoassay kit LincoPlex (Linco Research, St. Charles, MO, USA) with intra- and inter-assay precision values (VC) < 10 % and < 12 %, respectively. All experiments were conducted in duplicate. A standard curve was obtained based on the standards provided by the manufacturer. The values obtained below the standard range were considered a non-measurable value.

Statistical analysis

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS v.13.0 for Windows). Results are expressed as mean ± SEM and $p<0.05$ was considered statistically significant. All data were tested for their normal distribution (Kolmogorov-Smirnov test). The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analyzed were the stage (S) and the exercise (E). When significant effects were found, a one-way ANOVA was used to determine the differences between the data-groups involved.

RESULTS

The exercise performed in the different stages induced an increase in myoglobin levels as a marker of muscular damage, MDA levels as a marker of lipid peroxidation, and nitrite levels as a marker of NO synthesis (Figure 2). All these markers gave a sawtooth-type representation, with post-race values higher than respective basal ones. On each following day, the elevated post-race levels decreased returning to basal levels, although MDA and nitrite tended to maintain their basal levels slightly higher than the basal levels on the last day. This accumulative pattern in MDA levels was evidenced in post-race values of the third stage which was significantly higher with respect to the values of the first stage.

A similar profile pattern was also observed in CK and LDH activities and in creatinine levels (Table 2). CK activity, significantly affected by exercise and the stage, progressively increased after each stage with the highest values at the end of the third stage. LDH activity and creatinine levels were significantly influenced by exercise but not by the stage. LDH activity only reported significant differences after the first stage, whereas creatinine levels reported significant differences between the first and third stages. The cellular damage markers AST, ALT and AP were not affected by the physical activity performed in the different stages and did not present an accumulative effect over the cycling competition. Total protein (TP) presented lower levels as of the third stage, with a post-race value significantly lower than the first and second stages. Moreover, the post-race levels of the two first stages presented a higher concentration of TP in serum, but not significantly. Cortisol levels were significantly influenced by an interaction between the exercise and the stage factors, with the highest values before the beginning of the fourth stage.

Plasma cytokine levels corresponding to basal first stage and post-race third stage are shown in Table 3. The exercise increased TNFa levels 2.7 times, IL6 levels 1.4 times, IL2 levels 2.2 times, and IFNy levels 2.0 times. IL10 was not affected by the exercise.

DISCUSSION

The present results reveal that well-trained elite cyclists respond to a strenuous, long-duration, repetitive exercise such as a 4-day cycling tour by increasing the plasma levels of inflammatory and oxidative stress markers. Exercise-induced muscle damage is succeeded by biochemical changes within the circulation that can be detected by measuring muscle enzyme activities such as CK, LDH and myoglobin levels in serum, together with increases in pro-inflammatory cytokines [25, 33]. In the present study, competitive maximal exercise during a race competition was accompanied by increases in these muscle markers. Mechanical stress in addition to metabolic stress are believed to be the most important initial factors

leading to exercise-induced muscle damage [34]. As befits, our results show an increase in cortisol levels accompanying the increases in biochemical markers of muscular damage. The exercise and time-of-day not affect cortisol and IL6 [35]. Moreover the circadian rhythm of cortisol is abolished in elite athletes [36]. Circulating myoglobin and CK levels were significantly higher after the race and CK was also increased at rest with respect to the previous day. These competition-induced changes in biochemical markers could probably be related to subsequent muscle repair processes and local inflammatory responses. Accordingly, it is well established that muscle repair following acute muscle injury includes an initial pro-inflammatory phase during which the muscle releases cytokines and the site of damage is infiltrated by immune cells that coordinate the removal of dead muscle cells [37]. Even though this is a desirable response in terms of muscle repair, and probably also muscle adaptation [38], it may trigger further muscular inflammatory processes and damage, partly through the increased formation of ROS [39].

In this study, we have also reported significant increases in IL6, TNFa, IFNy and IL2. High concentrations of interleukin-6 in skeletal muscle after exercise are a consistent finding in the literature [19, 25, 26, 40, 41]. However, the appearance of IL6 in the circulation depends on several factors, including the type, intensity, and duration of exercise. Plasma IL6 has been found to be higher during running than during cycling, and has been associated with the recruitment of more muscle groups in the former [42]. Moreover, the peak IL6 increase also occurred within the 5 to 20 min period following exercise [43], this maximal signal decrease with time [44]. This observation may account for the moderate increase in plasma IL6 found in our study [45]. It has been evidenced that muscle in contraction produces IL6, which was the first myokine described with a function related to obtaining energy substrates by the muscle and maintaining muscular contraction [18]. TNFa in plasma does not change after acute exercise in most exercise studies [18]. An anti-inflammatory response to acute exercise initiated by IL6 muscle secretion which in turn activates the secretion of IL10 and interleukine-1 receptor antagonist (IL1ra) by immune cells that could avoid the inflammatory effects of TNFa has

been indicated [21]. IL10 suppresses the production of inflammatory cytokines and an anti-inflammatory agent, IL1ra. In addition, the suppressive effects of IL10 result in the inhibition of the functions mediated by T cells, monocytes/macrophages and natural killer (NK) cells [46-48]. The simultaneous presence of increased levels of IL6 and TNFa are indicative of an inflammatory situation [20]. The increased IL6 levels found after a cycling stage are associated to the IL6 function in terms of energy availability to maintain muscle contraction by stimulating both glucose and lipid metabolism [49, 50]. However, the high elevated levels of TNFa, IL2 and INFy could be related to an inflammatory response induced by exhaustive exercise. Acute exercise induces an anti-inflammatory response, but we detected an inflammatory response induced after repetitive sessions of exhaustive, prolonged exercise such as a cycling competition [20]. In this way, the observations of increased values of TNFa after exercise are scarce and only evident when exercise is very intense [51-54]. Several factors of stress such as heat shock protein 60 (HSP60) have been revealed to be able to activate cytokine production by the same pathway as lipopolysaccharide (LPS) [55, 56]. Although these factors were not analyzed in this study, we did observe significant signs of muscle damage after the cycling stage that could be related to the synthesis of stress factors by the muscle. In fact, the inflammatory response to LPS induces the synthesis of INFy and IL2 by Th1 cells [57]. Similarly, we detected increased plasma levels of INFy and IL2 after the third cycling stage. Thus, our results suggest changes in pro-inflammatory cytokines after exercise that may reflect an inflammatory state which is the consequence of an excessive amount of exercise, although the stimuli that induce the production of pro-inflammatory cytokines by immune cells after exhaustive exercise are unknown.

Despite the fact that skeletal muscle is relatively resistant to exercise-induced oxidative damage, it is clear that intense and/or prolonged muscular activity can result in harmful outcomes [58]. In addition, exercise causes an increase in immunological and biochemical markers of muscle damage, and also increases the production of ROS. Damaging exercise also induces an inflammatory

response, which further increases ROS formation [59]. Single bouts of aerobic and anaerobic exercise can induce an acute state of oxidative stress [60]. Although a single bout of exercise often leads to acute oxidative stress, in accordance with the principle of hormesis, such an increase appears to be necessary for an up-regulation of endogenous antioxidant defences [1]. We have observed an increased lipid oxidative damage level induced by acute exercise after each cycling stage but this oxidative damage marker regained the basal values after 18 hours of recovery. Non-muscle-damaging exercise induces alterations in redox homeostasis that last a few hours post exercise with a rapid return of redox biomarkers to the resting values. However, the alterations in redox biomarkers persist for and/or appear several days after muscle-damaging exercise [6]. Numerous recent studies in healthy sedentary humans [43, 61, 62] and also in patients with chronic fatigue syndrome [63] or chronic respiratory insufficiency [64], have demonstrated that the maximal signal of thiobarbituric acid reactive substances (TBARS, another marker of lipid peroxidation than MDA) occurs 5-10 min after exercise and decrease with time, although we detect elevated MDA levels after 3 h post-exercise. The accumulative effect of daily repetitive stages on lipid oxidative damage markers was evidenced in the plasma levels of MDA which was significantly higher after the third stage than after the first stage. Plasma levels of nitrite, a marker of NO synthesis, were also significantly increased after each cycling stage, returning to initial basal levels after 18 hours of recovery. NO synthesis is activated during each cycling stage. In this way, Cazzola et al. evidenced that the increase in the levels of oxidative damage after a football match occurred despite resting conditions in players regularly involved in training and competition when compared with sedentary controls [65]. McBreide et al. reported that plasma MDA was also found to be elevated after heavy resistance exercise involving upper and lower body muscles in recreational weight training exercises [66]. Along these lines, we have previously observed that oxidative damage markers are directly correlated with antioxidant enzyme activities after a cycling stage [32, 67].

In summary, the cyclist stages induced oxidative stress, evidenced by an increase in biochemical markers which could be related to subsequent muscle repair processes and probably to local inflammatory responses, and which is partially recovered to basal values by the next morning. Repetitive stages during the cycling competition accumulated plasma oxidative markers and pro-inflammatory cytokines. The changes in pro-inflammatory cytokines and plasma oxidative damage markers after exercise may reflect an inflammatory state as a consequence of an excessive, repetitive amount of exercise.

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CONFLICTS OF INTEREST

The authors declare that they do not have any conflict of interest.

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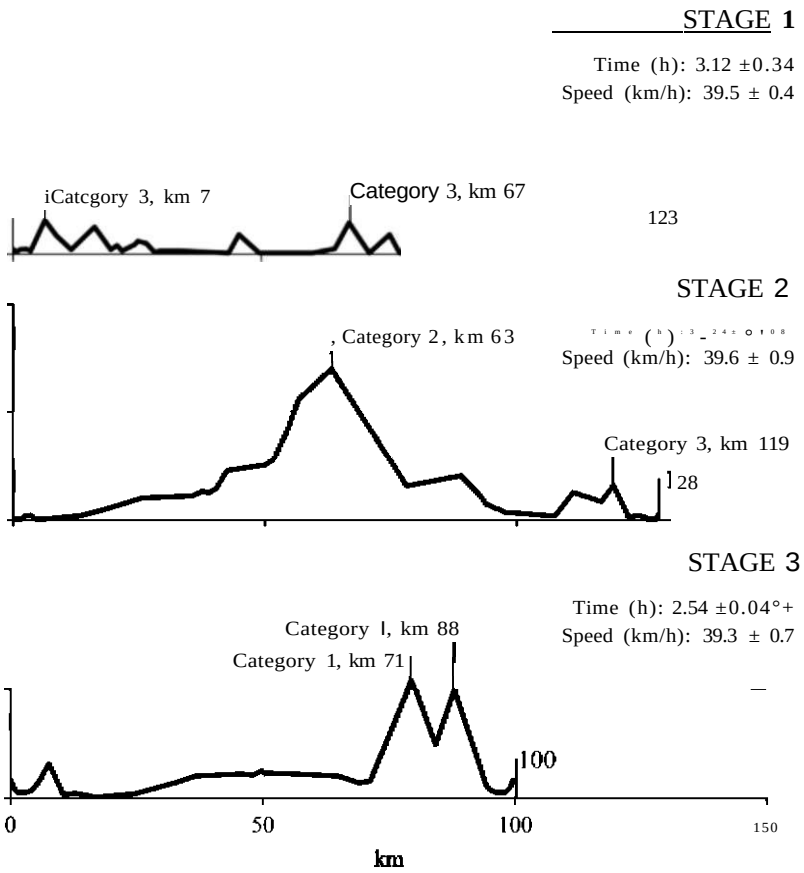

Figure 1. Statistical analysis: One-way ANOVA, $p<0.05$. (°) Significant differences between first stage, (t) Significant differences between second stage.

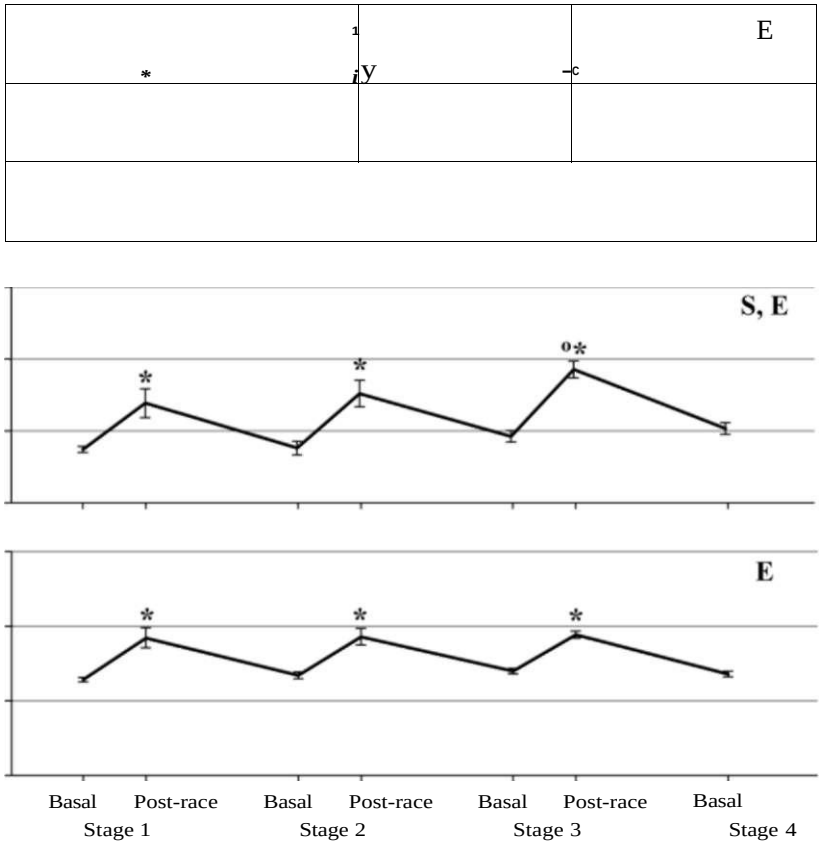

Figure 2. Statistical analysis: Two-way ANOVA, $p<0.05$. (S) Significant effect of stage, (E) Significant effect of exercise. (SxE) Significant interaction between factors. One-way ANOVA, $p<0.05$. (°) Significant differences between first stage, (t) Significant differences between second stage, (t) Significant differences between third stage, (*) Significant differences between basal and post-race.

Table 1. Physical characteristics of cyclists

PARAMETER	MEAN	±	SD
Age (years old)	20.3	±	0.9
Height (cm)	178	±	3
Weight (kg)	68.8	±	4.3
Body fat percentage (%)	9.50	±	0.70
VO_2 max (mL- kg^{-1}-min^{-1})	73.2	±	6.7

Table 2. Biochemical analysis

		STAGE				ANOVA		
		1	2	3	4	S	E	SxE
CK	Basal	94.4 ± 17	141 ± 17	166 ± 14°	172 ± 18°	X	X	
(U/L)	Post-race	125 ± 16	181 ± 15°	198 ± 26°				
LDH	Basal	222 ± 22	265 ± 18	243 ± 29	257 ± 8.4		X	
(U/L)	Post-race	296 ± 23*	307 ± 21	307 ± 23				
AST	Basal	20.6 ± 2.0	22.2 ± 2.5	24.6 ± 2.5	24.9 ± 2.4			
(U/L)	Post race	23.1 ± 1.9	25.7 ± 2.4	27.6 ± 3.0				
ALT	Basal	14.8 + 2.2	15.8 + 1.6	17.0 + 2.1	16.1 + 1.9			
(U/L)	Post race	17.1 ± 2.0	17.5 ± 1.8	18.4 ± 2.3				
AP	Basal	63.8 + 5.0	63.7 ± 4.8	63.2 + 4.4	63.5 + 4.5			
(U/L)	Post-race	69.8 ± 5.1	71.2 ± 4.6	66.1 ± 4.4				
Creatinine	Basal	0.82 ± 0.04	0.86 + 0.06	0.92 + 0.06	0.98 + 0.09		X	
(mg/dL)	Post-race	1.07 ± 0.07*	0.98 ± 0.10	0.99 ± 0.08*				
Protein	Basal	7.28 ± 0.18	7.18 + 0.10	6.78 + 0.19	6.94 + 0.14	X		
(g/dL)	Post-race	7.72 ± 0.11	7.64 ± 0.09	6.72 ± 0.36°*				
Cortisol	Basal	11.1 ± I.Oac	17.1 ± I.Oab	13.9 ± I.Oabc	18.2 ± 2.9b			X
(Hg/dL)	Post-race	18.4 ± 3.5b	15.7 ± 3.8abc	9.44 ± 1.6c				

Statistical analysis: Two-way ANOVA, $p<0.05$. (S) Significant effect of stage, (E) Significant effect of exercise. (SxE) Significant interaction between factors. One-way ANOVA, $p<0.05$. (°) Significant differences between first stage, [$) Significant differences between second stage, (t) Significant differences between third stage, (*) Significant differences between basal and post-race.

Table 3. Cytokines

	Basal	Post-race
TNFa (pg/mL)	1.41 ± 0.35	3.84 ± 0.75*
IL1ß (pg/mL)	0.433 ± 0.054	0.375 ± 0.055
IL6 (pg/mL)	8.08 ± 0.87	11.4 ± 4.2*
IL2 (pg/mL)	0.866 ± 0.450	1.88 ± 0.36*
IFNy (pg/mL)	2.65 ± 0.34	5.19 ± 0.54*

Data correspond to basal first stage and post-race third stage. Statistical analysis: (*) One-way ANOVA, $p<0.05$.

MANUSCRIPT II

Effect of omega-3 fatty acids diet supplementation on plasma fatty acids availability and oxidative stress during training season and acute exercise

EFFECT OF OMEGA-3 FATTY ACIDS DIET SUPPLEMENTATION ON PLASMA FATTY ACIDS AVAILABILITY AND OXIDATIVE STRESS DURING TRAINING SEASON AND ACUTE EXERCISE

ABSTRACT

Purpose: diet supplementation with omega-3 fatty acids (omega3-FAs) could influence the plasmatic fatty acid profile and the oxidative balance. The aim was to determine the effects of the diet supplementation with 1.14 g/day of docosahexaenoic acid (DHA) on the plasma oxidative balance after training and acute exercise.

Methods: fifteen volunteer male soccer players were randomly assigned to either a placebo group that consumed an almond-based beverage or to an experimental group that consumed the same beverage enriched with DHA for eight weeks. Blood samples were taken at basal conditions at the beginning of the nutritional intervention and after eight weeks of training in basal and in post¬ exercise conditions.

Results: the experimental beverage increased the plasma DHA availability in non-esterified fatty acids (NEFAs) and triglyceride fatty acids (TGFAs) and increased the polyunsaturated fatty acid (PUFA) fraction of NEFAs but had no effects on the biomarkers for oxidative balance in plasma. During the training season, plasma markers for oxidative damage of proteins, the degree of haemolysis and the antioxidant enzyme activities increased, but did not affect lipid oxidative damage. Acute exercise did not alter the basal levels of plasma markers for oxidative and nitrosative damage of proteins and lipids, nor the antioxidant enzyme activities.

Conclusion: the training season and acute exercise, but not the DHA diet supplementation, altered the pattern of plasma oxidative damage, as the plasma antioxidant system proved sufficient to prevent the oxidative damage induced by acute exercise in well-trained soccer players.

Keywords: omega-3, DHA, plasma, oxidative stress, exercise, training

INTRODUCTION

Regular physical activity invokes a healthy body adaptation against elevated oxidant levels by increasing the cellular and plasma antioxidant capability [1, 2]. Acute exercise induces lesser adaptation than regular exercise against oxidative stress [3], and antioxidant capability can be overwhelmed by oxidant production. A long-term stimulation of the endogenous defense mechanism by regular physical activity results in a constant presence of physiological oxidant stimuli which can overload the antioxidant system [4], although it also can induce an adaptive response to counteract the oxidative stress. This adaptation process is performed by different mechanisms, such as an increase in antioxidant enzyme activities [5-7] and a regulation in mitochondrial quality, i.e., the mitochondrial life cycle span, biogenesis, maintenance, and clearance [8]. Omega-3 fatty acids (omega3-FAs) are polyunsaturated fatty acids (PUFAs) that have greater oxidation capabilities than saturated fatty acids (SFA) [9, 10]. Omega3-FAs or their derivatives can exert their functions by interacting with different nuclear transcription factors as PPARs, NFKB, (HNF)-4a, LXR, SREBP [11], which also mediate gene expression of antioxidant enzymes [12, 13]. An increase in omega3-FAs intake and alteration of the cellular membrane composition to a more unsaturated FA component could affect cellular oxidative equilibrium, mainly when a physiological situation increases the production of reactive oxygen species (ROS), which is what occurs during intense acute exercise [3, 14]. Paradoxically, a diet rich in omega3-FAs seemed to favor the maintenance of antioxidant status and the reduction of oxidative damage markers [15-17]. Nonetheless, several studies with male athletes point out pro-oxidant effects of omega-3 diet supplementation [18]. An experiment using an omega-3 supplement in male athlete nutrition showed a significant increase in oxidative stress at rest and after a judo-training session [18]. Supplementation with omega3-FAs alone significantly increased F(2)-isoprostanes after exhaustive exercise [19] although combining flavonoids and antioxidants with omega3-FAs was effective in reducing the immediate postexercise increase in F(2)-isoprostanes [20].

The aim of the present study was to determine the effects of diet supplementation with DHA on the plasma NEFAs and TGFAs composition and on the plasma oxidative balance in soccer players during a training season and after acute exercise. We determined the influence of diet supplementation with DHA, training and acute exercise on the plasma antioxidant enzyme activities, on the oxidative and nitrosative damage markers, on the cellular damage plasma markers, on the plasma NEFAs and TGFAs composition and on the plasma markers of lipid, nitrogen and carbohydrate metabolism.

MATERIALS AND METHODS

Subjects and study design

The study was done with 15 male soccer players (Real Mallorca B team), at the beginning of their annual sport season. Participants in the study were professional, federated soccer players, 19.7 ± 0.4 years old and 76.5 ± 2.5 kg of weight. All the participants followed a Mediterranean diet with a similar energy, carbohydrate and lipid intake.

Subjects were randomly selected for participation for one of two groups: placebo and experimental. The placebo group was made up of 6 subjects who took one liter of a placebo drink five times a week, while the experimental group was made up of 9 other subjects who consumed one liter of a DHA enriched experimental drink rich for five times a week, over a total period of 8 weeks. There were no differences in anthropometric characteristics (height, waist circumference, hip circumference, systolic blood pressure, diastolic blood pressure, body mass index, waist-hip ratio and fat mass and fat-free mass) or physical activity capabilities (VO_2max, intense physical activity time and moderate physical activity time) between the placebo and experimental group of the soccer players. All subjects were informed of the purpose and demands of the study before giving their written consent to participate. The study protocol was in accordance with the Helsinki Declaration for research on human subjects and was approved by the Ethical Committee of Clinical

Investigation of the Comunidad Autonoma de les Illes Balears (Palma de Mallorca, Balearic Islands, Spain).

Drink composition

The two drinks were elaborated by Liquats Vegetals S.A. (Girona, Spain) and were composed of 3.0% almond and 0.8% sucrose and the rest was water, flavor and the added oils and vitamin E. Moreover, the placebo contained 0.8% refined olive oil and the experimental contained 0.6% refined olive oil and 0.2% DHA-S Market (Market Biosciences Corporation, Columbia, EEUU). Externally the two beverage types were identical in their taste and visual appearance.

Experimental procedure

Three different blood samples were obtained for each subject. One blood sample was taken under basal conditions at the beginning of the nutritional intervention, and another two blood samples were taken at the end of the nutritional intervention, under basal and post¬ exercise conditions. The exercise consisted of a 2 hour habitual physical training session. Briefly, after 15 min of warm-up, the players performed the Leger Boucher test to indirectly determine VO2max. After that, the players did a recovery exercise of control-passing for 15 min. The main body of the training session was characterized by small-sided soccer games. Briefly, the first exercise consisted of a 5 player vs 5 player possession exercise in an area of 20 x 15 m (4 repetitions of 5 min with 30 sec of recovery between repetitions); the second was a 6 vs 6 possession exercise in an area of 30 x 20 m (3 repetitions of 6 min with 1 min of recovery between repetitions), and finally, the players played a football match 5 vs 5 in 30 x 40 m for 20 min.

Venous blood samples were obtained from the antecubital vein of control and experimental men with suitable vacutainers containing EDTA as anticoagulant. Plasma was obtained after centrifugation (900 x g, 30 min, 4°C). Serum was obtained after centrifugation (1,000 x g, 30 min, 4°C) of another vacutainer sample without anticoagulant, obtained as described above.

Fatty Acids determinations

Beverage (5 uL) lipid content was extracted with 5 mL of chloroform-methanol mixture (2:1v/v) by the method of Folch et al. [21], containing 0.01% butylated hidroxyanisole (BHA) as antioxidant and 2 uL of n-heptadecanoic acid (C17:0, 15 mM) as internal standard. Plasma NEFAs (100 uL) were diluted in 5 mL methanol:water (1:1 v/v) containing 0.01 % BHA, 2 uL of p-toluenesulphonyl fluoride (TSF, 50 mM) as lipase inhibitor and 2 uL of C17:0 (15 mM). Total plasma FAs (10 uL) were used for reaction with 200 uL Triglyceride Reagent (SIGMA) containing lipase and diluted in 4 mL methanol:water (5:3 v/v) containing 0.01 % BHA and 2 uL of C17:0 (15 mM). The NEFAs and total FAs were extracted by a column chromatographic method, with a Carbopack™ 60/80 (Supelco) as a stationary phase and 10 mL chloroform:methanol (1:1 v/v) as an eluent. The resultant organic phases were evaporated under a nitrogen stream at 55°C. The dry residue was dissolved in 75 uL of n-hexane and 25 uL of the derivatization reagent Meth-Prep™ II (GRACE) was added. An aliquot of 1 uL was injected into the gas chromatograph with helium as a mobile phase. The gas chromatograph was a Agilent 5890 model with a flame ionization detector (FID) and the column was a Supelcowax® 10 Capillary GC column, 30 m x 0.53 mm, d_f 0.50 u.m. Individual fatty acids and mix of fatty acid methyl esters (Supelco®) were used for the identification of the chromatography peaks .

Quantification was performed from the internal standard (C17:0) and the responses of the different fatty acids in the FID was corrected by a response factor calculated from the areas of the standard fatty acids of separate chromatograms with several fatty acid concentration standards and the area of the C17:0 obtained from the same chromatograms. The TGFAs were calculated resting NEFAs from total FAs.

Malondialdehyde determination

Malondialdehyde (MDA), a marker of lipid peroxidation, was analyzed in plasma using a colorimetric assay kit (Calbiochem). Briefly, samples and standards were placed in eppendorfs containing n-

methyl-2-phenylindole (10.3 mM) in acetonitrile:methanol (3:1, v/v). HCl 12 N was added and the samples were incubated for 1 h at 45°C. Absorbance was measured at 586 nm. This method is specific for MDA determination.

Assay of Nitrotyrosine and Protein Carbonyls

Plasma protein carbonyl derivates (10 u.g of protein) and nitrotyrosine (150 u.g) were determined by immunological methods using the OxiSelect™ Protein Carbonyl Immunoblot Kit (Cell Biolabs, INC) and OxiSelect™ Nitrotyrosine Immunoblot Kit (Cell Biolabs, INC) following the manufacturer's instructions. Total protein concentrations were measured by the Bradford method [22]. Samples were transferred to a nitrocellulose membrane by the dot blot method. Image analysis was performed using Quantity One-1D analysis software (Bio-Rad Laboratories).

Nitrite and Nitrate determination

Serum samples were centrifuged (15,000 x g, 30 min, 4°C) in 10 K filters (Amicon® Ultra; Millipore) to remove proteins. The supernatants was recovered and used to measure nitrite and nitrate concentration by detecting the liberated NO in the gas-phase chemiluminescence reaction with ozone, with the use of a NO analyzer (NOA 280i; Sievers).

Enzymatic determinations

Catalase (CAT) activity was measured in plasma by the spectrophotometric method of Aebi [23]. Superoxide dismutase (SOD) activity was measured in plasma by an adaptation of the method of McCord and Fridovich [24]. All activities were determined with a Shimadzu UV-2100 spectrophotometer at 37°C.

Vitamin E determinations

Vitamin E was determined in beverages and plasma samples. The extraction of the liposoluble vitamins was carried out using n-hexane after deproteinization with ethanol containing 0.2% butylated hydroxytoluene (BHT). Vitamin E concentration was determined after

drying the samples under a nitrogen current and dissolving them in methanol. The mobile phase consisted of acetonitrile:tetrahydrofuran:water (550:370:80, v/v/v). The HPLC was a Shimadzu equipped with a diode array detector and a Nova Pak C_{18}, 3-9 x 150 mm column with a-tocopherol was determined at 290 nm. Quantification was done with external patron (Sigma-Aldrich).

Serum markers of lipid, nitrogen and carbohydrate metabolism

The serum determinations of glucose, urea, creatinine, uric acid, total cholesterol, triglycerides (TG), cholesterol HDL and LDL, bilirubin, total protein and albumin were done in an autoanalyser (Technicon DAX System). Plasma volume loss was calculated from the total protein concentrations at pre and post-exercise [25]. This value was used to calculate the plasma concentration of all parameters analyzed in order to avoid the effects of plasma volume loss on the concentration of the parameters after exercise.

Plasma cellular damage and inflammatory markers

The serum activities of aspartate aminotransferase (AST), alanine transaminase (ALT), gamma-glutamyl transpeptidase (YGT), lactate dehydrogenase (LDH), creatine phosphokinase (CPK) and C-reactive protein (CRP) were and one by autoanalyser (Technicon DAX System).

Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS v.15.0 for Windows). Results are expressed as mean ± SEM and $p<0.05$ was considered statistically significant. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). Two of these statistical tests are realized independently, one test analyzed the statistical factors supplementation (S) and time of supplementation and training (T) in basal conditions at the initial and final period of supplementation. The other test analyzed S and the acute exercise (E) realized at the final of the experiment. When significant effects were found, a one-way

ANOVA was used to determine the differences between the groups involved. All the data were tested for their normal distribution.

RESULTS

The FA composition of beverages reflected the enrichment of the experimental beverage with DHA-S Market oil, rich in C22:6n3 (Table 1). The experimental beverage presented significantly higher concentrations of the FAs C20:3, C22:0, C22:5 and C22:6n3 whereas they were undetected in placebo beverage. Additionally, the experimental beverage presented significantly higher concentrations of FAs C16:0, C16:1 and C20:1 than the placebo beverage. The daily beverage intake of the experimental group was about 1.14 g of DHA in addition to the basal diet omega3-FA intake whereas for the placebo group, the only omega-3 intake was from the diet. The daily intake of vitamin E from the diet and from the supplemented beverages was similar in both placebo and experimental group during the nutritional intervention. The beverage supplement provided a 2.6 times higher dose than the recommended dietary vitamin E allowance (RDA) for the general population in both the placebo and experimental group.

The overall composition of plasma NEFAs remained practically unchanged amongst the group with nutritional intervention with DHA enriched or the placebo beverages, although some individual fatty acids changed (Table 2). The C16:0, C20:0 and the total of saturated NEFA were lower at the end of nutritional intervention than at the beginning, independent of the type of placebo or experimental beverage supplemented. The plasmatic non-esterified monounsaturated fatty acids (MUFA) levels remained at the initial values after the nutritional intervention throughout 8 week treatment, however the plasma C16:1 NEFA increased in both placebo and experimental groups. The basal plasma non-esterified PUFAs content decreased mainly in placebo group at the final of the nutritional intervention; in contrast, the trend of change of experimental group was to maintain the initial non-esterified PUFA levels. The plasma non-esterified PUFAs levels of the experimental group were significantly higher than the placebo one at the final of

nutritional intervention. In fact, the plasma content of C22:6n3 NEFA significantly increased at the final of nutritional intervention respect to the initial values. The total plasma NEFAs content did not change for the 8 week treatment nor was influenced by the intake of beverage type. Acute exercise increased the total plasma non-esterified MUFA and the C16:1 and C18:1 NEFAs contents in both the placebo and experimental group. Acute exercise maintained the basal plasma SFAs, PUFAs, and total NEFAs levels. The diet supplement influenced the plasma levels of some NEFAs. The plasma NEFAs C16:0, C22:0 and C22:6n3, PUFA and total NEFAs content were significantly higher in the experimental group.

The composition of plasma TGFAs presented minor changes in contrast to the plasma NEFAs throughout the experiment (Table 3). The SFA, MUFA, PUFA and total TGFAs did not change during nutritional intervention or exercise, independently of placebo type or experimental drink. The consumption of the two beverages during the 8 week period increased the C16:1 TGFAs in a way similar to the C16:1 NEFA. Significant differences were observed between groups for the C18:2 and C18:3n3 TGFAs from the very beginning of the nutritional intervention. The C22:6n3 TGFA showed a similar pattern as the C22:6n3 NEFA, increasing in levels in the experimental group during the nutritional intervention and continued to rise in post-exercise conditions. The C20:4n6 TGFA of the experimental group presented higher levels than the placebo group, and these were significant in post-exercise conditions.

The training season significantly affected the plasma basal values of the carbonyl index (a marker of protein modification) in both groups (Table 4). The carbonyl index increased about 1.6 and 1.7 times in the placebo and experimental groups, respectively. This protein oxidation was accompanied by a significant increase in plasma catalase activity, (about 3.0 in placebo and 4.6 times in experimental groups, respectively), and with a significant increase in SOD activity (2.9 and 3.4 times, in placebo and experimental group, respectively). The basal MDA levels (marker of lipid peroxidation) at the end of the nutritional intervention presented values similar to those of the initial basal levels in both groups. The nitrotyrosine index (a marker for

nitrosative damage), nitrite, nitrate and vitamin E levels were not affected by training season, supplement or beverage consumption. Acute exercise performed after 8 weeks of intervention only significantly affected the nitrite and nitrate levels, increasing nitrite 1.6 and 1.4 times, and nitrate 1.3 and 1.2 times, in the placebo and the experimental groups, respectively.

The serum markers of cellular damage, and lipid, carbohydrate and nitrogen metabolism are shown in table 5. In basal conditions, the muscular damage markers, creatinine and uric acid, significantly decreased their levels after 8 weeks of training and supplementation period. Increased fat intake from diet and the different beverages did not affect the levels of TGs. The experimental group presented higher levels of cholesterol and cholesterol LDL than placebo group at the start and throughout the nutritional intervention, and continued under post-exercise conditions. The acute exercise provoked a hypoglycemia after 2 h post-exercise in the soccer players and an increase in the uric acid levels. Moreover, the acute exercise induced a hemoconcentration reflected by the greater concentration of total protein levels in post-exercise conditions, for this reason all post¬ exercise data were corrected with the correspondent basal protein levels to eliminate the effects of plasma volume loss.

Serum markers of cellular damage such as: LDH, AST, ALT, Y G T , CPK, and the serum PCR levels as a marker of inflammation, are shown in table 6. The basal levels of LDH after 8 weeks of training were higher than the basal initial values, and this increase was higher in the placebo group (1.3 times) than in the experimental group (1.1 times). However, neither acute exercise nor DHA diet supplementation influenced the serum activity of LDH. AST, ALT, Y G T , CPK and PCR were not influenced by training season, DHA diet supplementation nor acute exercise. It is not able that all soccer players in all experimental conditions showed CPK values higher than the normal limit (30 - 200 U/L) and also of those of the post-exercise levels of LDH (125 - 243 U/L).

DISCUSSION

The daily intake of one liter of the experimental drink provides an additional 1.14 g/day DHA to the diet. The daily intake of PUFAs is about 11 g/day for people with similar characteristics that follow the habits of the Mediterranean diet [26]. The diet supplementation with 1 L daily of the beverage provided an increase of about 12% of the total lipid intake. The amount of DHA intake in the experimental beverage is similar to those reported in several nutritional intervention studies [18, 27], although lower than those used in other nutritional intervention studies [17, 19, 28-31]. Nutritional intervention trials with omega3-FAs supplements in male athletes indicated the effects on the plasma FA composition [17, 19, 27, 31], but they did not differentiate the NEFA from TGFA or the phospholipids, although some studies performed with healthy yet not athletically active people-differentiated these two fractions of plasma FAs [32-34]. Nutritional intervention with placebo and experimental beverages had an influence on the FAs composition of plasma TGFAs and also changed the profile of plasma NEFAs. The DHA diet supplementation was reflected in the plasma by an increase in DHA levels in the experimental group with both an increase of NEFAs and TGFAs. This specific increase of DHA has been observed in other studies after diet supplementation with fish oil and DHA-enriched oil [16]. Plasma albumin carries FAs in the two forms, as lysophosphatidylcholine (LPC), which delivers into the erythrocytes, and as NEFA which supplies this FA mainly to platelets [35], although it is also available as fuel for exercising muscle [36]. Supplementation with omega3-FAs increased the structural FAs, such as the membrane phospholipids [37], but also increased the availability of these FAs for energetic functions. Regular exercise training *per se* influences the phospholipid FAs composition of muscle membranes, with the phospholipids richer in DHA, but has no effect on the composition of the FAs stored in TG within the muscle [38]. The changes in composition of the NEFA fraction as a result of the nutritional intervention and training increased the energy available to maintain muscle contraction more in the experimental group than the placebo one because the long-chain PUFAs have a higher energetic density

than SFAs [39, 40]. During light exercise, adipose tissue-derived NEFAs make up the majority of the oxidative fuel used by muscle, and the importance of plasma NEFAs increases when exercise is prolonged [36]. Moreover, DHA diet supplementation in the experimental beverage altered the pattern of change in the NEFAs induced by training. The consumption of omega3-FAs could alter the use of FAs as fuel to support the physical activity by altering the muscular availability of FAs that are needed to induce muscle adaptation for a more efficient use of FAs.

Acute exercise does not influence the TGFAs but does increase the total plasma MUFAs content in the NEFAs fraction. Exercise mobilizes the fatty stores in order to facilitate NEFAs availability to contracting muscle [36, 41, 42]. The different pattern of change induced by acute exercise in the FAs of NEFA fraction and TGs, together with the lack of effects of acute exercise on the levels of plasma TGs, could reflect a limited use of TGFAs to sustain the energy demands of exercise. Levels of plasma C16:0, C22:0, C22:6n3, PUFA and total NEFAs content are higher in the experimental group than placebo group. The consumption of the experimental beverage increases the availability of plasma NEFAs to sustain the energy demands of exercise. However, the plasma NEFAs availability did not prevent the decrease in plasma glucose levels 2 hours after acute exercise.

Training season and acute exercise, but not DHA supplementation, altered the plasma markers of oxidative damage. The higher presence of MUFAs and PUFAs in circulating lipids of the experimental group did not influence the degree of oxidative damage in the lipid fraction during training season or after acute exercise. It is in agreement with other studies that have described a lack of changes in lipid oxidation after diet supplementation with PUFA [43, 44]. However, the blood antioxidant system cannot avoid the oxidative damage to plasma proteins during the training season. The carbonyl groups could be formed by direct oxidation of some side chain amino acids, but also by the reaction with advanced glycation or advanced peroxidation end products [45, 46]. Thus, the formation of carbonyl proteins could be not parallel to the formation of MDA; and the

increase in the oxidative damage in proteins, but not in the lipid fraction during training season, could indicate a greater susceptibility of proteins to direct oxidation of some side chain amino acids or by a result of the reaction with advanced glycation end products that results from lipid peroxidation end products. The acute exercise practiced by trained soccer players did not alter the basal levels of plasma markers of oxidative and nitrosative damage of the proteins and lipids. The endogenous and exogenous plasma antioxidant system is sufficient to avoid oxidative damage induced by acute exercise of a normal training session in well-trained footballers. The accumulation of workouts and matches during the training season increases the plasma antioxidant enzyme activities. It has been described that acute exercise in soccer players increases lipid oxidative markers [47], but regular training reduces plasma MDA levels [48]. Regular exercise, on the basis of its possible hormesis effects, acts as antioxidant and could reduce the oxidative damage [49]. Our findings are in agreement with previous studies reporting increased antioxidant enzymes in plasma in response to extreme exercise [50, 51]. In the present work, the increased antioxidant activity could be produced by the antioxidant content of both the placebo and experimental beverages (40 mg/day of vitamin E and the antioxidant phenols of the almonds). In previous studies, an antioxidant diet supplement attenuated the appearance of oxidative damage markers and increased the antioxidant enzyme activities in neutrophils [14, 52, 53]. The training season reduced the plasma uric acid levels, which are only statistically significant in the placebo group. Uric acid is the end product of purine nucleotide catabolism during exercise, which increases the degradation of adenine nucleotides and the transformation of xanthine dehydrogenase into xanthine oxidase [54]. Plasma xanthine oxidase activity generates plasma uric acid, together with the production of the superoxide anion. Plasma uric acid levels respond to intense exercise and to vitamin C diet supplementation [55]. It cannot be discarded that a decrease in plasma ROS production by xanthine oxidase during the training season could be reflected by the significant decrease in the plasma uric acid concentration. Acute exercise increases the plasma uric acid levels [50, 54-56] that could be indicative of an increased anion superoxide production by plasma

xanthine oxidase during acute exercise. Moreover, the acute exercise increased the serum nitrite and nitrate levels. It is suggested that the nitrate-nitrite-NO pathway is mainly activated under anaerobic and acidic conditions [57]. Nitrite is a sensitive marker of NO synthesis which provides a nitric oxide synthase (NOS) independent source for NO generation [57, 58].

Exercise-induced muscle damage has been reported in several studies [59-61]. Muscle damage leads to a temporary loss of the exercising capacity for force production and has implications for increases in muscle post-exercise muscle soreness [62]. The training season increased the levels of some serum markers of hemolysis and muscular damage such as serum LDH activity. Serum CPK activity, a marker for muscle protein release, presented higher levels than the high-normal limit of the population but was not affected by the training season. Moreover, acute exercise maintains the high basal values of these serum muscle damage markers. The increased LDH activity and the maintenance of the serum CPK activity by the training season could reflect hemolysis rather than muscle damage after training season. The high activity of serum CPK from the beginning could a result the muscle mass of the footballers. In fact, female athletes with less muscular mass have been shown to have lower CPK serum activities than male athletes [63]. Neither acute exercise nor t diet supplementation with DHA altered the serum cellular damage markers of well-trained footballers. The surplus intake of DHA did not influence the adaptations to exercise practiced along the eight weeks, except by a lower increment of LDH activity and the higher acid uric levels in experimental the group.

In summary, the diet supplementation with a beverage rich in DHA during the regular training season of soccer players increased the DHA availability in plasma in both NEFAs and TGFAs fractions. The change in plasma NEFAs profile induced by the DHA diet supplementation was more energetic than the induced by the placebo. Acute exercise did not influence the FAs composition of plasma TGs but it increased the availability of plasma MUFAs content of the NEFA fraction. The training season and acute exercise, but not the DHA diet supplementation, altered plasma oxidative damage. The

endogenous and exogenous plasma antioxidant system was enough to avoid oxidative and nitrosative damage induced by acute exercise in well-trained footballers.

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CONFLICTS OF INTEREST

The authors declare that they do not have any conflict of interest.

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Table 1. Fatty acid composition of beverages

	Placebo			Experimental		
C16:0 (uM)	1799	±	173	3607	±	620 *
C16:1 (uM)	275	±	23	591	±	115 *
C18:0 (uM)	1135	±	156	1928	±	423
C18:1 (uM)	10635	±	2602	22503	±	5605
C18:2 (uM)	5459	±	1161	12177	±	2996
C18:3n6 (uM)	104	±	38	298	±	152
C18:3n3 (uM)	394	±	55	455	±	44
C20:0 (uM)	92.4	±	4.9	116	±	7
C20:1 (uM)	19.4	±	1.4	35.9	±	2.3 *
C20:2 (uM)	277	±	20	282	±	14
C20:3 (uM)		ND		20.9	±	2.2 *
C20:4n6 (uM)	161	±	10	140	±	9
C22:0 (uM)		ND		75.6	±	1.7 *
C22:5 (uM)		ND		1715	±	70 *
C22:6n3 (uM)		ND		3457	±	117 *
SFA (%)	11.9	±	2.0	9.90	±	1.15
MUFA (%)	57.4	±	7.2	51.7	±	5.0
PUFA (%)	30.7	±	2.7	38.3	±	4.4 *
Vitamin E (mg/L)	41.6	±	17.8	45.7	±	27.7

ND non detect. Statistical analysis: Student's t-test for unpaired data. (*) Significant differences between placebo and experimental, $p < 0.05$.

Table 2. Composition of plasma non-esterified fatty acids

		Initial	Final		ANOVA			ANOVA		
		Basal	Basal	Post-exercise	S	T	SxT	S	E	SxE
14:0	Placebo	6.9S ± 1.21	7.29 ± 2.72	4.64 ± 0.80						
<HM)	Experimental	5.22 + 0.29	4.27 ± 0.38	7.13 ± 1.12						
16:0	Placebo	165 ± 32	66.6 ± 5.8 #	84 + 8		X		X		
(p.M)	Experimental	116 ± 14	102 ± 16	120 ± 15						
16:1	Placebo	3.55 ± 0.69	6.33 ± 0.71 #	13.4 ± 3.4		X			X	
(|iM)	Experimental	3.50 ± 0.55	7.79 + 1.15 #	17.5 ± 3.5$						
18:0	Placebo	91.0 + 9.8	92.6 + 34.4	70.2 ± 4.2						
(HM)	Experimental	93.5 ± 5.9	75.3 ± 12.4	74.9 ± 7.2						
18:1	Placebo	60.6 ± 11.2	43.0 ± 6.0	93.0 ± 14.3$					X	
(p.M)	Experimental	70.7 ± 5.9	63.9 ± 7.9	113 ±19$						
18:2	Placebo	140 ± 21	83.7 ± 5.5	108 ± 14						
(p.M)	Experimental	120 ± 14	128 + 30	121 ± 12						
18:3n6	Placebo	59.7 ± 16.5	10.9 ± 2.6	12.6 ± 7.2						
(p.M)	Experimental	34.3 ± 7.6	48.7 ± 9.1	22.7 ± 11.0						
18:3n3	Placebo	3.48 ± 0.78	3.82 ± 0.61	4.85 ± 0.98						
(p.M)	Experimental	4.84 ± 1.32	10.7 ± 6.5	6.77 ± 0.96						
20:0	Placebo	6.11 ± 1.15	1.97 ± 0.72 ***it***	1.77 ± 0.34		X				
(HM)	Experimental	5.67 ± 1.02	1.69 ± 0.36 #	2.67 ± 0.27						
20:3	Placebo	9.48 ± 1.30	5.00 ± 0.88	5.07 ± 0.98						
(p.M)	Experimental	7.92 ± 1.60	7.78 ± 2.02	5.88 ± 0.45						
20:4n6	Placebo	53.3 ± 7.1	31.9 ± 4.9	32.0 ± 2.8						
(p.M)	Experimental	44.4 ± 7.3	61.0 ± 17.9	47.9 ± 4.7						
22:0	Placebo	1.70 ± 0.47 a,b	1.15 ± 0.12 a	1.49 ± 0.18			X	X		
(HM)	Experimental	1.13 ± 0.16 a	2.13 ± 0.30 b	2.56 ± 0.57						
22:6n3	Placebo	11.1 + 1.9	12.6 + 3.4	13.1 ± 1.8	X	X		X		
(HM)	Experimental	12.3 ± 2.3	25.8 ± 4.3 *,#	38.2 ± 3.6*						
SFA	Placebo	270 ± 41	169 + 34	162 ± 12		X				
(p.M)	Experimental	222 ± 17	186 ± 26	207 ± 23						
MUFA	Placebo	64.2 ± 11.7	49.3 ± 6.6	106 ±17$					X	
(p.M)	Experimental	74.2 ± 6.4	71.7 + 8.4	131 ± 22 $						
PUFA	Placebo	277 ± 41 a	140 ± 10 b	176 ± 13			X	X		
(p.M)	Experimental	224 ± 23 a,b	267 + 53 a	243 ± 27						
Total	Placebo	611 ± 91	358 ± 32	444 ± 40				X		
(HM)	Experimental	519 ± 39	524 ± 77	580 + 62						

Statistical analysis: Two-way ANOVA, p<0.05. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between factors. (E) Significant effect of exercise, (SxE) Significant interaction between factors. One-way ANOVA, p<0.05. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period, ($) Significant differences between basal and post-exercise.

Table 3. Fatty acid composition of plasma triglycerides

		Initial	Final		ANOVA	ANOVA
		Basal	Basal	Post-exercise	S T SxT	S E SxE
14:0	Placebo	19.2 ± 3.0	24.6 ± 6.7	20.6 ± 4.3		
(|iM)	Experimental	19.6 ± 2.5	18.0 ± 2.8	14.4 ± 0.8		
16:0	Placebo	395 ± 36	469 ± 39	502 ± 65		
(p.M)	Experimental	521 ± 35	449 ± 42	473 ± 19		
16:1	Placebo	20.9 ± 3.2	75.2 ± 14.5 #	54.4 ± 8.9	X	
(UM)	Experimental	21.6 ± 3.1	55.8 ± 11.0 #	58.4 ± 5.5		
18:0	Placebo	310 ± 34	334 ± 22	295 + 24		
(UM)	Experimental	374 ± 37	307 ± 12	348 ± 23		
18:1	Placebo	428 ± 64	547 ± 44	501 ± 67		
((iM)	Experimental	555 ± 54	568 ± 55	547 ± 54		
18:2	Placebo	1030 ± 104	1170 ± 121	1071 ± 117	X	
(UM)	Experimental	1353 ± 112	1309 ± 77	1329 ± 99		
18:3n6	Placebo	324 ± 158	192 + 52	132 i 44		
(UM)	Experimental	128 ± 55	159 ± 36	69 ± 35		
18:3n3	Placebo	207 ± 33	179 ± 10	134 + 15	X	
(UM)	Experimental	157 ± 19	123 ± 23	137 ± 14		
20:0	Placebo	2.53 ± 0.8	8.90 ± 3.28	4.22 ± 0.98		
(|iM)	Experimental	4.36 ± 1.27	5.99 ± 2.36	8.64 ± 3.81		
20:3	Placebo	31.2 ± 11.1	30.7 ± 5	32.3 ± 4.9		
(\iM)	Experimental	38.7 ± 11.1	50.4 ± 16.2	42.5 ± 8.1		
20:4n6	Placebo	216 ± 38	238 ± 35	192 ± 47		X
(UM)	Experimental	403 ± 80	353 ± 111	414 ± 61 *		
22:0	Placebo	47.9 ± 10.9	49.9 ± 8.7	46.3 ± 8.3		
((iM)	Experimental	47.5 ± 6.4	49.2 ± 11.2	59.9 ± 7.1		
22:6n3	Placebo	59.9 ± 10.9	60.7 ± 9.3	56.2 ± 10.0	X X	X
(UM)	Experimental	49.8 ± 10.0	120 ± 39 *,#	115 ± 16*		
SFA	Placebo	773 ± 45	826 ± 95	867 ± 97		
(jiM)	Experimental	966 ± 70	718 ± 111	902 ± 45		
MUFA	Placebo	449 ± 66	622 ± 55	556 ± 76		
(|iM)	Experimental	576 ± 57	623 ± 61	605 ± 57		
PUFA	Placebo	1635 + 149	1670 ± 247	1614 ± 247		
(jiM)	Experimental	2021 ± 193	2125 ± 300	2097 ± 162		
Total	Placebo	2857 + 239	3117 ± 374	3107 + 418		
(цM)	Experimental	3563 ± 308	3377 ± 178	3605 ± 260		

Statistical analysis: Two-way ANOVA, $p<0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between factors. (E) Significant effect of exercise, (SxE) Significant interaction between factors. One-way ANOVA, $p<0.05$. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period, ($) Significant differences between basal and post-exercise.

Table 4. Oxidative damage markers and antioxidant parameters

^		Initial	Final	ANOVA S T SxT
MDA	Placebo	1.51 ± 0.12	0.687 ± 0.090	
(mM)	Experimental	1.41 ± 0.10	1.64 ± 0.97	
Carbonyl index	Placebo	100 ± 17	159 ± 19 #	X
(%)	Experimental	78.4 ± 9.1	133 ± 15 #	
N-Tyr index	Placebo	100 ± 9	102 ± 6	
(%)	Experimental	100 ± 10	107 ± 9	
Nitrite	Placebo	178 ±8	170 ± 11	
(nM)	Experimental	166 ±9	171 ± 6	
Nitrate	Placebo	28.3 ± 3.7	24.1 ± 2.3	
(HMJ	Experimental	32.6 ± 3.6	29.7 ± 3.1	
Catalase	Placebo	176 ± 55	528 ± 158	X
(kat/Lofblood)	Experimental	134 ± 40	625 ± 292	
SOD	Placebo	206 ± 86	601 ± 98	X
(pkat/Lofblood)	Experimental	137 ± 57	465 ± 55	
Vitamin E	Placebo	9.26 ± 2.06	11.6 ± 1.3	
((iM)	Experimental	12.8 ± 1.93	13.1 ± 1.6	
^		Basal	Post-exercise	ANOVA S E SxE
MDA	Placebo	0.687 ± 0.090	0.703 ± 0.068	
(mM)	Experimental	1.64 ± 0.97	0.912 ± 0.124	
Carbonyl index	Placebo	100 ± 12	80.6 ± 9.2	
(%)	Experimental	84.0 ± 9.7	78.6 ± 9.0	
N-Tyr index	Placebo	100 ± 6	124 ± 17	
(%)	Experimental	105 ±9	104 ± 7	
Nitrite	Placebo	170 ± 11	274 ± 22 #	X
(nM)	Experimental	163 ± 9	229 ± 20 #	
Nitrate	Placebo	24.1 ± 2.3	32.1 ± 3.4	X
(UM)	Experimental	29.7 ± 3.1	35.5 ± 3.0	
Catalase	Placebo	528 ± 158	525 ± 60	
(kat/Lofblood)	Experimental	625 ± 292	1193 ± 266	
SOD	Placebo	601 ± 98	393 ± 89	
(pkat/Lofblood)	Experimental	465 ± 55	535 ± 89	
Vitamin E	Placebo	11.6 ± 1.3	12.5 ± 1.9	
(UM)	Experimental	13.1 + 1.6	17.4 ± 1.9	

(A) Data study initial and final, in basal conditions. (B) Final study data, for basal and post-exercise conditions. Statistical analysis: Two-way ANOVA, $p<0.05$. (S) Significant effect of supplementation, (E) Significant effect of exercise, (SxE) Significant interaction between factors. One-way ANOVA, $p<0.05$. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period or significant differences between basal and post-exercise.

Table 6. Serum markers of cellular damage and Inflammation

		Initial	Final		ANOVA	ANOVA
		Basal	Basal	Post-exercise	S T SxT	S E SxE
AST	Placebo	31.3 + 5.5	29.0 + 4.1	30.4 + 3.2		
(U/L)	Experimental	31.1 + 2.7	27.3 + 1.9	29.6 + 2.2		
ALT	Placebo	19.5 + 2.8	20.5 + 3.0	19.7 + 3.1		
(U/L)	Experimental	22.9 + 2.2	22.4 + 2.0	22.8 + 1.9		
VGT	Placebo	14.2 + 3.7	16.2 ± 2.9	16.3 + 3.0		
(U/L)	Experimental	12.7 + 0.9	14.9 + 0.8	15.1 + 0.9		
LDH	Placebo	189 + 9	242 + 26 #	248 + 19		
(U/L)	Experimental	206 + 13	230 + 15	251 ± 13	X	
CPK	Placebo	388 + 120	300 + 69	438 ± 70		
(U/L)	Experimental	402 + 86	358 + 92	469 ± 101		
CRP	Placebo	0.148 ± 0.023	0.233 + 0.133	0.193 ± 0.118		
(mg/dL)	Experimental	0.153 ± 0.038	0.233 ± 0.096	0.232 ± 0.096		

Statistical analysis: Two-way ANOVA, p<0.05. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between factors. (E) Significant effect of exercise, (SxE) Significant interaction between factors. One-way ANOVA, p<0.05. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period ($) Significant differences between basal and post-exercise.

(mg/dL)	Experimental	0.689 + 0.134	0.789 + 0.095	0.950 + 0.102		
Protein	Placebo	75.2 + 1.4	75.0 + 1.4	79.3 ± 2.0		X
(g/L)	Experimental	76.4 + 1.4	75.6 + 1.3	79.4 ± 1.7		
Albumin	Placebo	49.7 + 0.5	45.6 + 1.2	46.1 ± 1.2		
(g/L)	Experimental	50.2 + 1.0	46.1 + 0.7	47.1 ± 0.7		

Statistical analysis; Two-way ANOVA, p<0.05. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between factors. (E) Significant effect of exercise, (SxE) Significant interaction between factors. One-way ANOVA, p<0.05. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period (S) Significant differences between basal and post-exercise.

MANUSCRIPT III

Docosahexaenoic acid dietary supplementation provides better erythrocyte antioxidant defenses and less protein nitrosative damage in professional soccer players

DOCOSAHEXAENOIC ACID DIETARY SUPPLEMENTATION PROVIDES BETTER ERYTHROCYTE ANTIOXIDANT DEFENSES AND LESS PROTEIN NITROSATIVE DAMAGE IN PROFESSIONAL SOCCER PLAYERS

ABSTRACT

Omega-3 fatty acids modulate the inflammatory responses, with beneficial effects for patients suffering from various disorders such as cardiovascular disease. The aim was to determine the influence of long-term DHA dietary supplementation on the erythrocyte fatty acid profile and the oxidative balance in soccer players after training and acute exercise. Fifteen volunteer male soccer players were randomly assigned to a placebo group that consumed an almond-based beverage or experimental group that consumed the same beverage enriched with DHA for eight weeks. Blood samples were taken in basal conditions at the beginning of the nutritional intervention and after eight weeks of training in basal and in post-exercise conditions. The training season increases the activity of antioxidant enzymes in erythrocytes. The experimental beverage changes the erythrocyte membrane composition increasing DHA levels, modulates the antioxidant defences and markers of oxidative damage. DHA supplementation increases the catalytic activity of superoxide dismutase and provokes a reduction of the peroxidative damage induced by training or exercise. Moreover, the glutathione reductase increases its protein levels as a result of the interaction of training and DHA dietary supplementation, pointing to an influence of DHA on the maturation of reticulocytes to erythrocytes. In conclusion, long-term dietary supplementation with DHA provides a better antioxidant defenses and less protein peroxidative damage in professional soccer players during training season and after exercise.

Keywords: omega-3 fatty acids; antioxidant enzymes; oxidative damage; exercise training

INTRODUCTION

Regular physical activity for a healthy body induces an adaptation against elevated oxidant levels by increasing the cellular and plasma antioxidant capability [1, 2]. However, with acute exercise, this adaptation is minimal and antioxidant capability can be overwhelmed by oxidant production [3]. The production of reactive oxygen species (ROS) serves as a double edged sword, as they have a role in producing both deleterious and signaling molecules. Evidence for this was found in HL60 cell cultures stimulated with an acute level of hydrogen peroxide or with a continuous low level production of hydrogen peroxide [4]. Acute exercise involves a kinetic shift probably via the allosteric activity of enzymes, which may not be sufficient to restore an oxidant-antioxidant redox balance [5, 6]. A long-term stimulation of endogenous defense mechanism due to regular physical activity provokes the continuous presence of physiological oxidant stimuli resulting in an antioxidant adaptation and greater protection against oxidative challenges [4, 7-9]. Moreover, extenuate and repetitive physical activity or exercise performed in extreme environmental conditions may cause muscle damage, overtraining and reduced athletic performance [3, 10-13].

Erythrocytes are one of the most susceptible blood cell types to suffer from oxidative stress and cell damage [14]. The high polyunsaturated free fatty acid content of the erythrocyte membranes and the high quantity of oxygen and haem iron in the cell produces conditions optimal for the production of ROS [15]. The inability to repair by resynthesis the damaged components caused by ROS [16] can increase the rate of haemolysis [17]. However, the ROS produced in the erythrocytes can be eliminated by the antioxidant defense system by the antioxidant enzymes: catalase, superoxide dismutase (SOD), glutathione reductase (GRd) and glutathione peroxidase (GPx) [6, 8, 14]; as well as the oxidized/reduced glutathione system and other low molecular weight antioxidants such as vitamins E and C [18].

Dietary intake of omega-3 fatty acids and specific supplementation with the docosahexaenoic acid (DHA, C22:6n3)

increases blood levels of this essential fatty acid [19]. A potential increase of the unsaturated lipid fraction enhances the oxidative susceptibility to produce lipid hydroperoxides and the oxidative-derivated products [20]. Paradoxically, beneficial effects have been pointed out, with for a diet rich in omega-3 fatty acids promoting the maintenance of the antioxidant status and the reduction of oxidative damage markers [21-23]. Moreover, it has been reported that the antioxidant effects of DHA supplement works by inhibiting lipid peroxidation in erythrocytes [24]. The effects of the DHA supplement on oxidative stress depends on the resting or post-exercise conditions [25], and on the antioxidant intake taken together with DHA [26]. In resting conditions, biomarkers of oxidative stress decrease with DHA treatment [25] whereas in post-exercise conditions, the effect of supplementation on stress oxidative parameters is not clearly known due to the different types and duration of supplementation and exercise [27-29].

The aim of the present study was to determine the effects of diet supplementation with DHA on the fatty acid composition of erythrocytes and the oxidative balance during a training season and after acute exercise in soccer players. The influence of training season; acute exercise and DHA dietary supplementation on the erythrocyte antioxidant enzyme activities and levels and on the oxidative and nitrosative damage markers has been determined.

MATERIALS AND METHODS

Subjects and study design

The study was performed with 15 male soccer players (Reial Mallorca B team), at the beginning of their annual sport season. The subjects were randomly selected to be included into two groups: placebo and experimental groups. The placebo group was constituted by 6 subjects who took one liter five times a week of a placebo drink and the experimental group was constituted by other 9 subjects who consumed the experimental drink rich in DHA one liter five times a week, for a period of 8 weeks. All the subjects were informed of the purpose and demands of the study before giving their written consent

to participate. The study protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the Comunidad Autónoma de les Illes Balears (Palma de Mallorca, Balearic Islands, Spain).

Drink composition

The two drinks were composed with 3.0% of almond and 0.8% of sucrose and the rest was water, flavor and the added oils and vitamin E. Moreover, the placebo was 0.8% of refined olive oil and the experimental was 0.6% of refined olive oil and 0.2% of DHA-S Market (Market Biosciences Corporation, Columbia, EEUU). The two almond drinks were elaborated by Liquats Vegetals S.A. (Girona, Spain) and was obtained by: bleaching the almonds, then crushing them almonds water, and then centrifuging of the mixture in order to eliminate insoluble materials. Natural cinnamon, and lemon flavors, sucrose, vitamin E and the respective oil for the experimental (olive oil plus DHA-S) or placebo (olive oil) was added. Finally the beverage were sterilized and packed. Externally the two type beverages were identical in taste and appearance.

Experimental procedure

For each subject, three different blood samples were obtained. One blood sample was taken in basal conditions at beginning of the nutritional intervention. Another two blood samples were taken at the end of the nutritional intervention, in basal and post-exercise conditions. The exercise consisted in a 2 hours habitual physical training session. Briefly, after a 15 min of warm-up, the players performed the Leger Boucher test to indirectly determine the VO_2 max. After that, players practised a recovery exercise of control-passing during 15 min. The main body of the training session was characterized by small-sided games. Briefly, the first exercise consists in 5 vs 5 possession exercise in an area of 20 x 15 m (4 repetitions of 5 min with 30 sec of recovery between repetitions); the second was a 6 vs 6 possession exercise in an area of 30 x 20 m (3 repetitions of 6 min

with 1 min of recovery between repetitions), and finally, the players played a football match 5 vs 5 in 30 x 40 m during 20 min.

Venous blood samples were obtained from the antecubital vein of control and experimental men and were used to purify erythrocytes following an adaptation of the method described elsewhere [30, 31].

Haematological analysis

Haematological parameters such as erythrocyte number, mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH), were determined in an automatic flow-cytometer analyser Technicon H2 (Bayer) VCS system. Haemoglobin concentration was determined using Drabkin reagent. This reagent oxidizes the haem group leading to the formation of methaemoglobin which reacts with potassium cyanide forming cyanmethaemoglobin, a stable pigment which can be detected spectrophotometrically at 540 nm.

Anthropometry measurements

Height was determined using a mobile anthropometer (Kawe 44444, Asperg, Germany) to the nearest millimetre, with the subject's head placed in the Frankfurt plane. Body weight was determined to the nearest 100 g using a digital scale (Tefal, sc9210, Rumilly, France). The subjects were weighed in bare feet and light underwear. Waist and hip perimeters were measured to the nearest 0.1 cm, using a non-stretchable measuring tape (KaWe, 43972, France). Triceps, subscapular, biceps, iliac crest, supraspinal, abdominal, thigh, and leg skinfold thickness were measured using a Holtain skinfold calliper (Tanner/Whitehouse, Crosswell, Crymych, UK), and a mean of three measurements was used [32]. The subjects were asked to stand erect in a relaxed position with both feet placed together on a flat surface.

Different anthropometric indexes were calculated using these measurements: body mass index [BMI = mass (kg)/ squared height (m)]; waist-hip index [waist perimeter (cm)/hip perimeter (cm)]; fat free mass [FFM = 100 - BF]. Body fat (BF) was measured from skinfold thickness according to Carter-Yuhasz equation [33]. All

anthropometric measurements were performed by one observer to avoid inter-observer variation.

Dietary intake

Dietary habits were assessed using a 7-day dietary record questionnaire completed at the beginning of the study. A qualified dietician verified and quantified the food records. All food items consumed were transformed into nutrients using a special computerized program based on the European and Spanish food composition tables [34].

Fatty Acids determinations

Erythrocyte or beverage (250 ul) lipid content was extracted with chloroform-methanol mixture (2:1v/v) by the method of Folch et al. [35], containing 0.01% butylated hidroxyanisole (BHA) as antioxidant and 20 u± of n-heptadecanoic acid (15 mM) as internal standard. The resultant organic phase was evaporated under a nitrogen stream at 55°C. The dry residue was dissolved in 225 u± of n-hexane and 25 u± of Meth-Prep™ II (GRACE) derivatization reagent was added. An aliquot of 1 ul was injected into the gas chromatograph. The mobile phase consisted of helium. The gas chromatograph was a Agillent 5890 model with a flame ionization detector (FID) and the column was a Supelcowax® 10 Capillary GC column, 30 m x 0.53 mm, d, 0.50 u.m. Individual fatty acids and a mix of fatty acid methyl esters (Supelco®) were used for the identification of the chromatography peaks.

Quantification was performed from the internal standard (C17:0) and the different responses of the fatty acids in the FID was corrected by a response factor calculated from the areas of the standard fatty acids of different chromatograms with different fatty acid concentrations and the area of the internal standard obtained from the same chromatograms.

Vitamin E determinations

Vitamin E was determined in the placebo and experimental drinks. The extraction of liposoluble vitamins was carried out using n-hexane after deproteinisation with ethanol containing 0.2% butylated hydroxytoluene (BHT). Vitamin E concentration was determined after drying the samples under nitrogen current and dissolving in methanol. The mobile phase consisted of acetonitrile:tetrahydrofuran:water (550:370:80, v/v/v). The HPLC was a Shimadzu with a diode array detector and the column was a Nova Pak, C_{18}, 3-9 x 150 mm. a-tocopherol was determined at 290 nm. The quantification was done with external patron (Sigma-Aldrich).

Malondialdehyde determination

Malondialdehyde (MDA) as a marker of lipid peroxidation was analyzed in 1/100 diluted erythrocytes using a colorimetric assay kit (Calbiochem). Briefly, samples and standards were placed in eppendorfs containing n-methyl-2-phenylindole (10.3 mM) in acetonitrile:methanol (3:1, v/v). HCl 12N was added and the samples were incubated for 1 h at 45°C. Absorbance was measured at 586 nm. The method used is specific for MDA determination.

Assay of Nitrotyrosine and Protein Carbonyls

Protein carbonyl derivatives and nitrotyrosine were determined by immunological methods using the OxiSelect™ Protein Carbonyl Immunoblot Kit (Cell Biolabs, INC) and OxiSelect™ Nitrotyrosine Immunoblot Kit (Cell Biolabs, INC) following the manufacturer's details. Total protein concentrations were measured by the method of Bradford [36]. Initially, samples (10 u.g or 150 u.g of protein for carbonyl or nitrotyrosine, respectively) were transferred onto a nitrocellulose membrane by the dot blot method. For carbonyl determination, the membrane was incubated in the presence of 2,4-dinitrophenylhydrazine (DNPH) after transference. Then, the membrane was incubated with the primary antibody, specific to DNP moiety proteins in the case of carbonyl determination or rabbit anti-nitrotyrosine antibody for nitrotyrosine determination. This step was followed by incubation with a horseradish peroxidase-antibody (goat

anti-rabbit IgG) conjugate directed against the primary antibody. The membrane was then treated with luminol, which is converted to a light-emitting form at wavelength 428 nm by the antigen/primary antibody/secondary antibody/peroxidase complex. The light was visualized and detected by short exposure to a Chemidoc XRS densitometer (Bio-Rad Laboratories). Image analysis was performed using Quantity One-ID analysis software (Bio-Rad Laboratories).

Enzymatic determinations

Erythrocyte catalase (CAT) activity was measured by the spectrophotometric method of Aebi [37]. Superoxide dismutase (SOD) activity was measured in erythrocytes by an adaptation of the method of McCord and Fridovich [38]. Glutathione reductase (GRd) activity was measured in erythrocytes by a modification of the Goldberg and Spooner spectrophotometric method [39]. Glutathione peroxidase (GPx) activity was measured using an adaptation of the spectrophotometric method of Flohé and Gunzler [40]. All activities were determined with a Shimadzu UV-2100 spectrophotometer at 37°C.

Western blot analysis

Antioxidant enzyme protein levels were determined by Western blot. Protein extracts were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). CAT (20 u.g of protein), Cu/Zn-SOD (10 u.g of protein) and GPx (200 u.g of protein) were loaded on a 12% agarose gel, whereas GRd (10 u.g of protein) was loaded on a 15% agarose gel. Following electrophoresis, samples were transferred onto a nitrocellulose membrane and incubated with a primary monoclonal antibody: anti-CAT antibody (Calbiochem), anti-Cu/Zn-SOD antibody (Sigma) and anti-GRd antibody and anti-GPx antibody (Santa Cruz). Then, incubation with a secondary peroxidase-conjugated antibody was performed. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad Laboratories) Western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and analysed with Quantity One-ID Software (Bio-Rad Laboratories).

Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS v.21.0 for Windows). Results are expressed as mean ± SEM. and $P < 0.05$ was considered statistically significant. The Shapiro-Wilk W-test was applied to assess the normal distribution of the experimental data. To test the effects of supplementation and training or supplementation and acute exercise, a two-way analysis of variance (ANOVA) was performed. When significant effects were found, one-way ANOVA was used to determine the differences between the groups involved.

RESULTS

The fatty acid composition of the experimental and placebo beverages are shown in Table 1. The experimental drink presented significant higher concentrations of the fatty acids C20:3, C22:0, C22:5 and C22:6n3 whereas they were undetected in placebo drink. The experimental beverage also presented significantly higher concentrations of C16:0, C16:1, and C20:1 fatty acids compared with the placebo beverage. There was no significant difference in the vitamin E concentration between the two drinks. An intake of 1 L five times a week of the respective beverage supposed a daily intake of about 1.14g of DHA additional to the basal dietary DHA intake in the experimental group.

There were no differences in the anthropometric characteristics and physical activity capabilities between the placebo and experimental group of soccer players at the beginning of the competitive season (Table 2). No differences were observed in nutrient intake between the placebo and experimental groups before their participation in the nutrition trial, except for the intake of fiber (Table 3). The daily intake of polyunsaturated lipids was about 11 g daily in both groups before the beginning of the nutritional intervention. The diet supplementation with 1 L daily of the beverage provided an increment of about 12% of the total lipid intake. The experimental group intakes were approximately 1.07, 4.64 and 3.93 g/day of additional SFA, MUFA and PUFA, respectively, whereas the

placebo group intake, additional SFA, MUFA and PUFA amount of 0.57, 2.21 and 1.28 g/day, respectively.

The overall erythrocyte composition was practically unchanged in the group with the DHA enriched beverage (Table 4). Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and total fatty acids in erythrocytes were not modified during the nutritional intervention for either group. The main effect found was for the erythrocyte content of DHA (C22:6n3 fatty acid), which significantly increased in the experimental group after the nutritional intervention, reaching values 1.3 times higher than placebo group. The DHA supplementation significantly affects the concentration of fatty acid C20:3, although this value was also different between groups at the beginning of the study. Exercise had almost no influence on erythrocyte fatty acid composition, irrespective to the placebo or experimental group (Table 5). The SFA, MUFA, PUFA and total fatty acids in erythrocytes maintained basal values, independently of the type of drink consumed during 8 weeks after exercise. However, a significant increase in C18:3n3 fatty acid concentration (about 63%) after exercise was detected in erythrocytes of the placebo but not in the experimental group. The differences in DHA (C22:6n3 fatty acid) content after the supplementation period were maintained after acute exercise. The C22:0 fatty acid concentration slightly increased only in the experimental group after exercise, resulting in significant differences respect to the placebo group. The concentration of C18:3n3 and C20:3 fatty acids were significantly lower (about 63% and 34% respectively) in the experimental than placebo group in basal conditions, whereas the concentration of C22:0 and C22:6n3 fatty acids were about 63% and 39% significantly higher in the experimental than the placebo group after exercise. The changes in the lipid composition of erythrocytes indicated that the subjects followed the prescribed beverage intake during the trial as well as demonstrating that supplementation with the experimental drink was effective in incorporating DHA into the erythrocytes.

The nutritional intervention or the training period did not affect the basal erythrocyte characteristics (Table 6). Erythrocyte

counts, haemoglobin, haematocrit, MCV, MCH, MCHC and RDW maintained the basal initial values after 8 weeks of nutritional intervention in both the placebo and the experimental groups. Acute exercise and dietary supplementation only induced higher a MCHC value after exercise in the experimental group.

The effects of dietary supplementation with DHA, the training season and the acute exercise on the oxidative and nitrosative damage of erythrocytes are shown in Table 7. The dietary supplementation with DHA did not affect MDA erythrocyte levels (an indicator of lipid peroxidation), carbonyl index (an indicator of protein modification) or nitrotyrosine index (an indicator of nitrosative damage) (Table 7A). However, MDA levels significantly increased 1.5 times in both the placebo and the experimental group during after the training season. The N-Tyr index, contrary to the erythrocyte MDA, significantly decreased 3 times at the end of the training season in both groups. Acute exercise and DHA diet supplementation with the experimental drink did not affect the MDA levels o and the carbonyl index. However, the N-Tyr index was affected by exercise and by the interaction between supplementation and exercise. The placebo group significantly increased the N-Tyr index after exercise about 2.5 times, whereas the experimental group maintained the basal values.

The DHA dietary supplementation and the training season significantly affected the basal activity and protein levels of the antioxidant enzymes in erythrocytes (Table 8 A). The catalase activity increased significantly at the end of the training period in the placebo and experimental groups without significant differences in protein levels. A significant interaction between DHA diet supplementation and training was observed in the activity of SOD and GPx. SOD activity was higher in the experimental group at the end of the training season, whereas GPx activity was higher in the placebo group. The protein levels of Cu/Zn-SOD and GPx were not affected significantly by supplementation and training season. GRd activity increased significantly at the end of the study in both groups; whereas its respective protein levels showed higher values in the experimental group at the end of the training season.

The Table 8B also shows the effects of acute exercise on antioxidant enzyme activities and the protein levels of the placebo and experimental groups at the end of the nutritional intervention. The erythrocytes of experimental group had lower catalase and GPx activities than placebo group in basal conditions; however the basal values of SOD and GR activities were similar in placebo and experimental groups. Acute exercise and diet supplementation significantly changed the GPx activity and maintained the catalase, SOD and GR the basal activities after exercise. GPx activity decreased its levels after exercise mainly in the supplemented group. The protein levels of these antioxidant enzymes were not significantly affected by dietary supplementation or acute exercise with the exception of GRd protein levels, which were influenced by DHA diet supplementation. The experimental group presented significantly higher erythrocyte GRd protein levels after exercise than the placebo group.

DISCUSSION

The nutritional intake of the athletes before nutritional intervention was similar in the two experimental groups. The energy intake was adjusted to the level recommended for the general population, although it was low for active people, whose energy and protein intake should be 20% higher than the general population [41]. The protein intake of the participants is greater than general recommendations and the carbohydrates and fiber are lower [41]. The subject diets have a high quantity of cholesterol and lipids with a decompensation of saturated/unsaturated fatty acids. These deviations have been also been observed in the general Balearic Island population [42]. The diet supplementation with the placebo and the experimental beverages increases diet PUFA intake; moreover, the daily intake of one liter of the experimental drink provides 1.14g/day DHA in addition to the provided by the diet. Supplemental intake with the experimental beverage of DHA is lower than that used in nutritional intervention studies with the general population [43] or trained men [23, 44] but it is similar to other nutritional intervention studies with health people [45]. The daily intake of experimental drink increases DHA levels in erythrocyte

membranes, in accordance with other studies [43, 45], whereas no changes were reported in the SFAs, MUFAs and PUFAs.

The membrane fatty acid composition is a dynamic system [46] and their control and regulation are not clearly understood [47]. The dietary fatty acid profile significantly influences the incorporation of fatty acids into the cell membranes influencing their function [43, 48, 49]. Exercise, independent off at intake, can modify cell fatty acid profile in different tissue types [50]. An increased fluidity of red blood cell membranes has been observed after chronic exercise [51] and it has been also reported that exercise, depending on the intensity and sport-type influences erythrocyte fatty acid composition profile [47, 51, 52]. We find a significant effect by exercise in the amount of C18:3n3 present in erythrocyte membranes. This is the first time that a rapid change in membrane composition has been observed in humans; however, several studies performed in rats showed an effect of acute exercise on the fatty acid composition of erythrocytes, which was influenced by aging, training status and body temperature [53, 54].

Oxidative damage is more prominent in red cells compared to other cells, probably due to their high iron and polyunsaturated fatty acid contents, their role as an oxygen transporter, and by their protection of the host in vivo by neutralizing exogenous and endogenous free radicals [14, 55]. The oxidative damage in the lipid fraction of erythrocytes slightly increased during diet intervention in both the placebo and the experimental groups, although the exercise practised at the end of the study did not affect this oxidative damage marker. We consider that this increment in oxidative damage is in parallel to the development of the football season with its accumulation of workouts and matches, and this is in accordance with previous studies [56-58]. It has been pointed out that regular exercise in young footballers may be beneficial by reducing the amount of lipid peroxidation and increasing the activity of the antioxidant enzymes [58-60]. The antioxidant enzyme activities increase in the erythrocytes after the training season of footballers, but it does not avoid a light increase in the oxidative damage in the lipid fraction of erythrocytes. Diet supplementation with DHA does not alter the basal pattern of

oxidative damage in erythrocytes observed in the footballers during the training season and after exercise training. The oxidative damage in erythrocytes depends on the exercise intensity. Intense exercise such as a mountain cycling stage or a maximal exercise test induces oxidative damage in erythrocytes [14, 61], whereas in submaximal exercise, no significant differences were obtained in MDA levels [61].

Neither training, exercise or DHA diet supplementation affected the protein oxidative damage measured as carbonyl index. The efficiency of dietary fish oil to reduce in vivo oxidative damage of proteins in rats, and such antioxidant activity may differ among different fish oil sources due to variations in EPA/DHA content [62]. Protein carbonylation is an important irreversible modification that increases during oxidative stress. ROS formed in the exercise initiates the peroxidation of PUFA to form lipid peroxidation end products such as MDA, which could add to protein side chains like lysine, and result in the formation of carbonyl groups in the protein. Therefore, the formation of carbonylated proteins could be not parallel to the formation of MDA. The absence of changes in carbonylated proteins, together with the slight increase in MDA, could indicate an increased susceptibility to oxidation by of lipids than proteins; and the amount of MDA produced is too scarce for reaction with the proteins. The results also suggest that the ROS produced are not enough to directly oxidize the proteins in the presence of the erythrocyte antioxidant environment.

Dietary supplementation with DHA reduces the nitrosative capability of exercise because the nitrotyrosine index is maintained at the basal level after exercise, whereas the placebo group increased this parameter. Superoxide anion produced in the erythrocytes can react with nitric oxide to form peroxynitrite, which can react with peptide-bound tyrosine to form nitrotyrosine [63]. In a situation of increased erythrocyte production of oxide nitric in conditions such as after exercise [31], the elimination of superoxide anion is of great importance in order to reduce the peroxynitrite and nitrotyrosine production. Superoxide dismutase eliminates the anion superoxide necessary for the formation of peroxynitrite and nitrotyrosine. The increment of SOD activity during the training season for the

attainment of higher basal values in the DHA supplemented group could explain the low nitrotyrosine index in erythrocytes at the end of training period, as well as the maintained value observed in the experimental group after exercise. The effects of dietary supplementation with DHA on the increase erythrocyte SOD activity cannot be attributed to changes in the protein level of Cu/Zn-SOD because this protein level remained unchanged in erythrocytes.

The erythrocyte antioxidant enzyme activities increased as result of the training season, in accordance with a preventive erythrocyte antioxidant protection induced by the regular exercise [57, 59, 64, 65]. Increased levels of blood antioxidant activity at rest were found in subjects undergoing training routines [65]. Dietary supplementation with DHA exerts a differential influence on the basal and post-exercise erythrocyte antioxidant enzyme activities. DHA dietary supplementation enhances the effect of training season on erythrocyte SOD activity but diminishes the training effect on the GPx and the catalase activities. A previous study with long distance skiers in training routines revealed a decrease in erythrocyte SOD activities in the blood following an acute bout of exercise [66]. However, it was also reported that no changes were found in erythrocyte SOD activity in trained individuals following a duathlon competition [6]. The antioxidant enzyme activities in erythrocytes are modulated by acute exercise [6] and this modulation is influenced by the presence of low molecular weight antioxidants as vitamin C [18]. For SOD, GPx and catalase enzymes, the increased enzyme activity did not parallel enzyme protein content, suggesting posttranslational (activation) of the existing enzymes [67, 68]. These changes in the enzymatic capability of the antioxidant enzymes could be related to the effects of the ROS and low molecular weight antioxidants on the enzymatic protein [6, 56, 69]. No evidence for a direct action of DHA on the catalytic activity of antioxidant enzymes was found, but a reduction in catalase, GPx and GRd catalytic activities and an increase in SOD catalytic activity was evidenced in the DHA supplemented group. An important increase in the protein levels of GRd was appreciated as a result of the training season, mainly in the DHA supplemented group. These changes in GRd protein levels could be related to the process of

maturation of reticulocytes to erythrocytes, as this maturation has been reported to cause a significant decrease in the activities of antioxidant enzymes such as GRd, GPx and glutathione S-transferase, glucose-6-phosphate dehydrogenase and catalase [70]. It is possible that DHA dietary supplementation could induce higher production of GR in reticulocytes or that the GR reductase of reticulocytes was more protected from degradation in the maturation process of reticulocytes to erythrocytes.

In summary, the consumption of an enriched DHA almond drink for eight weeks by professional soccer players changed the erythrocyte membrane composition although did not alter the oxidative damage markers of the erythrocytes. The enhanced erythrocyte SOD activity induced by the DHA diet supplementation during training season reduces the peroxidative damage of erythrocyte proteins induced by training or exercise. The effects on the antioxidant activities are not induced by changes in the erythrocyte antioxidant enzyme proteins, which would have affected the catalytic capabilities of the antioxidant enzymes. However, GRd increased its protein levels as results of the interaction between training and DHA diet supplementation, pointing to an influence of DHA in the maturation of reticulocytes to erythrocytes. Dietary supplementation with DHA provides greater antioxidant defenses and less protein peroxidative damage in professional footballers during training season and after exercise.

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CONFLICTS OF INTEREST

The authors declare that they do not have any conflict of interest.

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Table 1. Beverages composition

	Placebo			Experimental		
C16:0 (uM)	1799	±	173	3607	±	620 *
C16:1 (uM)	275	±	23	591	±	115 *
C18:0 (uM)	1135	±	156	1928	±	423
C18:1 (uM)	10635	±	2602	22503	±	5605
C18:2 (uM)	5459	±	1161	12177	±	2996
C18:3n6 (uM)	104	±	38	298	±	152
C18:3n3 (uM)	394	±	55	455	±	44
C20:0 (uM)	92.4	±	4.9	116	±	7
C20:1 (uM)	19.4	±	1.4	35.9	±	2.3 *
C20:2 (uM)	277	±	20	282	±	14
C20:3 (uM)		ND		20.9	±	2.2 *
C20:4n6 (uM)	161	±	10	140	±	9
C22:0 (uM)		ND		75.6	±	1.7 *
C22:5 (uM)		ND		1715	±	70 *
C22:6n3 (uM)		ND		3457	±	117 *
Vitamin E (mg/L)	41.6	±	17.8	45.7	±	27.7

ND non detect. Statistical analysis: Student's t-test for unpaired data. (*) Significant differences between placebo and experimental, $p < 0.05$.

Table 2. Anthropometric parameters of the soccer players

	Placebo	Experimental
Age (years)	19.3 ± 0.4	20.4 ± 0.5
Weight (kg)	76.5 ± 1.8	76.4 ± 3.5
Height (cm)	179 ± 2	180 ± 3
Waist circumference (cm)	78.2 ± 0.8	78.5 ± 1.1
Hip circumference (cm)	97.0 ± 1.0	96.6 ± 1.4
Systolic blood pressure (mmHg)	117 ± 8	122 ± 3
Diastolic blood pressure (mmHg)	56.7 ± 5.9	66.7 ± 3.5
Body Mass Index (BMI, kg/m^2)	24.0 ± 0.6	23.5 ± 0.5
Waist-hip ratio (WHR)	0.805 ± 0.012	0.814 ± 0.012
Fat mass (Yuhasz, %)	7.53 ± 0.24	7.21 ± 0.25
Fat-free mass (%)	92.5 ± 0.2	92.8 ± 0.3
VO_2max (mL/kg min)	60.4 ± 1.8	62.0 ± 0.9
Intense physical activity time (min/day)	96.4 ± 57.9	50.4 ± 13.1
Moderate physical activity time (min/day)	68.6 ± 17.1	63.2 ± 14.6

Statistical analysis: Student's t-test for unpaired data. (*) Significant differences between placebo and experimental, $p < 0.05$.

Table 3. Daily nutrient intake of the athletes before supplementation

	Placebo			Experimental		
Energy (kcal)	2518	±	226	2215	±	210
Water (mL)	1880	±	287	1254	±	173
Proteins(g)	108	±	10	105	±	12
Carbohydrates (g)	282	±	37	225	±	26
Fiber (g)	18.8	±	2.3	13.9	±	0.9 *
Lipids (g)	106	±	9	99.2	±	8.1
Saturated fatty acids (g)	37.0	±	3.1	36.5	±	4.4
Monounsaturated fatty acids (g)	46.1	±	4.1	42.6	±	3.1
Polyunsaturated fatty acids (g)	11.9	±	1.5	10.7	±	0.9
Cholesterol (mg)	430	±	21	428	±	62
Alcohol (g)		NI			NI	
Protein energy (%)	17.5	±	0.8	18.8	±	0.7
Carbohydrate energy (%)	44.7	±	3.8	40.5	±	2.1
Lipid energy (%)	37.8	±	2.9	40.7	±	1.7
Saturated fatty acids energy (%)	13.1	±	1.0	14.8	±	0.9
Monounsaturated fatty acids energy (%)	16.3	±	1.1	17.6	±	1.1
Polyunsaturated fatty acids energy (%)	4.41	±	0.80	4.42	±	0.27
Alcohol energy (%)		NI			NI	
Sodium (mg)	2931	±	446	2293	±	182
Potassium (mg)	2860	±	219	3037	±	376
Magnesium (mg)	282	±	15	288	±	42
Phosphorous (mg)	1538	±	91	1543	±	219
Calcium (mg)	821	±	42	876	±	169
Iron (mg)	22.6	±	8.6	13.7	±	1.7
Copper (mg)	2.05	±	0.11	2.64	±	0.34
Selenium (ug)	94.8	±	13.1	99.5	±	12.4
Iodine (ug)	102	±	14	76.4	±	11.2
Zinc (mg)	13.1	±	1.3	12.6	±	2.5
Retinol (ug)	396	±	43	1421	±	783
Carotenes (ug)	1905	±	276	2534	±	751
Vitamin A (retinols equivalents)	787	±	56	1882	±	890
Thiamine (mg)	1.44	±	0.08	1.53	±	0.24
Riboflavin (mg)	1.88	±	0.05	2.17	±	0.40
Vitamin B_6 (mg)	2.44	±	0.29	2.07	±	0.35
Vitamin B_{12} (ug)	13.7	±	3.2	17.9	±	4.5
Vitamin C (mg)	80.2	±	12.3	89.2	±	25.0
Vitamin D (ug)	2.43	±	0.40	1.88	±	0.43
Vitamin E (mg)	8.06	±	0.70	6.99	±	0.57
Niacin (mg)	27.2	±	1.3	26.5	±	5.5
Pantothenic acid (mg)	5.52	±	0.18	6.45	±	1.23
Folic acid (ug)	262	±	11	255	±	44

NI non ingested. Statistical analysis: Student's t-test for unpaired data. (*) Significant differences between placebo and experimental, $p < 0.05$.

Table 4. Fatty acid composition in erythrocytes of the placebo and experimental groups, at the beginning and the end of the study, in basal conditions

		Initial	Final	ANOVA		
		(nmol/10⁸ erythrocytes)	(nmol/10⁸ erythrocytes)	S	T	SxT
C16:0	Placebo	62.7 ± 4.2	59.6 ± 5.8			
	Experimental	62.8 ± 5.8	56.8 ± 3.2			
C16:1	Placebo	2.02 ± 0.13	1.67 ± 0.17			
	Experimental	1.63 ± 0.23	1.55 ± 0.19			
C18:0	Placebo	122 ± 10	108 ± 13			
	Experimental	130 ± 10	110 ± 6			
C18:1	Placebo	41.3 ± 2.2	42.1 ± 3.2			
	Experimental	39.9 ± 4.5	40.6 ± 2.5			
C18:2	Placebo	51.3 ± 3.0	56.4 ± 3.7			
	Experimental	44.2 ± 5.6	48.7 ± 3.8			
C18:3n6	Placebo	4.39 ± 0.39	4.44 ± 0.51			
	Experimental	4.16 ± 0.43	4.39 ± 0.26			
C18:3n3	Placebo	12.5 ± 1.4	11.9 ± 1.6			
	Experimental	13.3 ± 1.5	14.1 ± 1.5			
C20:0	Placebo	1.79 ± 0.2	1.36 ± 0.18			
	Experimental	1.98 ± 0.18	1.83 ± 0.25			
C20:1	Placebo	3.24 ± 0.34	3.53 ± 0.32			
	Experimental	2.70 ± 0.29	3.25 ± 0.42			
C20:2	Placebo	5.17 ± 0.52	4.15 ± 0.46			
	Experimental	4.69 ± 0.53	4.35 ± 0.16			
C20:3	Placebo	8.28 ± 1.37	8.98 ± 1.38	X		
	Experimental	5.67 ± 0.78	5.91 ± 0.34 *			
C20:4	Placebo	117 ± 8	128 ± 12			
	Experimental	110 ± 14	120 ± 8			
C22:0	Placebo	0.827 ± 0.080	1.22 ± 0.18			
	Experimental	1.42 ± 0.35	1.54 ± 0.2			
C22:6n3	Placebo	29.0 ± 1.3	33.6 ± 3.1	X	X	
	Experimental	34.0 ± 3.6	43.0 ± 3.6 *			
SFA	Placebo	187 ± 14	170 ± 19			
	Experimental	196 ± 16	170 ± 9			
MUFA	Placebo	46.6 ± 2.6	47.3 ± 3.6			
	Experimental	44.3 ± 4.9	45.5 ± 3.0			
PUFA	Placebo	228 ± 14	247 ± 19			
	Experimental	204 ± 26	240 ± 14			
Total	Placebo	461 ± 29	464 ± 42			
	Experimental	445 ± 46	456 ± 26			

Statistical analysis: Two-way ANOVA, $p < 0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between both factors. One-Way ANOVA, $p < 0.05$. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period.

Table 5. Fatty acid composition in erythrocytes of the placebo and experimental groups, at the end of the study between basal and post-exercise conditions

		Placebo (nmol/10⁸ erythrocytes)	Experimental (nmol/10⁸ erythrocytes)	ANOVA S	E	SxE
C16-0	Basal	59.6 ± 5.8	56.8 ± 3.2			
	Post-exercise	53.4 ± 9.5	56.0 ± 2.4			
C16-1	Basal	1.67 ± 0.17	1.55 ± 0.19			
	Post-exercise	1.52 ± 0.24	1.76 ± 0.13			
C18-0	Basal	108 ± 13	110 ± 6			
	Post-exercise	100 ± 18	107 ± 6			
C18-1	Basal	42.1 ± 3.2	40.6 ± 2.5			
	Post-exercise	37.6 ± 6.7	42.9 ± 1.4			
C18-2	Basal	56.4 ± 3.7	48.7 ± 3.8			
	Post-exercise	48.8 ± 9.1	49.6 ± 1.8			
C18-3n6	Basal	4.44 ± 0.51	4.39 ± 0.26			
	Post-exercise	4.81 ± 0.31	4.35 ± 0.24			
C18-3n3	Basal	11.9 ± 1.6 "	14.1 ± 1.5 [a]		X	X
	Post-exercise	19.4 ± 1.7 [b]	14.0 ± 0.1 [a]			
C20-0	Basal	1.36 ± 0.18	12.32 ± 10.49			
	Post-exercise	1.35 ± 0.24	1.60 ± 0.07			
C20-1	Basal	3.53 ± 0.32	3.25 ± 0.42			
	Post-exercise	3.44 ± 0.68	3.62 ± 0.19			
C20-2	Basal	4.15 ± 0.46	4.35 ± 0.16			
	Post-exercise	4.01 ± 0.70	4.39 ± 0.16			
C20-3	Basal	8.98 ± 1.38	5.91 ± 0.34	X		
	Post-exercise	7.87 ± 1.59	6.22 ± 0.36			
C20-4	Basal	128 ± 12	120 ± 8			
	Post-exercise	113 ± 21	122 ± 6			
C22-0	Basal	1.22 ± 0.18	1.54 ± 0.20 *	X		
	Post-exercise	1.02 ± 0.13	1.66 ± 0.24 *			
C22-6n3	Basal	33.6 ± 3.1	43.0 ± 3.6 *	X		
	Post-exercise	29.0 ± 5.3	40.4 ± 3.0 *			
SFA	Basal	170 ± 19	180 ± 16			
	Post-exercise	156 ± 28	166 ± 8			
MUFA	Basal	47.3 ± 3.6	45.5 ± 3.0			
	Post-exercise	42.5 ± 7.6	48.2 ± 1.5			
PUFA	Basal	247 ± 19	240 ± 14			
	Post-exercise	227 ± 38	241 ± 10			
Total	Basal	464 ± 42	466 ± 29			
	Post-exercise	425 ± 72	455 ± 17			

Statistical analysis: Two-way ANOVA, $p < 0.05$. (S) Significant effect of supplementation, (E) Significant effect of exercise, (SxT) Significant interaction between both factors. One-Way ANOVA, $p < 0.05$. (*) Significant differences between placebo and experimental, (#) Significant differences between basal and post-exercise.

Table 6. Erythrocyte characteristics of the placebo and experimental groups

A		Initial	Final	ANOVA S T SxT
Erythrocytes (10^6/uL)	Placebo	5.43 ± 0.21	5.20 ± 0.16	
	Experimental	5.19 ± 0.11	5.17 ± 0.10	
Haemoglobin(g/dL)	placebo	± 03	15.4 ± ™	
	Experimental	15.5 ± 0.3	15.4 ± 0.3	
Haematocrit (/)	Placebo	46.9 ± 0.8	45.1 ± 1.1	
	Experimental	45.9 ± 0.9	45.5 ± 0.8	
Mean corpuscular volume (MCV) (fL)	Placebo	86.9 ± 2.6	87.0 ± 2.3	
	Experimental	88.4 ± 0.9	88.1 ± 1.0	
Mean corpuscular haemoglobin (MCH) (pg)	Placebo	29.7 ± 1.0	29.8 ± 1.0	
	Experimental	30.0 ± 0.4	29.7 ± 0.5	
Mean corpuscular Hb concentration (MCHC) (g/dL)	Placebo	34.1 ± 0.2	34.3 ± 0.3	
	Experimental	33.9 ± 0.1	33.7 ± 0.2	
Red blood cell distribution width (RDW) (%)	Placebo	11.7 ± 0.1	11.9 ± 0.1	
	Experimental	11.6 ± 0.1	11.6 ± 0.2	

B		Basal	Post-exercise	S E SxE
Erythrocytes (10^6/uL)	Placebo	5.20 ± 0.16	5.29 ± 0.20	
	Experimental	5.17 ± 0.10	5.12 ± ± 0.10	
Haemoglobin(g/dL)	¡ ; placebo	± 03	15Ü ± 03	
	Experimental	15.4 ± 0.3	15.5 ± 0.3	
Haematocrit (/)	Placebo	45.1 ± 1.1	45.7 ± 1.1	
	Experimental	45.5 ± 0.8	44.9 ± 0.9	
Mean corpuscular volume ($_{MCV}$) (f_L)	Placebo	87.0 ± 2.3	86.7 ± 2.3	
	Experimental	88.1 ± 1.0	87.8 ± 0.9	
Mean corpuscular haemoglobin (MCH) (pg)	Placebo	29.8 ± 1.0	30.3 ± 1.0	
	Experimental	29.7 ± 0.5	30.4 ± 0.4	
Mean corpuscular Hb concentration (MCHC) (g/dL)	Placebo	34.3 ± 0.3	35.0 ± 0.25	X
	Experimental	33.7 ± 0.2	34.6 ± 0.21 #	
Red blood cell distribution width (RDW) (/)	Placebo	11.9 ± 0.1	11.6 ± 0.1	
	Experimental	11.6 ± 0.2	11.4 ± 0.1	

(A) Data study initial and final, in basal conditions. (B) Final study data, for basal and post-exercise conditions. Statistical analysis: Two-way ANOVA, $p < 0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between supplementation and time, (E) Significant effect of exercise, (SxE) Significant interaction between supplementation and exercise. One-way ANOVA, $p < 0.05$. (*) Significant differences between placebo and experimental. (#) Significant differences between initial and final or significant differences between basal and post-exercise.

Table 7. Oxidative damage of the placebo and experimental groups

A		Initial	Final	ANOVA S T SxT
MDA	Placebo	2.30 ± 0.09	3.50 ± 0.21	X
(|imol/10⁶ erythrocytes)	Experimental	2.33 ± 0.12	3.63 ± 0.15	
Carbonyl index	Placebo	100 ± 29	150 ± 13	
(%)	Experimental	112 ± 14	127 ± 13	
N-Tyr index	Placebo	100 ± 29	29.3 ± 10.5 #	X
(%)	Experimental	123 ± 16	44.9 ± 7.1 #	
B		**Basal**	**Post-exercise**	**S E SxE**
MDA	Placebo	3.50 ± 0.21	3.74 ± 0.20	
(|imol/10⁶ erythrocytes)	Experimental	3.63 ± 0.15	3.84 ± 0.14	
Carbonyl index	Placebo	100 ± 13	113 ± 3	
(%)	Experimental	104 ± 8	126 ± 4	
N-Tyr index	Placebo	100 ± 36[a]	247 ± 33[b]	X X
(%)	Experimental	153 ± 24[a]	166 ± 18[a]	

(A) Data study initial and final, in basal conditions. (B) Final study data, for basal and post-exercise conditions. Statistical analysis: Two-way ANOVA, $p < 0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between supplementation and time, (E) Significant effect of exercise, (SxE) Significant interaction between supplementation and exercise. One-way ANOVA, $p < 0.05$. (*) Significant differences between placebo and experimental. (#) Significant differences between initial and final or significant differences between basal and post-exercise.

Table 8. Antioxidant enzyme activities and protein levels of the placebo and experimental groups

A		Initial	Final	ANOVA S	T	SxT
Catalase	Placebo	0.893 ± 0.262	8.38 ± 0.49 #		X	
(K/10⁶ erythrocytes)	Experimental	0.880 ± 0.220	7.14 ± 0.80 #			
SOD	Placebo	1.07 ± 0.07 "	7.52 ± 0.85 [b]	X	X	X
(pkat/10⁶ erythrocytes)	Experimental	1.48 ± 0.40 [a]	10.5 ± 0.35 [c]			
GRd	Placebo	0.679 ± 0.125	4.39 ± 1.46 #		X	
(nkat/10⁶ erythrocytes)	Experimental	0.871 ± 0.264	3.46 ± 0.89 #			
GPx	Placebo	2.83 ± 0.26 "	20.5 ± 2.6 [b]	X	X	X
fnkat/10⁶ erythrocytes)	Experimental	2.87 ± 0.30 [a]	13.3 ± 1.7 [c]			
Catalase	Placebo	100 ± 29	139 ± 50			
(%)	Experimental	106 ± 36	151 ± 62			
Cu/Zn-SOD	Placebo	100 ± 14	120 ± 32			
(%)	Experimental	117 ± 29	105 ± 40			
GRd	Placebo	100 ± 22 [a]	157 ± 46 "	X	X	X
(%)	Experimental	127 ± 22 [a]	400 ± 77 [b]			
GPx	Placebo	100 ± 11	99.7 ± 13.7			
(%)	Experimental	126 ± 18	114 ± 15			

B		Basal	Post-exercise	S	E	SxE
Catalase	Placebo	8.38 ± 0.49	9.68 ± 0.83	X		
(K/10⁶ erythrocytes)	Experimental	7.14 ± 0.80	7.93 ± 0.58			
SOD	Placebo	7.52 ± 0.85	7.90 ± 1.47			
(Dkat/10⁶ erythrocytes)	Experimental	10.5 ± 0.35	7.69 ± 1.43			
GRd	Placebo	4.39 ± 1.46	7.44 ± 1.76			
(nkat/10⁶ erythrocytes)	Experimental	3.46 ± 0.89	6.37 ± 1.63			
GPx	Placebo	20.5 ± 2.6 "	10.2 ± 1.0 [b]	X	X	X
(nkat/10⁶ erythrocytes)	Experimental	13.3 ± 1.7 [b]	10.3 ± 1.1 [b]			
Catalase	Placebo	100 ± 36	91.6 ± 25.5			
(%)	Experimental	108 ± 44	120 ± 36			
Cu/Zn-SOD	Placebo	100 ± 27	76.9 ± 13.5			
(%)	Experimental	87.4 ± 33.3	91.7 ± 20.9			
GRd	Placebo	100 ± 29 *	168 ± 6 *	X		
(%)	Experimental	255 ± 49 *	263 ± 56 *			
GPx	Placebo	100 ± 14	85.5 ± 14.7			
(%)	Experimental	117 ± 15	117 ± 10			

(A) Data study initial and final, in basal conditions. (B) Final study data, for basal and post-exercise conditions. Statistical analysis: Two-way ANOVA, $p < 0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between supplementation and time, (E) Significant effect of exercise, (SxE) Significant interaction between supplementation and exercise. One-way ANOVA, $p < 0.05$. (*) Significant differences between placebo and experimental. (#) Significant differences between initial and final or significant differences between basal and post-exercise.

MANUSCRIPTIV

Effects of docosahexaenoic acid diet supplementation, training and acute exercise on oxidative balance in neutrophils

EFFECTS OF DOCOSAHEXAENOIC ACID DIET SUPPLEMENTATION, TRAINING AND ACUTE EXERCISE ON OXIDATIVE BALANCE IN NEUTROPHILS

ABSTRACT

Diet supplementation with omega-3 fatty acids could influence the oxidative equilibrium, enhancing a pro-oxidant status. The aim was to determine the effects of diet supplementation with docosahexaenoic acid (DHA), training and acute exercise on oxidative balance in neutrophils. Fifteen volunteer male soccer players were randomly assigned to a placebo or experimental group. The placebo group was supplemented with an almond-based beverage whereas the experimental group was supplemented with the same beverage enriched with DHA, in addition to their Mediterranean type diet. Three blood samples were taken: in basal conditions at the beginning of the nutritional intervention and after eight weeks of training season in basal and post-exercise conditions. The training season significantly increased the antioxidant defenses of neutrophils, such as catalase, glutathione peroxidase and glutathione reductase enzyme activities; and decreased oxidative damage markers such as malondialdehyde (MDA), carbonyl and nitrotyrosine indexes. Oxidative damage markers decreased in neutrophils after acute exercise, which primed neutrophils to produce reactive oxygen and nitrogen species (RONS) after immune stimulation with zymosan or phorbol myristate acetate (PMA) in trained footballers. DHA supplementation resulted in no significant effects on oxidative stress balance in neutrophils. In conclusion, DHA supplementation did not modify the adaptive response of the antioxidant system of neutrophils to training or the production of RONS induced by immune stimulation after acute exercise.

Keywords: Antioxidant enzymes, DHA, neutrophil, oxidative stress, training

INTRODUCTION

Regular and moderate physical activity induces a healthy adaptation against elevated oxidant levels by increasing cellular and plasma antioxidant capability [1-3]. However, after acute, intense exercise, the antioxidant capability may not be enough to restore the oxidant-antioxidant redox balance, resulting in oxidative stress and a minimal adaptation [4]. Regular training brings about the continuous presence of physiological oxidant stimuli and adapts the body to exercise-induced oxidative stress, resulting in greater antioxidant protection against oxidative challenges [5] performed by different mechanisms, such as an increase in antioxidant enzyme activities and protein levels [6-8]. In accordance, it has been reported that the molecular mediators of endogenous ROS defense induced by exercise may be blocked by antioxidant supplementation [9, 10]. Moreover, it has been reported that regular exercise ameliorates type 2 diabetes mellitus, insulin resistance and increases mitochondrial formation [9].

Neutrophils play an important role as primary defense cells against infection in an acute inflammatory reaction. Acute exercise is known to mobilize peripheral neutrophils resembling the acute-phase immune response, similar to that induced after an infection [11-14]. Neutrophil defense mechanisms are characterized by the release of the content of neutrophil granules and the activation of the 'respiratory burst' which consumes molecular oxygen, resulting in the production of reactive oxygen species (ROS), reactive nitrogen species (RNS) and oxidized halides [15]. This increased cellular oxygen uptake results in toxic compounds that are used to kill the engulfed bacteria or are secreted to attack larger parasites outside the neutrophil.

Hypochlorous acid (HOCl), hydroxyl radical (-HO), nitric oxide (NO) and peroxynitrite derivates ($ONOO^-$) are the main oxidant agents produced during the oxidative burst process [16]. In the phagosome, HOCl is produced by myeloperoxidase (MPO) from hydrogen peroxide (H_2O_2) and Cl^- [17]. Plasma-membrane NADPH oxidase, when assembled, catalyzes the formation of superoxide anions ($O_2^{\cdot-}$), which then form H_2O_2, singlet oxygen ($^1O_2$) and -HO. Moreover, enhanced ROS production by neutrophils after intense exercise has been

associated with cellular and plasma oxidative damage and also contributes to the oxidative disequilibrium evidenced in sportsmen after exercise [18]. However, when the exercise is exhausting such as a marathon race it has been reported an increase in circulating cytokines than participate in the regulation of neutrophil surface antigen expression, such decreasing the expression of CD16 which involves a decrease in phagocytic activity with a consequent minor ROS production and decrease in neutrophil oxidative burst activity [19]. Many studies have reported a transient decrease in immune function in response to exhaustive exercise which could be related with an increase in susceptibility to upper respiratory illness [20, 21].

Omega-3 fatty acid such as docosahexaenoic acid (DHA) have been reported to exert anti-inflammatory properties [22]. A diet rich in omega-3 increases the blood levels of these fatty acids [23], the unsaturated lipid fraction, and enhances the oxidative capability to produce lipid hydroperoxides and oxidative-derived products [24, 25]. DHA is the longest, most unsaturated and hence most oxidizable polyunsaturated fatty acid (PUFA) commonly found in nature [26]. Paradoxically, beneficial effects of a diet rich in omega-3 fatty acids have been pointed out for the maintenance of an antioxidant status and reduction of oxidative damage markers [22, 25, 27-30]. DHA is capable of stimulating superoxide production in neutrophils both directly and synergically in concert with other agonists [31]. This increased generation of superoxide after addition of DHA is a consequence of an increase in the activity of neutrophil NADPH-oxidase probably via protein kinase C activation [32]. DHA has been found to increase phagocytic and fungal activity in isolated neutrophils of rats without altering NO production, to increase the production of H_2O_2 and $O_2^{\cdot}$ in the presence of phorbol myristate acetate (PMA), but not to increase $O_2^{\cdot}$ production in the presence of zymosan [33]. In goats, DHA was shown to increase neutrophil defensive functions by up-regulating their phagocytosis activity and down-regulating ROS production, thus reducing excessive tissue damage [34]. In humans, DHA did not affect the neutrophil N-formyl-met-leu-phe-induced chemotaxis nor radical superoxide radical production [35]. Moreover, the neutrophil generation of

inflammatory precursors not seems been modulated by DHA [36]. Nitric oxide and other ROS modulate inflammation and the immune function [37].

Acute exercise primes neutrophils for the oxidative burst and induces an acute phase immune response affecting the oxidative balance in neutrophils which could be modulated by DHA. We investigate the combined effects of DHA and training and not just the effects of DHA alone in elite athletes on the neutrophil oxidative equilibrium. There are several studies analyzing the effects of both DHA and EPA (together or individually) and many other using fish oil supplementation. In the present study we aimed to evaluate the effects of DHA incorporated in a functional food and not as a pill or fish oil supplement which has a pool of different polyunsaturated fatty acids in variable proportion depending of the source of fish fatty acids [30, 38]. The primary outcome of the present study was to determine the capability of diet supplementation with DHA to increase its levels in the erythrocyte cellular membranes of soccer players during a training season. The secondary outcomes were to determine the effects of DHA diet supplementation, the training season and acute exercise on oxidative and nitrosative damage markers and antioxidant enzyme activities, and protein levels in neutrophils. Moreover, the possible DHA modulator effects on the capability of ROS production were analyzed when neutrophils were activated with PMA or zymosan.

MATERIALS AND METHODS

Subjects and study design

The study was performed with 15 male soccer players (Reial Mallorca B team), at the beginning of their annual sport season. The sample size calculation was performed taking into account a statistical power of 80% and, the DHA enrichment of the erythrocyte membranes was estimated as 25% for the experimental group. The required sample size was estimated in 7 subjects each group. We estimated that only 75% of subjects that begin the study will finish it and, consequently the recruitment of 20 subjects will be enough to

fulfill the minimal requirement of sample size. At the beginning of the study, 22 subjects were recruited but 6 of them left the football team during the experimental time to go play with the first and professional team and one broke the anterior cruciate ligament of the knee as it was explained in a consort flow diagram (Figure 1). Participants in the study were professional, federated soccer players 19.7 ± 0.4 years old and weighing 76.5 ± 2.5 kg. There were no differences in anthropometric characteristics (height, waist circumference, hip circumference, systolic blood pressure, diastolic blood pressure, body mass index, waist-hip ratio, fat mass and fat-free mass) or physical activity capabilities (VO_2max, intense physical activity time and moderate physical activity time) between the placebo and experimental group. All the participants followed a Mediterranean diet under medical supervision, with a similar intake of energy, carbohydrates and lipids.

All subjects were informed of the purpose and demands of the study before giving their written consent to participate. The study protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the Autonomic Community of the Balearic Islands N° IB 994/08 PI (Palma de Mallorca, Balearic Islands, Spain).

Subjects were double-blind randomly selected to be included in two groups: placebo and experimental groups. The selection was done using the correlative dorsal number and its random assignation to one of the two groups. The placebo group was made up of 6 subjects who took one liter of a placebo drink five times a week and the experimental group was made up of 9 other subjects who consumed one liter of an experimental drink rich in DHA five times a week, over a total period of 8 weeks. The intake for five times a week of the 1L respective beverage entailed a daily intake of about 1.14 g DHA as well as the basal DHA intake from the diet in the experimental group, whereas the placebo only ingested omega-3 from the diet. The DHA from the diet is between 171 - 376 mg/day, calculated from the fish intake in people from Balearic Islands with the same range of age than the soccer players [39] and, considering the different DHA

content depending on the fish species [40]. The DHA intake from the experimental beverage could triple the DHA from the diet. The dose of DHA assayed is similar to those used in other studies [41, 42] but is lower to the doses assayed in others [23, 28-30].

Drink composition

Both placebo and supplemented drinks were composed of 3.0% almond and 0.8% sucrose and the rest was water, flavor and the added oils and vitamin E. Moreover, the placebo drink contained 0.8% refined olive oil whereas the experimental drink contained 0.6% refined olive oil and 0.2% DHA-S Market (Market Biosciences Corporation, Columbia, EEUU) rich in C22:6 and C22:5. The two almond drinks were elaborated by Liquats Vegetals S.A. (Girona, Spain) and the procedure for obtaining them were: bleaching of the almonds, crushing of the almonds in water, centrifuging of the mixture in order to eliminate insoluble materials, addition of cinnamon and lemon natural flavors, sucrose, vitamin E and the respective oil for the experimental (olive oil plus DHA-S) or placebo (olive oil) drink. Finally, the beverages were sterilized and packed. Externally, both types of beverages were identical in both taste and visual appearance. The fatty acid composition of beverages was determined following the same procedure used to determine erythrocyte fatty acid composition as described below. The beverages, placebo and experimental, contained, respectively: 9.45 ± 0.91 and 7.95 ± 1.37 of C16:0 (%), 5.96 ± 0.82 and 4.25 ± 0.93 of C18:0 (%), 55.9 ± 13.7 and 49.6 ± 12.3 of C18:1 (%), and 28.7 ± 6.1 and 26.8 ± 6.6 of C18:2 (%). Moreover, the enrichment of the experimental beverage with DHA was reflected with 3.78 ± 0.15 (%) of C22:5 and 7.62 ± 0.26 (%) of C22:6 in the final composition of the drink, whereas they were undetected in the placebo drink.

Experimental procedure

For each subject, three different blood samples were obtained from the antecubital vein. One blood sample was taken in basal conditions at the beginning of the nutritional intervention. Another two blood samples were taken at the end of the nutritional

intervention, in basal and post-exercise conditions. The post-exercise sample was taken 2 h after the exercise because is coincident with an increment in circulating neutrophils, with changes in antioxidant enzymes activities and in markers of oxidative damage [12, 43]. The exercise consisted of a 2-hour habitual physical training session. After 15 min of warm-up, the players performed the Leger Boucher test to indirectly determine the VO_2max. After that, the players did a recovery exercise of control-passing for 15 min. The main body of the training session was characterized by small-sided games. Briefly, the first exercise consisted of a 5 v 5 possession exercise in an area of 20 x 15 m (4 repetitions of 5 min with 30 sec of recovery between repetitions); the second was a 6 v 6 possession exercise in an area of 30 x 20 m (3 repetitions of 6 min with 1 min of recovery between repetitions), and finally, the players played a football match 5 v 5 in 30 x 40 m for 20 min. Serum was obtained after centrifugation (1,000 x g, 15 min, 4°C) of the blood samples. Erythrocyte samples were obtained after blood centrifugation (900 x g, 30 min, 18°C) and were reconstituted and hemolyzed with distilled water in the same volume as plasma.

Neutrophil purification

The neutrophil fraction was purified following an adaptation of the method described elsewhere [44, 45]. Blood was carefully introduced on Ficoll in a proportion of 1.5:1 and was then centrifuged at 900 x g, at 4°C for 30 min. The precipitate containing the erythrocytes and neutrophils was incubated at 4°C with 0.15 M ammonium chloride to hemolyze the erythrocytes. The suspension was centrifuged at 750 x g, at 4°C for 15 min and the supernatant was then discarded. The neutrophil phase at the bottom was washed first with ammonium chloride and then with phosphate-buffered saline (PBS), pH 7.4. Finally, the neutrophils were lysed with distilled water for most of the determinations or were resuspended in Hank's balanced salt solution (HBSS) for luminol assays or resuspended in RIPA buffer for western blot assays. The neutrophils were quantified in fresh blood using the autoanalyzer system Technicon H2 (Bayer).

Erythrocyte Fatty Acid determination

Erythrocyte (250 ul) lipid content was determined by lipid extraction with a modification of the method of Folch [46] followed by derivatization with the reagent Meth-Prep™ II (GRACE) and gas chromatographic separation. The gas chromatograph was an Agilent 5890 model with FID detector and the column was a Supelcowax® 10 Capillary GC column, 30 m x 0.53 mm, d_f 0.50 u.m. Quantification was performed with an internal standard (C17:0). Individual fatty acids results were expressed as a percentage of the total fatty acid pool.

Nitrate determination

The nitrate levels were measured in an aliquot of neutrophils lysed with distilled water and in serum samples. After centrifugation at 900 x g at 4°C for 30 min, the supernatants were recovered and used to measure nitrate levels by detection of liberated NO by gas¬ phase chemiluminescence reaction with ozone using a nitric oxide analyzer (NOA) 280i (Sievers). Nitrate levels were determined following an adaptation of the method described by Braman [47]. The purge vessel was loaded with a saturated VCl_3 solution in 1M HCl and tempered to 90°C with a current of hot water. To prevent damage to the NOA from the hydrochloric acid vapor, a gas bubbler filled with 1M NaOH was installed between the purge vessel and the NOA. A nitrate standard (1 - 50uM) was used to calculate nitrate concentration. 25 uL of sample or standard were injected into the purge vessel and the area under the curve of NO peaks was recorded and processed using Liquid software. The measurement of nitric oxide metabolites as a surrogate for NO production is well established [48, 49].

Luminol assay to measure neutrophil ROS production

Opsonized zymosan (OZ) was used as a neutrophil stimulant. Zymosan A (Sigma) was suspended in HBSS at a concentration of 1 mg/mL and incubated with 10% human serum at 37°C for 30 min to opsonize the zymosan, followed by centrifugation at 750 x g for 10 min at 4°C. The precipitate was washed twice in HBSS and finally resuspended in HBSS at 1 mg/mL.

OZ suspension (100 ul) was added to a 96-well microplate containing 50 ul neutrophil suspension and 50 ul luminol solution (2 mM in PBS, pH 7.4). Luminol is cell permeable and reacts with intra- and extra- cellular ROS. Chemiluminescence was measured at 37°C for 90 min in FLx800 Microplate Fluorescence Reader (Biotek Instruments, Inc.). Each sample was determined in duplicate.

Dichlorofluorescein assay to measure neutrophil ROS production

ROS production in neutrophils was measured before and after stimulation with PMA using 2,7-dichlorofluorescein-diacetate (DCFH-DA) as indicator. DCFH-DA (30 u.g/mL) with PBS was added to a 96-well microplate containing neutrophil suspension. PMA (3 u.mol/L) prepared with HBSS or HBSS alone was added to the wells and fluorescence (Ex 480 nm; Em 530 nm) was recorded at 37°C for 1 h in a FLx800 Microplate Fluorescence Reader (Biotek Instruments, Inc., USA). DCFHDA is deacetylated by esterases into neutrophils to form 2,7-dichlorofluorescein (DCFH) which reacts with intracellular ROS producing a fluorescent derivative.

Malondialdehyde determination (MDA)

MDA, as a marker of lipid peroxidation, was analyzed in neutrophils using a colorimetric assay kit (Calbiochem). Briefly, samples and standards were placed in eppendorfs containing n-methyl-2-phenylindole (10.3 mM) in acetonitrile:methanol (3:1, v/v). HCl 12N was added and samples were incubated for 1 h at 45°C. Absorbance was measured at 586 nm.

Protein carbonyl and nitrotyrosine determination

Protein carbonyl derivatives and nitrotyrosine were determined by immunological methods using the OxiSelect™ Protein Carbonyl Immunoblot Kit (Cell Biolabs, INC) and OxiSelect™ Nitrotyrosine Immunoblot Kit (Cell Biolabs, INC) following the manufacturer's details. Total protein concentrations were measured by the method of Bradford [50]. Initially, samples (10 u.g or 150 u.g of protein for carbonyl or nitrotyrosine, respectively) were transferred onto a nitrocellulose membrane by the method of dot blot. For

carbonyl determination, the membrane was incubated in the presence of 2,4-dinitrophenylhydrazine (DNPH) after transference. Then, the membrane was incubated with the primary antibody, specific to DNP moiety proteins in the case of carbonyl determination or rabbit anti-nitrotyrosine antibody for nitrotyrosine determination. This step was followed by incubation with a horseradish peroxidase-antibody (goat anti-rabbit IgG) conjugate directed against the primary antibody. The membrane was then treated with luminol, which is converted to a light-emitting form at wavelength 428 nm by the antigen/primary antibody/secondary antibody/peroxidase complex. The light was visualized and detected by short exposure to a Chemidoc XRS densitometer (Bio-Rad Laboratories). Image analysis was performed using Quantity One-ID analysis software (Bio-Rad Laboratories).

Enzymatic determinations

The activities of catalase (CAT), myeloperoxidase (MPO), glutathione peroxidase (GPx) and glutathione reductase (GRd) in neutrophils were determined with a Shimadzu UV-2100 spectrophotometer at 37°C.

CAT activity was measured in neutrophils by the spectrophotometric method of Aebi [51] based on the decomposition of H_2O_2. MPO activity of neutrophils was measured by guaiacol oxidation [52]. The reaction mixture contained sodium phosphate buffer, pH 7.0 and 13.5 mM guaiacol. The reaction was initiated by adding 300 uM H_2O_2, and changes at 470 nm were monitored. GPx activity was measured using an adaptation of the spectrophotometric method of Flohe and Gunzler [53]. This activity was determined both with H2O2 and cumene hydroperoxide as substrates and with GRd and NADPH as enzyme indicators. GRd activity was measured in neutrophils by a modification of the Goldberg and Spooner spectrophotometric method [54], which required oxidized glutathione as the substrate.

Western blot analysis

Catalase, Mn-superoxide dismutase (Mn-SOD), glutathione peroxidase, glutathione reductase, thioredoxin reductase 1 (TrxRl), uncoupling protein 2 (UCP2) and uncoupling protein 3 (UCP3) protein levels were determined by Western blot. Protein extracts were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). CAT (20 u.g of protein), Mn-SOD (160 u.g of protein), GPx (160 u.g of protein), GRd (20 u.g of protein), TrxRl (80 u.g of protein), UCP2 (160 u.g of protein) and UCP3 (20 u.g of protein) were loaded onto a 12% agarose gel. Following electrophoresis, samples were transferred onto a nitrocellulose membrane and incubated with a primary monoclonal antibody: anti-CAT antibody and anti-Mn-SOD antibody (Calbiochem), anti-GPx antibody, anti-GRd antibody, anti-TrxRl antibody and anti-UCP2 antibody (Santa Cruz) and anti-UCP3 antibody (Chemicon International). Then, incubation with a secondary peroxidase-conjugated antibody, anti the primary antibody, was performed. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad Laboratories) western blotting detection systems. The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and analyzed with Quantity One-1D Software (Bio-Rad Laboratories).

Neutrophil RNA extraction and real-time RT-PCR assay

CAT, MPO, Mn-SOD, GPx and GRd mRNA expression was determined by multiplex real time PCR using human 18S rRNA as reference. For this purpose, total RNA was isolated from neutrophils by Tripure extraction (Roche Diagnostics, Germany). RNA (1u.g) from each sample was reverse transcribed using 50 U of Expand Reverse Transcriptase (Roche Diagnostics, Germany) and 20 pm oligo (dT) for 60 min at 37°C in a 10 u± final volume, according to the manufacturer's instructions. The resulting cDNA (2.5 u±) was amplified using the Light Cycler Fast Start DNA Master PLUS SYBR Green kit (Roche Diagnostics, Germany). Amplification was performed at 55°C and 45 cycles. Relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. Antioxidant enzyme levels were normalized to the invariant control 18S rRNA. The 18S rRNA was

selected as a reference gene because we previously tested several housekeeping genes (18S rRNA, actin and GAPDH) for stability in immune cells, resulting the 18S rRNA the most stable gene. mRNA levels at placebo and basal conditions were arbitrarily referred to as 1. Primers used are listed in Table 1.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS v.15.0 for Windows). Results are expressed as mean ± SEM. and $P < 0.05$ was considered statistically significant. A Kolmogorov-Smirnov test was applied to assess the normal distribution of the data. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analyzed were beverage supplementation and the training period or acute exercise. The sets of data in which there was a significant interaction between the factors analyzed were tested by one-way ANOVA. When significant effects of one factor were found, a Student's t test for paired data was used to determine the differences between the groups involved.

RESULTS

DHA supplementation with the experimental beverage significantly increased the levels of this fatty acid in the membranes of erythrocytes (7.93 ± 0.54 % initial, 10.8 ± 0.5 % end) whereas the placebo group maintained the initial DHA levels after 8 weeks of supplementation (7.37 ± 0.27 % initial, 8.40 ± 0.41 % end). No effects of either beverage were reported on the amounts of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) or PUFA of the erythrocytes.

Basal neutrophil counts were unchanged after the training season or diet supplementation with DHA (Table 2), whereas the acute exercise performed after the intervention period had a significant effect increasing neutrophil counts 2.0 and 2.3 times, in the placebo and experimental group, respectively, without significant differences between groups.

The training season had significant effects on oxidative damage markers (MDA, carbonyl index and nitrotyrosine index) in neutrophils (Table 3) whereas DHA diet supplementation did not report a significant effect. The training season significantly decreased MDA levels and the carbonyl index in neutrophils of the supplemented group, whereas the decrease in the placebo group was not statistically significant. Acute exercise had a significant effect on MDA and carbonyl index. The exercise significantly decreased MDA levels about 0.4 and 0.5 times in the placebo and experimental group, respectively. The carbonyl index decreased about 0.4 times in the placebo group and 0.7 times in the experimental group in post-exercise conditions. No statistical effects were found in the nitrotyrosine index.

The neutrophils' capabilities to produce ROS were measured after their stimulation with PMA or zymosan (Table 4). Neither the training season nor DHA supplementation reported significant effects in the production of ROS by neutrophils after their activation with zymosan or PMA. However, acute exercise significantly affected ROS production of neutrophils in response to PMA or zymosan stimulation. Acute exercise significantly increased maximal ROS production about 1.3 times in the placebo group and 1.2 times in the experimental group when neutrophils were activated with zymosan, and about 2.7 times in the placebo and 2.6 in the experimental when they were activated with PMA. Exercise also significantly decreased the time at which maximal ROS production was attained after neutrophil stimulation with zymosan, which was shorter after exercise. Moreover, there was an interaction between acute exercise and DHA diet supplementation in the time of the neutrophil response to stimulation by zymosan. DHA diet supplementation significantly decreased the time at which maximal ROS production by neutrophils was attained after exercise.

Neutrophil production of nitric oxide was assessed by determining the ratio of nitrate concentration between neutrophils and serum (Table 5). The nitrate concentration in neutrophils was about 50-170 times higher than in serum, indicating a possible efflux of nitrate to plasma. DHA supplementation or training season did not

affect basal nitrate levels either in neutrophil, in serum or in neutrophil/serum ratio. However, acute exercise performed at 8 weeks had a significant effect on nitrate levels in neutrophils and serum, significantly decreased nitrate levels in neutrophils but significantly increased it in serum, in both placebo and experimental groups. Moreover, the neutrophil/serum ratio decreased in post¬ exercise conditions, but it was significant in the experimental group.

The effects of DHA diet supplementation, the training season and acute exercise on the antioxidant enzymes of neutrophils are shown in Table 6. The training season significantly affected basal activity (CAT, GPx and GRd) and protein levels (GPx, TrxR1 and UCP3) of some antioxidant enzymes in neutrophils, but these were not significantly affected by DHA supplementation (Table 6A). The basal activities of CAT, GPx and GRd were significantly increased at the end of the training season in both the placebo and experimental groups, whereas MPO activity remained unchanged. The training season affected GPx protein levels, inducing a significant decrease in their levels. The differences observed in the pattern of change of antioxidant enzyme activities and in their respective protein levels indicated increases in the specific activity (i.e. enzymatic activity per unit of enzymatic protein) of GPx and GRd induced by training season, acute exercise or DHA diet supplementation. Mn-SOD and UCP2 protein levels were not affected by DHA diet supplementation or training season. The training season but not DHA diet supplementation significantly affected the neutrophil protein levels of Trx1 and UCP3, increasing theirs protein levels.

The effects of acute exercise and DHA diet supplementation on neutrophil antioxidant enzyme activities and protein levels are reported in Table 6B. Acute exercise had significant effects on GPx activity, increasing activities in both placebo and experimental groups, whereas no significant effects were evidenced in CAT, MPO or GRd activities. CAT and GPx maintained the basal protein levels whereas GRd significantly increased its levels after acute exercise in both placebo and experimental groups. This different pattern of change in protein levels and their activity produced alterations in the specific activity of these enzymes. CAT maintained its specific activity after

acute exercise, whereas the specific activity of GPx and GRd decreased after acute exercise.

Acute exercise and supplementation had a significant interaction on Mn-SOD protein level - increasing significantly 3.6-fold in the placebo group after exercise - whereas the increase in the experimental group - about 1.5-fold - was not statistically significant. DHA diet supplementation had a significant effect on UCP3 protein level, this protein level presented lower values in experimental than placebo group, both in basal and post-exercise conditions. Neither TrxR1 nor UCP2 protein levels were significantly affected by DHA diet supplementation or acute exercise.

Neither acute exercise nor DHA diet supplementation significantly affected the gene expression of the neutrophil enzymes CAT, MPO, Mn-SOD, GPx and GRd (Table 7).

DISCUSSION

The present study evidenced that diet supplementation with DHA had little influence on the oxidant/antioxidant response of neutrophils to training season and acute exercise, although the supplementation was able to incorporate the DHA into erythrocyte membranes. Erythrocyte membrane is often used as an indicator of omega-3 fatty acid supplementation because it reflects its incorporation into other cell types [55, 56] such as brain cells [57] or peripheral mononuclear cells [58]. Previous studies reported that fish oil supplementation has no effect on neutrophil function in untrained people after acute exercise [38, 59, 60]; however, this study investigated the combined effect of DHA and training in elite and well-trained subjects and not just the effect of DHA alone which could be responsible of the different results between other studies. No effects of DHA diet supplementation were evidenced on the neutrophil antioxidant response, on nitrosative or oxidative damage markers or on the stimulated neutrophil ROS production of soccer players. The only effects of DHA diet supplementation were a reduction in the time to attain the maximum neutrophil production of ROS after zymosan stimulation, and an attenuated response of UCP3

and Mn-SOD protein levels after an acute bout of exercise. The training season reduced nitrosative and oxidative damage markers, without affecting the neutrophil capability to produce RONS and increase antioxidant protection. Moreover, acute exercise decreased oxidative but not nitrosative damage markers: the neutrophils are primed to produce ROS after exercise, together with lower specific activities of glutathione-dependent antioxidant enzymes, but a higher protein level of Mn-SOD. No changes were reported in the expression of any of the genes analyzed in neutrophils.

The main effect of acute exercise on neutrophils was the increase of circulating counts, as has been previously well established [11, 12, 14, 61], whereas no changes were evidenced as a consequence of the training season. Mobilization of neutrophils from the marginated pools is mediated by exercise intensity-dependent secretion of stress hormones (catecholamines, cortisol and growth hormone) [62]. Neutrophilia is associated with exercise intensity and duration and also with central body temperature attained during exercise [63]. The neutrophil increase reported after acute exercise was lower than that evidenced in other exercise protocols and competitions, indicating an exercise of moderate intensity in accordance with previous studies [3, 12, 14, 43, 64-66].

The training season decreased oxidative and nitrosative damage markers as a reflection of the antioxidant effects of the regular sport training sessions during the 8 weeks of the study. In this way, it was noticed that regular exercise, on the basis of its possible hormesis effects, really act as an antioxidant and could reduce oxidative damage [10]. The accumulation of workouts and matches throughout the training season seems to augment antioxidant activity and to decrease oxidative and nitrotyrosine damage in neutrophils. In accordance, it has been evidenced that low-volume exercise training (walking 30-60 min, 2 days each week for 12 weeks) reduces the concentration of ROS derivatives and increases thioredoxin concentration and GPx activity in neutrophils of older adults in basal conditions [67]. The increase in enzyme activities was not parallel to increased enzyme protein content, suggesting post-translational activation of the existing enzyme molecules [68, 69]. In the case of

GPx this post-translation process was more accentuated because the enzyme activity increased whereas the protein content decreased. These changes in enzymatic capability of the antioxidant enzymes of neutrophils could be related to the direct effects of ROS and low molecular weight antioxidants on the enzymatic protein [70, 71]. DHA supplementation did not seem to affect the catalytic activity of antioxidant enzymes in basal conditions in neutrophils.

The training season significantly increased the protein level of the antioxidant TrxR1, although no effects of acute exercise were evidenced. Increases in TrxR and CAT activities in neutrophils were described in post-diving conditions [72]. TrxR plays an important role in the defense against oxidative stress regenerating disulfide sites in thioredoxin which in turn reduces disulfide sites in oxidized proteins [73]. Uncoupling proteins are mitochondrial proteins that belong to non-enzymatic defense preventing the excessive production of free radicals in the mitochondria [74, 75]. The training season significantly increased the protein levels of UCP3 in basal conditions mainly in the placebo group. Acute exercise performed after 8 weeks of intervention did not affect this protein level, with the UCP3 levels remaining higher in the placebo group. The training season induced a neutrophil adaptive response in order to reduce oxidative and nitrosative damage. Diet supplementation with DHA did not alter these patterns induced by training.

Acute exercise practiced at the end of the training season also brought about a decrease in MDA levels and carbonyl index in both placebo and experimental groups. It has been evidenced that acute exercise such as a football match, a cycling stage, a duathlon and triathlon competition increase lipid oxidative markers [61, 76, 77]. Regular training has also been evidenced to reduce plasma MDA levels in footballers [78] or to report no effects of acute exercise on neutrophil MDA [3] or carbonyl index in females, but increases in males [43]. The increased antioxidant capability of neutrophils after the training season could counteract the production of ROS induced by acute intense exercise. Neutrophils are a cell type with a high resistance to oxidative damage induced by acute exercise, although neutrophils also have a high capability to produce important amounts

of ROS when activated. Neutrophil production of ROS after stimulation with opsonized zymosan or PMA was not affected either by DHA diet supplementation or training season, but was significantly increased after acute exercise. Opsonized zymosan is a pathogen-associated molecular pattern (PAMPs) recognized by toll-like receptor 2 (TLR2) [79]. The binding of PAMPs to TLRs causes inflammation and the establishment of adaptive immunity [80]. PMA interacts with protein kinase C reactive to activate lisosomal NADPH-oxidase and induces degranulation [81]. However, oxidative damage markers were reduced after exercise in the current work. These two facts could be interpreted as a lack of stimulation of circulating neutrophils after acute exercise (then neutrophils did not increase the production of ROS) or as the existence of antioxidant processes that avoid oxidative damage when ROS are produced. On the other hand, neutrophil nitrate concentration and the neutrophil serum ratio decreased after exercise, indicating lower NO synthesis 2 hours after exercise. Nitric oxide is produced by activated macrophages to kill microorganisms and to eliminate nitrosylated macromolecules and, in this process, NO is rapidly oxidized to nitrites or nitrates [37]. Moreover, humans can reduce nitrate to nitrite, NO, and other bioactive nitrogen oxides [82]. The exercise provokes a blood cell NO synthesis [82, 83]. Hence, neutrophils were probably primed to oxidative burst after exercise, but the lack of immune stimulus kept the neutrophils at a lower level of ROS and NO production, even resulting in a decrease in oxidative damage markers. The main effect of DHA diet supplementation on neutrophil capabilities to produce ROS was on the time for the maximal production of ROS to be attained after neutrophil stimulation with opsonized zymosan. The experimental group showed a shorter time at which maximal chemiluminescence levels was attained, but similar maximal ROS production, indicating that the neutrophils in the experimental group responded faster to zymosan than in the placebo one after exercise.

Intense exercise such as a mountain cycling stage reduces GPx enzyme activity of neutrophils [18]. The exercise performed after the supplementation intervention in the present work affected only GPx activity significantly, reducing its activity, but this was not

accompanied by a decrease in protein levels, suggesting a deactivation of this enzyme. The rise in Mn-SOD could increase the production of H_2O_2 and, in accordance with previous studies, reporting a function of H_2O_2 as an activator of peroxidases, this could result in a reactivation of GPx activity after exercise [84, 85]. Neither was GRd enzyme activity affected by exercise, contrary to GRd protein levels which significantly increased mainly in the DHA group. In a previous study, the enzyme activity of GPx decreased with an increase in the enzyme activity of GRd, after a football match [3]. Protein levels of UCP3 were higher in the placebo group than in the experimental group, both in basal and post-exercise conditions at the end of the supplementation. In this way, free fatty acids are considered UCP regulators, and the different profile of the fatty acids of the different beverages may be responsible for the different UCP3 response [75, 86]. However, no consequences on oxidative damage markers were observed in neutrophils of the DHA diet supplemented group due to low UCP3 protein levels.

In conclusion, diet supplementation with DHA had little influence on the oxidative balance of neutrophils, contrary to training which had a good influence on the oxidative stress of neutrophils, increasing antioxidant defenses and decreasing oxidative and nitrosative damage markers. The increased neutrophil antioxidant capabilities of trained football players seemed to counteract the ROS and RNS neutrophil production induced by acute intense exercise, even decreasing the markers of oxidative damage in neutrophils.

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DECLARATION OF INTEREST

The authors declare that they do not have any conflict of interest.

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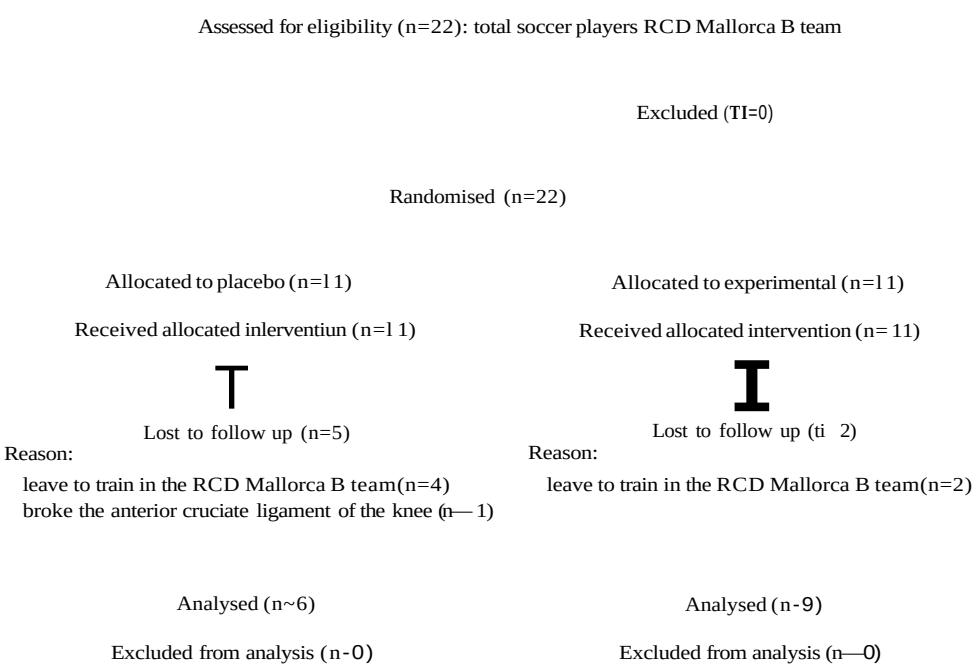

Figure 1. Consort flow diagram showing the movement of participants in the study.

Table 1. Primers and conditions used in real-time PCRs

Gene	Primers	Conditions
	Fw: 5'-ATG TGA AGT CAC TGT GCC AG-3'	95°C 10 s
18S	**Rv:** 5'-GTG TAA TCC GTC TCC ACA GA-3'	60°C 10 s
		72°C 12s
	Fw: 5'-TTT GGC TAC TTT GAG GTC AC-3'	95°C 10 s
CAT	**Rv:** 5'-TCC CCA TTT GCA TTA ACC AG-3'	60°C 10 s
		72°C 15 s
	Fw: 5'-TGAACATGGGGAGTGTTTCA-3'	94°C 15 s
MPO	**Rv:** 5'-CCAGCTCTGCTAACCAGGAC-3'	61°C 30 s
		72°C 60 s
	Fw: 5'-CGTGCTCCCACACATCAATC-3'	95°C 10 s
Mn-SOD	**Rv:** 5'-TGAACGTCACCGAGGAGAAG-3'	60°C 10 s
		72°C 12 s
	Fw: 5'-TTC CCG TGC AAC CAG TTT G-3'	94°C 10 s
GPx	**Rv:** 5'-TTC ACC TCG CAC TTC TCG AA-3'	63°C 10 s
		72°C 15s
	Fw: 5'-TCACGCAGTTACCAAAAGGAAA-3'	95°C 10 s
GRd	**Rv:** 5'-CACACCCAAGTCCCCTGCATAT-3'	63°C 10 s
		72°C 15 s

Table 2. Effects of training season, acute exercise and DHA diet supplementation on neutrophils

				ANOVA
A		**Initial**	**Final**	**S T SxT**
Neutrophils	Placebo	4.13 ± 1.12	3.47 ± 0.53	
(10^6 cells/mL blood)	Experimental	3.10 ± 0.28	2.80 ± 0.12	
B		**Basal**	**Post-exercise**	**S E SxE**
Neutrophils	Placebo	3.47 ± 0.53	6.82 ± 0.28 #	**X**
(10^6 cells/mL blood)	Experimental	2.80 ± 0.12	6.49 ± 0.55 #	

(A) Data study initial and final, in basal conditions, (B) Final study data, in basal and post-exercise conditions. Statistical analysis: Two-way ANOVA, $p<0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between supplementation and time, (E) Significant effect of exercise, (SxE) Significant interaction between supplementation and exercise. One-way ANOVA, $p<0.05$. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period or significant differences between basal and post-exercise.

Table 3. Oxidative damage of the placebo and experimental groups

				ANOVA
A		**Initial**	**Final**	**S T SxT**
MDA	Placebo	2.87 ± 0.59	2.17 ± 0.20	**X**
(u.mol/10^{9}cell)	Experimental	3.93 ± 0.60	2.37 ± 0.20 #	
Carbonyl index	Placebo	100 ± 27	68.4 ± 17.7	**X**
(%)	Experimental	120 ± 12	63.8 ± 12.4 #	
N-Tyr index	Placebo	100 ± 37	75.9 ± 14.0	**X**
(%)	Experimental	107 ± 18	51.0 ± 5.9	
B		**Basal**	**Post-exercise**	**S E SxE**
MDA	Placebo	2.17 ± 0.20	0.930 ± 0.034 #	**X**
(u.mol/10^{9}cell)	Experimental	2.37 ± 0.20	1.10 ± 0.104 #	
Carbonyl index	Placebo	100 ± 8	43.0 ± 9.8	**X**
(%)	Experimental	93.4 ± 18.6	61.7 ± 13.0	
N-Tyr index	Placebo	100 ± 18	65.0 ± 20.9	
(%)	Experimental	67.6 ± 7.8	116 ± 25	

(A) Data study initial and final, in basal conditions, (B) Final study data, in basal and post-exercise conditions. Statistical analysis: Two-way ANOVA, $p<0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between supplementation and time, (E) Significant effect of exercise, (SxE) Significant interaction between supplementation and exercise. One-way ANOVA, $p<0.05$. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period or significant differences between basal and post-exercise.

Table 4. Luminol and dichlorofluorescein assay

A		Initial	Final	ANOVA T	S	TxS
Neutrophils Zymosan	Placebo	1,527 ± 150	1,659 ± 99			
(RLU/10^6 cells)	Experimental	1,479 ± 101	1,581 ± 113			
Neutrophils Zymosan	Placebo	34.4 ± 0.9	33.3 ± 1.0			
Time (min)	Experimental	35.4 ± 1.2	34.9 ± 0.7			
Neutrophils PMA	Placebo	6,334 ± 796	6,723 ± 768			
(RFU/10^6 cells)	Experimental	5,809 ± 322	5,975 ± 398			
B		**Basal**	**Post-exercise**	**S**	**E**	**ExS**
Neutrophils Zymosan	Placebo	1,659 ± 99	2,200 ± 72*	X		
(RLU/10^6 cells)	Supplemented	1,581 ± 113	1,985 ± 94*			
Neutrophils Zymosan	Placebo	33.3 ± 1.0 a	31.4 ± 0.8 a	X		X
Time (min)	Experimental	34.9 ± 0.7 a	29.5 ± 0.7 b			
Neutrophils PMA	Placebo	6,723 ± 768	17,821 ± 951*	X		
(RFU/10^6 cells)	Experimental	5,975 ± 398	15,584 ± 652*			

(A) Data study initial and final, in basal conditions, (B) Final study data, in basal and post-exercise conditions. Statistical analysis: Two-way ANOVA, $p<0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between supplementation and time, (E) Significant effect of exercise, (SxE) Significant interaction between supplementation and exercise. One-way ANOVA, $p<0.05$. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period or significant differences between basal and post-exercise.

Table 5. Nitrate levels

A		Initial	Final	ANOVA S	T	SxT
Neutrophil	Placebo	0.492 ± 0.059	0.635 ± 0.086			
(u.mol/10⁹cells)	Experimental	0.543 ± 0.074	0.669 ± 0.118			
(mM)	Placebo	1.64 ± 0.20	2.12 ± 0.29			
	Experimental	1.81 ± 0.34	2.23 ± 0.39			
Serum	Placebo	28.3 ± 3.7	24.1 ± 2.3			
(uM)	Experimental	32.6 ± 3.6	29.7 ± 3.1			
Neutrophil/Serum	Placebo	58.0 ± 18.6	88.0 ± 13.1			
	Experimental	55.7 ± 15.2	75.1 ± 17.3			

B		Basal	Post-exercise	S	E	SxE
Neutrophil	Placebo	0.635 ± 0.086	0.404 ± 0.082	X		
(umol/10⁹cells)	Experimental	0.669 ± 0.118	0.452 ± 0.086			
(mM)	Placebo	2.12 ± 0.29	1.35 ± 0.39	X		
	Experimental	2.23 ± 0.39	1.51 ± 0.29			
Serum	Placebo	24.1 ± 2.3	32.2 ± 3.3	X		
(uM)	Experimental	29.7 ± 3.1	37.1 ± 3.1			
Neutrophil/Serum	Placebo	88.0 ± 13.1	41.9 ± 10.1	X		
	Experimental	75.1 ± 17.3	40.7 ± 9.2 #			

(A) Data study initial and final, in basal conditions, (B) Final study data, in basal and post-exercise conditions. Statistical analysis: Two-way ANOVA, $p<0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between supplementation and time, (E) Significant effect of exercise, (SxE) Significant interaction between supplementation and exercise. One-way ANOVA, $p<0.05$. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period or significant differences between basal and post-exercise.

Table 6. Antioxidant enzyme activities and protein levels of the placebo and experimental groups

A		Initial	Final	ANOVA S	T	SxT
Catalase	Placebo	32.3 ± 8.0	98.5 ± 23.8		X	
(K/10⁹ cells)	Experimental	36.8 ± 3.7	69.4 ± 9.2			
MPO	Placebo	336 ± 104	234 ± 65			
(ukat/10⁹ cells)	Experimental	397 ± 111	337 ± 45			
GPx	Placebo	130 ± 31	365 ± 63		X	
(nkat/10⁹ cells)	Experimental	116 ± 11	300 ± 45			
GRd	Placebo	169 ± 72	284 ± 79		X	
(nkat/10⁹ cells)	Experimental	109 ± 30	388 ± 125			
Catalase	Placebo	100 ± 26	146 ± 67			
(%)	Experimental	80.6 ± 15.5	104 ± 32			
Mn-SOD	Placebo	100 ± 30	55.2 ± 15.2			
(%)	Experimental	75.0 ± 6.6	101 ± 26			
GPx	Placebo	100 ± 26	27.1 ± 8.7 #		X	
(%)	Experimental	134 ± 20	29.5 ± 4.1 #			
GRd	Placebo	100 ± 30	70.2 ± 11.8			
(%)	Experimental	70.9 ± 13.8	67.3 ± 10.9			
TrxRI	Placebo	100 ± 38	166 ± 51		X	
(%)	Experimental	78.9 ± 13.3	165 ± 42			
UCP2	Placebo	100 ± 46	115 ± 46			
(%)	Experimental	40.7 ± 10.9	75.5 ± 19.1			
UCP3	Placebo	100 ± 19	249 ± 65 #		X	
(%)	Experimental	99.0 ± 29.6	159 ± 21			

B		Basal	Post-exercise	S	E	SxE
Catalase	Placebo	98.5 ± 23.8	31.6 ± 9.2			
(K/10⁶ cells)	Experimental	69.4 ± 9.2	93.5 ± 35.4			
MPO	Placebo	234 ± 65	253 ± 32			
(u.kat/10⁶ cells)	Experimental	337 ± 45	224 ± 46			
GPx	Placebo	365 ± 63	106 ± 7	X		
(nkat/10⁶ cells)	Experimental	300 ± 45	149 ± 18			
GRd	Placebo	284 ± 79	271 ± 47			
(nkat/10⁶ cells)	Experimental	388 ± 125	293 ± 55			
Catalase	Placebo	100 ± 46	48.1 ± 11.4			
(%)	Experimental	71.2 ± 22.2	102 ± 29			
Mn-SOD	Placebo	100 ± 28 a	364 ± 56 b	X		X
(%)	Experimental	183 ± 48 a,c	271 ± 20 b,c			
GPx	Placebo	100 ± 40	183 ± 53			
(%)	Experimental	124 ± 27	136 ± 20			
GRd	Placebo	100 ± 17	175 ± 49	X		
(%)	Experimental	95.8 ± 15.6	201 ± 34 #			
TrxR1	Placebo	100 ± 31	165 ± 25.2			
(%)	Experimental	99.4 ± 25.2	87.2 ± 17.5			
UCP2	Placebo	100 ± 40	135 ± 50			
(%)	Experimental	65.9 ± 16.7	74.8 ± 10			
UCP3	Placebo	100 ± 26	109 ± 24	X		
(%)	Experimental	63.9 ± 8.5	76.3 ± 11.2			

(A) Data study initial and final, in basal conditions, (B) Final study data, in basal and post-exercise conditions. Statistical analysis: Two-way ANOVA, $p<0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between supplementation and time, (E) Significant effect of exercise, (SxE) Significant interaction between supplementation and exercise. One-way ANOVA, $p<0.05$. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period or significant differences between basal and post-exercise.

Table 7. Gene expression before and after exercise

				ANOVA
		Basal	**Post-exercise**	**S E SxE**
CAT	Placebo	1.00 ± 0.53	2.35 ± 1.41	
(AU)	Experimental	1.21 ± 0.55	2.25 ± 1.21	
MPO	Placebo	1.00 ± 0.26	1.85 ± 1.10	
(AU)	Experimental	1.08 ± 0.52	0.55 ± 0.20	
Mn-SOD	Placebo	1.00 ± 0.26	0.93 ± 0.18	
(AU)	Experimental	1.22 ± 0.37	1.73 ± 0.55	
GPx	Placebo	1.00 ± 0.28	2.35 ± 1.41	
(AU)	Experimental	2.29 ± 1.05	2.26 ± 1.21	
GRd	Placebo	1.00 ± 0.37	1.85 ± 1.10	
(AU)	Experimental	1.15 ± 0.35	0.55 ± 0.20	

Final study data, in basal and post-exercise conditions. (AU) Arbitrary Units. Statistical analysis: Two-way ANOVA, $p<0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between supplementation and time, (E) Significant effect of exercise, (SxE) Significant interaction between supplementation and exercise. One-way ANOVA, $p<0.05$. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period or significant differences between basal and post¬ exercise.

MANUSCRIPT V

Diet supplementation with DHA enriched food during training season in soccer players enhances the mitochondrial antioxidant capabilities in PBMCs

DIET SUPPLEMENTATION WITH DHA ENRICHED FOOD DURING TRAINING SEASON IN SOCCER PLAYERS ENHANCES THE MITOCHONDRIAL ANTIOXIDANT CAPABILITIES IN PBMCS

ABSTRACT

Exercise induces oxidative stress and causes adaptations in antioxidant defences. The aim of the present study was to determine the effects of a 2 month diet supplementation with DHA on the pro-oxidant and antioxidant status of peripheral blood mononuclear cells (PBMCs) during soccer training and after acute exercise. 15 male soccer players, in a randomized double-blind trial, were treated with either a beverage enriched with DHA or a placebo for 8 weeks. Blood samples were collected in basal conditions before and after the training period and after an acute and intense exercise. The training season increased the carbonyl and nitrotyrosine index but decreased the MDA levels. Basal catalase activity decreased in both groups after 8 weeks of training, whereas glutathione peroxidase activity increased mainly in the placebo group. Protein levels of UCP2, UCP3 and iNOS significantly increased after the training period. Acute exercise induced redistribution in the number of circulating cells, increased the MDA levels and N-Tyr index and decreased the levels of nitrate. Acute exercise also increased the activity of antioxidant enzymes as well as the protein levels without affecting their expression. Acute exercise increased PBMCs ROS production after immune stimulation. Diet supplementation with DHA significantly increased the UCP3 levels of after training and the SOD protein levels after acute exercise, and reduced the production of ROS after acute exercise. In conclusion, DHA increases the antioxidant capabilities while reducing the mitochondrial ROS production in a regular soccer training period, and reduces the oxidative damage markers in response to acute exercise.

Keywords: DHA, PBMC, antioxidant enzymes, oxidative stress, training, exercise, ROS production, PMA

INTRODUCTION

Intense acute exercise changes the oxidative balance as result of oxygen overconsumption [1]. The oxygen consumption during physical activity is associated with high rate of production of reactive oxygen species (ROS) [2] which can overwhelm the antioxidant defences, resulting in an oxidative stress status [3-5]. The high production of ROS during exhaustive exercise causes oxidative changes on lipids, proteins and nucleic acids [6-9]. However, the practice of regular exercise leads to an adaptation of the body to high oxidant levels and improves exercise performance [10-13]. These adaptive responses include the NFkB pathway, which activates target genes related to antioxidant defenses [14-16], inducible nitric oxide synthase (iNOS) [17] and uncoupling protein (UCP) [18]. The changes in the enzymatic activity designated to reduce the deleterious effects of ROS coexist with oxidative damage in lipids [19]. Consequently, ROS participate in both the effects on the antioxidant gene expression and the effects on the oxidative changes of lipids, proteins and nucleic acids.

Omega-3 fatty acids are essential for various physiological processes in humans [20-24]. Increasing the amount of omega-3 fatty acids in the diet led to a marked incorporation of these fatty acids into membrane phospholipids [25-27]. Docosahexaenoic acid (DHA), an omega-3 fatty acid abundant in marine foods, is an important component of neuronal membranes where it has a clear structural function [22] and may affect the speed of signal transduction [23]. Omega-3 fatty acids exert anti-inflammatory properties after eccentric exercise in untrained men [28]. Moreover, omega-3 fatty acid also promotes the gene expression of key enzymes to introduce fatty acid into the mitochondria and their use as energetic fuel in the respiratory chain [29, 30]. However, the polyunsaturated fatty acids (PUFAs) are highly susceptible to oxidation, which is directly related to their degree of unsaturation [31]. Studies about the pro-oxidant or antioxidant properties of omega-3 fatty acids are controversial. Some studies have reported that supplementation with high concentrations of omega-3 fatty acids, increases oxidative stress in both animals and humans [32], whereas other studies observed a protective effect of

PUFAs against oxidative stress [33, 34] and other studies reported no clear effects [35].

The aim of the present study was to determine the effects of the consumption of a functional drink enriched with moderate levels of DHA on the oxidative balance status during exercise as well as on the adaptive response of the lymphocyte oxidative balance to training. Our aim is to determine if a DHA diet supplementation has protective effects on exercise-induced oxidative stress and if it affects the antioxidant adaptations involved in regular training.

MATERIALS & METHODS

Subjects and study design

The study group consisted of 15 male soccer players, at the start of the competition season. There were no differences in the anthropometric characteristics and physical activity capabilities between the placebo and experimental groups of soccer players. Participants in the study were professional and federated soccer players 19.7 ± 0.4 years old, 76.5 ± 2.5 kg of weight, and 179.5 ± 2.5 cm of height. The waist circumference was 78.4 ± 0.9 cm, the hip circumference was 97.4 ± 1.2 cm and the waist-hip ratio (WHR) was 0.809 ± 0.012. The value of systolic blood pressure was 119.5 ± 6.5 mmHg, and 61.7 ± 4.7 mmHg for the diastolic blood pressure. The body mass index (BMI) was 23.7 ± 0.55 kg/m^2. The football players had 92.6 ± 0.2% fat-free mass. The VO_2max was 61.4 ± 1.35 mL/kg min. Finally, the intense physical activity time was 73.4 ± 36.5 min/day, and 65.9 ± 15.8 min/day of moderate physical activity time. The subjects were healthy, non-smokers. Subjects were randomly selected to be included in two groups: placebo and experimental groups, and there were no differences between groups in the anthropometric characteristics and physical activity. The placebo group was made up of 6 subjects who took one liter of a placebo drink five times a week and the experimental group was made up of 9 other subjects who consumed one liter of an experimental drink rich in DHA five times a week, over a total period of 8 weeks. All the subjects were informed of the purpose and demands of the study before giving their

written consent to participate. The study protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the Comunitat Autonoma de les Illes Balears (Palma de Mallorca, Balearic Islands, Spain).

Drink composition

The two drinks were composed of 3.0% almond, 0.8% sucrose and 0.8% of different lipids in function of placebo or experimental drink and the rest was water, flavor and vitamin E. The lipid content of the placebo drink was 0.8% refined olive oil and the experimental drink was 0.6% refined olive oil and 0.2% DHA-S Market (Market Biosciences Corporation, Columbia, EEUU). The two almond drinks was elaborated for Liquats Vegetals S.A. (Girona, Spain) and it was by: bleaching the almonds, then crushing them in water, centrifuging of the mixture to eliminate insoluble materials, and the addition of cinnamon and lemon natural flavors, sucrose, vitamin E and the respective oil for the experimental (olive oil plus DHA-S) or placebo (olive oil) drink. Finally the beverage were sterilized and packed. Externally the two beverage types were identical in their taste and appearance.

The experimental drink presented significant concentrations of the fatty acids C20:3, C22:0, C22:5 and C22:6n3 which were undetected in placebo drink. Both beverage types have a similar fatty acid composition; however, as the experimental beverage was enriched with DHA, there were slight differences between the compositions of the two drinks. The experimental drink had significantly higher concentrations of the fatty acids C20:3 (21uM), C22:0 (76 uM), C22:5 (1,715 uM) and C22:6n3 (3,457 uM) although these fatty acids were undetected in the placebo beverage. The concentration of vitamin E in both placebo and experimental drinks is equivalent to 0.4 mg/mL of a-tocopherol acetate. Daily intake of 1 liter of beverage during five days of the respective beverage supposed a daily intake of 1.14 g of DHA, to which must be added the intake of omega-3 in the diet, on the other hand the placebo group only received their omega-3 intake the from the diet.

Experimental procedure

Three blood samples were obtained from each subject. One sample was extracted in basal conditions before starting the nutritional intervention, and another two blood samples were taken at the end of the nutritional intervention, in basal and post-exercise conditions.

The exercise consisted of a 2 h habitual physical training session. After 15 min of warm-up, the players performed the Leger Boucher test to indirectly determine the VO_2max. After that, the players did a recovery exercise of control-passing for 15 min. The main body of the training session was characterized by small-sided games. Briefly, the first exercise consists of a 5 vs 5 possession exercise in an area of 20 x15 m (4 repetitions of 5 min with 30 sec of recovery between repetitions); the second was a 6 vs 6 possession exercise in an area of 30 x 20 m (3 repetitions of 6 min with 1 min of recovery between repetitions), and finally, the players played a football match of 5 vs 5 in 30 x 40 m for 20 min.

Venous blood samples were obtained from the antecubital vein of sportsmen in suitable vacutainers with EDTA as anticoagulant. Venous blood samples were obtained after 12 h, overnight, fasted conditions (basal sample), and 2 h after finishing the training. Since it is well known that the posture (hydrostatic pressure gradients) has an impact on the plasma volume after exercise [36], subjects remained seated 0.5-1 h prior to blood sample collection.

Dietary intake

Dietary habits were assessed using a 7-day dietary record questionnaire completed at the beginning of the study. A qualified dietician verified and quantified the food records. All food items consumed were transformed into nutrients by a special computerized program based on the European and Spanish food composition tables [37].

The subjects followed a Mediterranean diet during the sport season. The energy intake of 2,518 kcal was also consumed on

average, 108 g protein, 282 g of carbohydrates, fats and 106 g water 1,880 mL in the placebo group, meanwhile the experimental group, ingested 105 g of protein, 225 g of carbohydrate, fat and 99.2 g water 1,254 mL, and the average of energy consumed was 2,215 kcal. No differences were observed in nutrient intake between the placebo and experimental groups.

PBMCs purification

The PBMC fraction was purified following an adaptation of a method described [38]. Blood was carefully introduced on Ficoll in a proportion of 1.5:1 and was then centrifuged at 900 x g, at 4°C for 30 min. The PBMCs layer was carefully removed. The plasma and the Ficoll phases were discarded. The PBMCs slurry was then washed twice with PBS and centrifuged for 10 min at 1,000 x g, 4°C. This process was performed in triplicate, one of the samples was destined to obtaining RNA, another sample was lysed with distilled water, and the other was preserved with RIPA.

Fatty Acids determination

The fatty acid extraction of erythrocyte samples (250 uL) was done by a modification of method of Folch [39]. Initially, 250 u± of erythrocyte samples were diluted in 5 mL of Cl_3CH:CH_3OH (2:1, v/v) BHA 0.01% containing 20 uL of n-heptadecanoic acid (15 mM) as internal standard for posterior fatty acids quantification. The final organic phase was evaporated under a nitrogen stream at 55°C. The dry residue was dissolved in 225 uL of n-hexane and 25 uL of Meth-Prep™ II (GRACE), derivatization reagent, and 1 ul was injected into the gas chromatograph. The mobile phase consisted of helium. The gas chromatograph was a Agillent 5890 model with a flame ionization detector (FID) and the column was a Supelcowax® 10 Capillary GC column, 30 m x 0.53 mm, d_f 0.50 u.m.

Malondialdehyde assay

Malondialdehyde (MDA) as a marker of lipid peroxidation was analyzed PBMCs by a colorimetric assay for MDA determination based on the reaction of MDA with a chromogenic reagent to yield a stable

chromophore with maximal absorbance at 586 nm. Briefly samples or standards were placed in glass tubes containing n-methyl-2-phenylindole (10.3 mM) in acetonitrile:methanol (3:1). HCl 12N was added and the samples were incubated for 1 h at 45°C. Absorbance was measured at 586 nm.

Protein carbonyl and nitrotyrosine determination

Protein carbonyl derivatives and nitrotyrosine were determined by immunological methods using the OxiSelect™ Protein Carbonyl Immunoblot Kit (Cell Biolabs, INC) and OxiSelect™ Nitrotyrosine Immunoblot Kit (Cell Biolabs, INC) by following the manufacturer's details. Total protein concentrations were measured by the method of Bradford [40]. Initially, samples (10 ug or 150 ug of protein for carbonyl or nitrotyrosine, respectively) were transferred onto a nitrocellulose membrane by the method of dot blot. For carbonyl determination, the membrane was incubated in the presence of 2,4-dinitrophenylhydrazine (DNPH) after transference. Then, the membrane was incubated with the primary antibody, specific to DNP moiety proteins in the case of carbonyl determination or rabbit anti-nitrotyrosine antibody for nitrotyrosine determination. This step was followed by incubation with a horseradish peroxidase-antibody (goat anti-rabbit IgG) conjugate directed against the primary antibody. The membrane was then treated with luminol, which is converted to a light-emitting form at wavelength 428 nm by the antigen/primary antibody/secondary antibody/peroxidase complex. The light was visualized and detected by short exposure to a Chemidoc XRS densitometer (Bio-Rad Laboratories). Image analysis was performed using Quantity One-1D analysis software (Bio-Rad Laboratories).

Hydrogen peroxide production

H_2O_2 production in PBMCs was measured before and after stimulation with phorbol myristate acetate (PMA) or lipopolysaccharide (LPS) using 2,7-dichlorofluorescin-diacetate (DCFH-DA) as indicator. A stock solution of DCFH-DA (1 mg/mL) in ethanol and PMA (1 mg/ mL) in DMSO were prepared and stored at 20°C until

analysis. DCFH-DA (30 Sg/mL) in PBS was added to a 96-well microplate containing 50 EL PBMCs suspension. PMA (10 ng/mL) or LPS (1 Eg/mL) prepared in HBSS or HBSS alone was added to the wells and the fluorescence (Ex, 480 nm; Em 530 nm) was recorded at 37°C for 1 h in FLx800 Microplate Fluorescence Reader (Bio-tek Instruments, Inc.).

Comet assay

Assessment of DNA damage was carried out using the alkaline comet assay method. Briefly, slides were prepared by adding purified PBMCs, mixed with 0.6% low-melting-point agarose. For DNA release, cells were lysed by immersing slides in lysing solution (2.5 mol/L NaCl, 100 mmol/L EDTA disodium salt, 10 mmol/L Tris, 1% Triton X-100 and 10% DMSO, pH 10.0) at 4°C for 40 min. After removal from the lysing solution, the slides were placed in an electrophoresis trough containing an alkaline electrophoresis buffer (300 mmol/L NaOH, 1 mmol/L EDTA). A current of 25 V and 300 mA was applied for 30 min. The slides were then removed and Tris buffer 0.4 mol/L adjusted to pH 7.5 with concentrated HCl was added onto the slides to neutralize excess alkali. DNA was stained by adding ethidium bromide. Comet measurements were made by image analysis using a fluorescence microscope equipped with an excitation filter of 450-490 nm and a barrier filter of 520 nm and the Comet software (TriTek CometScoreTM). Images of 50 random nuclei were taken at 200x magnification and were analysed for each sample. The comet measurements that were recorded and subsequently used for analysis were: percentage DNA in tail (tail intensity) and tail moment (tail intensity x tail length).

Nitrate determination

An aliquote of PBMCs was used to measure nitrate (100 ul) concentration by detecting liberated nitric oxide (NO) in a gas-phase chemiluminescence reaction with ozone using a NO analyzer (NOA 280i; Sievers) following an adaptation of the described method [41]. The area under the curve of NO peaks was recorded and processed using Liquid software.

Enzyme activities

We determined the catalase, GRd and GPx activities in PBMCs. All activities were determined with a Shimadzu UV-2100 spectrophotometer at 37°C. Catalase activity was measured by the spectrophotometry method of Aebi [42] based on the decomposition of H_2O_2. Glutathione reductase activity was measured by a modification of the Goldberg and Spooner [43] spectrophotometry method. Glutathione peroxidase activity was measured using an adaptation of the spectrophotometric method of Flohe and Gunzler [44].

Western blot analysis

Antioxidant enzyme protein levels were determined by Western blot. Protein extracts were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Catalase (20 u.g of protein), Cu/Zn-SOD (10 u.g of protein), Mn-SOD (20 u.g of protein), thioredoxin reductase 1 (TrxRI) (20 u.g of protein), UCP3 (20 u.g of protein) and GPx (200 u.g of protein) were loaded on a 12% agarose gel, UCP2 (20 u.g of protein) and iNOS (20 u.g of protein) were loaded on 10% agarose gel, whereas GRd (10 u.g of protein) and Cu/Zn-SOD (10 u.g of protein) were loaded on a 15% agarose gel. Following electrophoresis, samples were transferred onto a nitrocellulose membrane and incubated with a primary monoclonal antibody: anti-catalase antibody and anti-Mn-SOD antibody (Calbiochem), anti-Cu/Zn-SOD antibody (Sigma) anti-GRd antibody, anti-GPx antibody anti-TrxR1 antibody and anti-UCP2 antibody (Santa Cruz), anti-UCP3 antibody (Chemicon International) and anti-iNOS antibody (Stressgen). Blots were then incubated with a secondary peroxidase-conjugated antibody anti the primary antibody was performed. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad Laboratories) Western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and analyzed with Quantity One-1D Software (Bio-Rad Laboratories).

PBMCs RNA extraction and relative quantitative RT-PCR assay

Catalase, Cu/Zn-SOD, Mn-SOD, GPx, UCP3, mRNA expression was determined by multiplex real time PCR using human 18S rRNA as reference. For this purpose, total RNA was isolated from PBMCs by Tripure extraction (Roche Diagnostics, Germany). RNA (1 ug) from each sample was reverse transcribed using 50 U of Expand Reverse Transcriptase (Roche Diagnostics, Germany) and 20 pmol oligo (dT) for 60 min at 37°C in a 10 u± final volume, according to manufacturer instructions. The resulting cDNA (2.5 uI) was amplified using the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Diagnostics, Germany). Amplification was performed at 55°C and 45 cycles. The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. Antioxidant enzyme levels before and alter the stage were normalized to the invariant control 18S rRNA. mRNA levels at the beginning of the stage were arbitrarily referred to as 1. Primers used are listed in Table 1.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS 9 for Windows). Results are expressed as means ± S.E.M. and $p<0.05$ was considered statistically significant. All the data were tested for their normal distribution. Student's t-test for paired data was used to determine the significance of the data.

RESULTS

The nutritional intervention changed the lipid composition of erythrocyte membranes of footballers (Table 2). The percentage of DHA in erythrocyte membranes significantly increased about 1.2 times in the experimental group after 8 weeks of dietary intervention, whereas the placebo group maintained the initial values. In parallel, the enrichment of the erythrocytes membrane in DHA produced a light decrease in the proportion of eicosatrienoic and arachidonic acids in the experimental group.

Blood cell distribution is reported in Table 3. The lymphocyte counts significantly increase, 1.2 times during the training season for

both the experimental and placebo groups (Table 3A). Similarity, PBMCs counts also increased in a similar way as lymphocytes although this increase was not statistically significant in the placebo group. The basophil counts were that presented the highest increase after training season both in placebo (about 2.3 times) and experimental (about 2.8 times) groups. Acute exercise significantly increased the number of leukocytes, about 1.5 times in the experimental group and 1.4 times in the placebo, and the number of monocytes about 1.5 times in both groups (Table 3B). A significant lymphopenia was observed after exercise, decreasing about 0.89 times and 0.70 times the lymphocyte counts in placebo and experimental groups respectively. The number of basophils was significantly decreased after acute exercise-about 0.58 times and 0.54 in the placebo and experimental group, respectively.

The effects of diet supplementation with DHA, the training season and the acute exercise on the oxidative and nitrosative damage of PBMCs are shown in Table 4. No effects of DHA diet supplementation on the oxidative damage markers in PBMCs were observed; however both the training season and acute exercise significantly affects some key markers of oxidative damage. The basal MDA levels of PBMCs significantly decreased about 0.72 times in placebo and about 0.45 times in experimental groups at the end of the training season. Inversely, the basal values of carbonyl index and N-Tyr index significantly increased in PBMCs about 9.8 times and 6.2 times in placebo and about 8.4 times and 6.5 times in experimental group respectively. The basal nitrate levels significantly decreased about 0.52 times in the placebo and 0.31 times in the experimental group at the final of training season. Training season did not alter the initial status of oxidative damage in nucleic acids of PBMCs.

Acute exercise significantly increased the values of the MDA in PBMCs, about 2.1 times both in placebo and experimental groups. Similarity, the nitrosative damage marker of PBMCs proteins only increased significantly in the experimental group about 1.6 times, whereas the increase was not significant in the placebo group. The nitrate levels significantly decreased in PBMCs about 0.39 times in placebo although they were only non-significantly decreased by 0.64

times in experimental group after exercise. No significant influences by acute exercise or DHA diet supplementation were evidenced in the carbonyl index. Moreover, the acute exercise significantly increased the % tail in 1.4 times in both the experimental and placebo groups.

No significant effects of training season or DHA diet supplementation were evidenced on the ROS production by PBMCs stimulated with PMA (Figure 1A). Acute exercise significantly increased the ROS production in both groups. In a similar way, the training season didn't influenced the ROS production by PBMCs when stimulated with LPS, but acute exercise and DHA diet supplementation significantly affected the PBMCs ROS production after stimulation with LPS (Figure 1B). The acute exercise significantly increased ROS production by PBMCs, and this increase was significantly higher in the placebo (20%) than the experimental group.

The training season significantly influenced the basal catalase and GPx activities and the basal levels of antioxidant proteins UCP2 and UCP3 and, the iNOS levels (Table 5A). The DHA diet supplementation significantly influenced the basal GPx activity and the basal protein levels of UCP3 in PBMCs. The training season significantly reduced the catalase activity in PBMCs by 0.29 times in the placebo group, whereas the reduction about 0.43 times in the experimental group was not statistically significant. Inversely, the GPx activity significantly increased in PBMCs at the final of training season both in placebo (1.4 times higher) and in experimental (1.7 times higher) groups. No significant effects were evidenced in the GRd activity. These changes in the activities of catalase and GPx were not parallel to changes in the protein levels of these enzymes. The protein basal levels of catalase, Cu/Zn-SOD, Mn-SOD, GRd, GPx and TrxR1 in PBMCs maintained the initial values during the training season and supplementation. At the end of the training season the protein levels of UCP2 were about 3.6 times and 2.6 times higher than at the beginning in both the placebo and experimental group respectively. Similarity, the iNOS protein levels increased about 1.7 and 1.9 times in placebo and experimental groups, respectively. The UCP3 protein levels also significantly increased during the training season about 4.6 times in placebo group and 3.8 times in experimental group. DHA diet

supplementation also influenced the UCP3 protein levels, with basal levels 1.5 times significantly higher in experimental than placebo group.

The table 5B shows the effects of acute exercise and DHA diet supplementation on the antioxidant capabilities of PBMCs. The acute exercise significantly influenced the enzyme activities of catalase, GRd and GPx. The catalase activity significantly increased after the exercise about 2.1 times in the placebo and about 4.6 times in the experimental group. The GRd activity increased after exercise about 2.7 times in placebo and 2.1 times in experimental group, although the increase was only statistically significant in the placebo group. The GPx increased after exercise about 1.2 times in placebo and about 1.8 times in experimental groups, with significant differences only in the supplemented group. This pattern of these antioxidant enzymes was very similar to the pattern of change for their respective protein levels, with the exception of the GRd enzyme, which did not changed the protein levels. Moreover, the protein levels of Cu/Zn-SOD, TrxR1, UCP2, UCP3 and iNOS, but not Mn-SOD, were significantly increased after exercise in both the experimental and the placebo groups. The protein levels of Cu/Zn-SOD in PBMCs after acute exercise was influenced by DHA supplementation, with those of the experimental group significantly higher than those of the placebo group. Gene expressions of catalase, GPx, UCP3, Cu/Zn-SOD, Mn-SOD were not significantly affected by acute exercise or by DHA diet supplementation.

DISCUSSION

DHA diet supplementation with the enriched beverages favors the incorporation of this essential fatty acid into the erythrocyte membranes [25-27]; therefore, the PBMCs membranes of the soccer players should also incorporate the DHA supplemented by the enriched beverage [45]. We found that beverage intake by the participants in the trial is enough to alter the erythrocyte membrane composition, and increases the incorporation of DHA. This incorporation into erythrocyte membranes reduces the proportion of other fatty acids such as eicosatrienoic and arachidonic acids into the

membranes, which could alter the availability of pro- and anti-inflammatory lipid mediators in response to an immune stimulus.

Effects of training season on oxidative stress

Regular training induced greater protection against PBMCs lipid peroxidation in the footballers in accordance with a previous study [46], whereas no changes were observed in the percentage of DNA in tail and in the tail moment. However, the levels of nitrotyrosine and protein carbonyl derivates were increased. The higher nitrosative damage could reflect higher peroxynitrite production in PBMCs. Peroxynitrite results from the reaction between superoxide anion and NO [47], which could have increased by the higher levels of expression of iNOS at the final of training season. Peroxynitrite initiates lipid peroxidation [48] and reacts with sulfhydryls [49] and methionine [50]; however, in absence of direct targets, peroxynitrite can also discompose generating nitrate (70%) or undergoes homolytic rupturing (30%); generating hydroxyl radicals ($OH^{\cdot}$) and nitrogen dioxide ($NO_2^{\cdot}$) [51]. Lower nitrate levels, together the higher nitrotyrosine index in PBMCs at the end of the training season suggests that the peroxynitrite mainly reacts with proteins residues. Oxidative modifications of proteins comprise alterations of single amino acids, like the formation of protein carbonyls and methionine sulfoxide, or the aggregation of whole proteins [52]. The reaction between MDA and amino groups of proteins also introduce carbonyls groups into the protein [53]. We suggest that the lower levels of MDA in lymphocytes at the final of the training season could be result of the reaction of MDA with proteins increasing the carbonyl marker after training.

The training significantly affects the activity of antioxidant enzymes but it does affect the antioxidant protein levels. The changes observed in the catalase and GRd activities during the training season could reflect a change in the strategy to eliminate ROS from a catalase-based system to a glutathione-based system as observed in other works [54]. Catalase has a high Michaelis-Menten constant for the hydrogen peroxide, while for GPx is lower [55]. These adaptations could mean that lymphocytes produces ROS at lower rate that are

better removed by GPx. Moreover, after training, the lymphocytes present higher levels of UCP2 and UCP3, which can contribute as an antioxidant to reduce the rate of ROS production in mitochondria [56, 57]. It has been demonstrated that an increase in ROS production and, consequently, an increase in oxidative damage in muscle during a single bout of exercise, is accompanied by an increase in the expression of UCP3 [58]. This reduction in ROS production is important as different classes of signaling actions may be regulated by different ROS levels [59]: low/moderate rates involve processes such as proliferation and differentiation, and adaptive programs, including the transcriptional up-regulation of antioxidant genes, whereas, higher levels involves the initiation of senescence and cell death.

Effects of acute exercise on oxidative stress

Acute exercise causes significant changes in circulating cells increasing the number of leukocytes after an acute exercise such as after a duathlon [60] or a diving session [5]. Moreover, acute exercise causes a decrease in the number of circulating lymphocytes [13, 16] and a monocytosis [61].

Acute exercise, as a result of increased oxygen demand, induces oxidative stress and increases the markers of oxidative damage [4]. In addition, acute exercise causes an increase in the activity of antioxidant enzymes and protein levels, without affecting gene expression. After the acute exercise, the MDA marker increases in both groups, although less in the experimental group, which could reflect a protective effect of omega-3 fatty acids against oxidative stress, as has been shown to occur in rats and humans [62, 63]. No changes were detected in the index of carbonyls index due to intense physical exercise, which is contrary to the results obtained in other studies [16, 54, 64]. This response could be a consequence of the well-trained status of athletes with a higher degree of protection against oxidative damage or the intensity of exercise. The nitrotyrosine index significantly increases as a consequence of acute exercise, and these results coincide with the results obtained in neutrophils after a half marathon [65]. Moreover, nitrate levels significantly decrease in PBMCs after acute exercise, these results are

similar to those obtained in other study, carried out on plasma [66] and reinforce the idea of the distribution of peroxynitrite between its transformation into nitrate or its reaction with proteins.

The lymphocyte enzymatic antioxidant defenses have shown great adaptations to oxidative stress induced by exercise increasing their activities and protein levels [16, 60]. In this work a significant increase in PBMCs catalase, GPx and GRd activities were observed. This increase is reflected also in the catalase and GPx protein levels. Therefore, the increased activity of catalase, GPx and GRd after exercise could be attributed to the activation of preexisting enzymes. In accordance, exercise has been pointed out to induce the activation of some enzymes in erythrocytes and lymphocytes as a result of post translational regulation [67-69].

Furthermore, acute exercise induces the synthesis of other antioxidant proteins such as Cu/Zn-SOD, TrxR1, UCP2 in PBMCs to reinforce the antioxidant defenses. Thioredoxin reductase plays an important role in regenerating disulfide sites in oxidized proteins [70]. Therefore, TrxR1 has also an important role in mediating constitutive denitrosylation, thus, maintaining low levels of *S*-nitrosylation in response to exogenous and endogenous NO [71]. The UCP2 protein expression is increased with the increasing amount of ROS produced in the mitochondria [8]. In this way, the ROS production is significantly increased after acute exercise than when the PBMCs are activated with PMA. It is suggested that the monocytes or other PBMCs have greater capacity of ROS production after acute exercise in response to immune stimulus as PMA or LPS [72, 73]. However, we cannot discard that the high production of ROS after activation of post-exercise PBMCs was due to a change in the type of circulating cells. Acute exercise induce a greater number of circulating monocytes which could be activated by PMA in a greater extension than other PBMCs [74].

Effects of Omega-3 supplementation on oxidative stress

Diet supplementation with DHA modifies the oxidative adaptation of PBMCs to training and to acute exercise. DHA affects

the changes reported in the GPx activity and also in the UCP2 and UCP3 protein levels induced by training season. These effects on the UCP3 protein level are consistent with studies showing that omega-3 may increase the expression of UCP3 in mice cells [75]. The diet supplementation with DHA changes the oxidative balance in the PBMCs resulting in a more antioxidant protection at mitochondrial level. The increased UCP3 levels in the experimental group after DHA supplementation could increase the antioxidant capacity and could be related with the decrease in the mitochondrial ROS production after acute exercise in accordance with the lower production of ROS after immune stimulation with LPS. Circulating LPS binds Toll-Like Receptor 4 (TLR4) and activates signaling cascades such as the NFH3 that modulate expression of a variety of pro-inflammatory cytokines [76]. Furthermore, it has been shown that LPS induces cellular ROS production [77] and ROS, are involved in TLR4-associated activation of NFkB [78]. Moreover, the MDA levels in PBMCs tend to be lower in the supplemented group than in the placebo after acute exercise. There are some evidences that omega-3 fatty acids increase mitochondrial fat oxidations by increasing the expression of mitochondrial carnitine palmitoyl transferase I, UCP3 and peroxisomal acyl-CoA oxidase, enzymes involved in beta-oxidation in the skeletal muscle [79].

Conclusion

In conclusion, the training season causes a change in the strategy of elimination of ROS from catalase-based system to a glutathione-based system and increases the defenses against mitochondrial ROS production. Although the training season induces a greater protection against PBMCs lipid peroxidation it also causes an increase in the oxidative damage to proteins, indicating an insufficient adaption of the antioxidant machinery. Acute exercise results in oxidative stress, which induces antioxidant adaptations in PBMCs in order to avoid oxidative damage via increases in both enzymatic activities and protein levels. However, these adaptations did not prevent the occurrence of oxidative damage in PBMCs even in the well-trained footballers. Diet supplementation with a functional food rich in DHA during the training season of footballers influences the

mitochondrial oxidative balance of PBMCs increasing the antioxidant capabilities and reducing the production of ROS.

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CONFLICTS OF INTEREST

The authors declare that they do not have any conflict of interest.

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Table 1. Primers and conditions used in real-time PCRs

Gene		Primer	Conditions	
18S	Fw:	5'-ATG TGA AGT CAC TGT GCC AG-3'	95°C	10 s
	Rv:	5'-GTG TAA TCC GTC TCC ACA GA-3'	60°C	10 s
			72°C	12 s
Catalase	Fw:	5'-TTT GGC TAC TTT GAG GTC AC-3'	95°C	10 s
	Rv:	5'-TCC CCA TTT GCA TTA ACC AG-3'	60°C	10 s
			72°C	15 s
Cu/Zn-SOD	Fw:	5'-TCA GGA GAC CAT TGC ATC ATT-3'	95°C	10 s
	Rv:	5'-CGC TTT CCT GTC TTT GTA CTT TCT TC-3'	63°C	10 s
			72°C	15 s
Mn-SOD	Fw:	5' -CGTGCTCCCACACATCAATC-3'	95°C	10 s
	Rv:	5'-TGAACGTCACCGAGGAGAAG-3'	60°C	10 s
			72°C	12 s
GPx	Fw:	5'-TTC CCG TGC AAC CAG TTT G-3'	94°C	10 s
	Rv:	5'-TTC ACC TCG CAC TTC TCG AA-3'	63°C	10 s
			72°C	15 s
UCP3	Fw:	5'- CGT GGT GAT GTT CAT AAC CTA TG-3'	95°C	5 s
	Rv:	5'- CGG TGA TTC CCG TAA CAT CTG-3'	60°C	7 s
			72°C	10 s

Table 2. Fatty acid composition in erythrocytes in basal conditions

		Initial	Final	ANOVA S	ANOVA T	ANOVA SxT
Eicosenoic	Placebo	1.97 ± 0.07	2.01 ± 0.12			
(%)	Experimental	1.79 ± 0.12	1.82 ± 0.17			
Eicosadienoic	Placebo	3.16 ± 0.15	2.33 ± 0.13 *		X	
(%)	Experimental	2.86 ± 0.15	2.52 ± 0.13			
Eicosatrienoic	Placebo	4.97 ± 0.47	4.95 ± 0.47	X		
(%)	Experimental	3.78 ± 0.26 #	3.41 ± 0.22 #			
Arachidonic	Placebo	71.9 ± 0.3	71.6 ± 0.6	X	X	
(%)	Experimental	71.8 ± 1.4	68.0 ± 0.9 *,#			
Docosahexaenoic	Placebo	18.0 ± 0.6	19.1 ± 0.9	X	X	
(%)	Experimental	19.8 ± 1.4	24.3 ± 0.9 *,#			

Statistical analysis: Two-way ANOVA. (S) Significant effect of supplementation, (T) Significant effect of training, (SxT) Significant interaction between both factors. (*) Significant differences between placebo and experimental. (#) Significant differences between the beginning and end of the nutritional intervention, $p<0.05$.

Table 3. Cellular counts and distribution

A		Initial	Final	ANOVA S T SxT
Leukocytes	Placebo	6.84 ± 1.14	6.52 ± 0.35	
(10^3 cells/uL)	Experimental	6.30 ± 0.38	7.01 ± 0.39	
Lymphocytes	Placebo	1.98 ± 0.18	2.48 ± 0.18 #	x x
(10^3 cells/uL)	Experimental	2.49 ± 0.17	3.10 ± 0.330*,#	
Monocytes	Placebo	0.480 ± 0.054	0.500 ± 0.050	
(10^3 cells/uL)	Experimental	0.430 ± 0.040	0.510 ± 0.035	
Eosinophils	Placebo	0.231 ± 0.083	0.282 ± 0.078	
(10^3 cells/uL)	Experimental	0.270 ± 0.121	0.291 ± 0.083	
Basophils	Placebo	0.032 ± 0.005	0.072 ± 0.012#	x
(10^3 cells/uL)	Experimental	0.032 ± 0.009	0.090 ± 0.010#	
PBMC	Placebo	2.79 ± 0.16	3.34 ± 0.15	x x
(10^3 cells/uL)	Experimental	3.23 ± 0.29	3.99 ± 0.34#	

B		Basal	Post-exercise	S E SxE
Leukocytes	Placebo	6.52 ± 0.35	9.96 ± 0.40#	x
(10^3 cells/uL)	Experimental	7.01 ± 0.39	9.55 ± 0.47#	
Lymphocytes	Placebo	2.48 ± 0.18	2.21 ± 0.14#	x
(10^3 cells/uL)	Experimental	3.10 ± 0.33	2.17 ± 0.14#	
Monocytes	Placebo	0.500 ± 0.050	0.770 ± 0.052#	x
(10^3 cells/uL)	Experimental	0.510 ± 0.035	0.740 ± 0.051#	
Eosinophils	Placebo	0.282 ± 0.078	0.174 ± 0.041	
(10^3 cells/uL)	Experimental	0.291 ± 0.083	0.163 ± 0.050	
Basophils	Placebo	0.072 ± 0.012	0.042 ± 0.008#	x
(10^3 cells/uL)	Experimental	0.090 ± 0.010	0.049 ± 0.011#	
PBMC	Placebo	3.34 ± 0.15	3.19 ± 0.15	
(10^3 cells/uL)	Experimental	3.99 ± 0.34	3.13 ± 0.16	

(A) The initial and final data studies, in basal conditions, (B) Final study data, in basal and post-exercise conditions. Statistical analysis: Two-way ANOVA, $p<0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between supplementation and time, (E) Significant effect of exercise, (SxE) Significant interaction between supplementation and exercise. One-way ANOVA, $p<0.05$. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period or significant differences between basal and post¬ exercise.

Table 4. Oxidative and nitrosative damage of PBMC

A		Initial	Final	ANOVA S	T	SxT
MDA	Placebo	0.36 ± 0.04	0.26 ± 0.02#		X	
(umol/10^{3}cells)	Experimental	0.44 ± 0.06	0.20 ± 0.06#			
Carbonyl index	Placebo	100 ± 23	975 ± 9 5 #		X	
(%)	Experimental	109 ± 19	922 ± 86 #			
N-Tyr index	Placebo	100 ± 35	621 ± 117 #		X	
(%)	Experimental	76.9 ± 13.8	501 ± 39 #			
Nitrate	Placebo	1.97 ± 0.73	1.03 ± 0.15		X	
(umol/10^{3}cells)	Experimental	3.51 ± 0.72	1.10 ± 0.15#			
Comet assay						
% Tail	Placebo	4.39 ± 0.34	4.17 ± 0.39			
	Experimental	4.13 ± 0.32	3.94 ± 0.39			
Tail moment	Placebo	0.32 ± 0.03	0.34 ± 0.06			
	Experimental	0.34 ± 0.05	0.36 ± 0.07			
B		**Basal**	**Post-exercise**	**S**	**E**	**SxT**
MDA	Placebo	0.26 ± 0.05	0.58 ± 0.11 #		X	
(umol/10^{3}cells)	Experimental	0.20 ± 0.02	0.40 ± 0.06 #			
Carbonyl index	Placebo	100 ± 10	114 ± 4			
(%)	Experimental	94.4 ± 8.8	105 ± 4			
N-Tyr index	Placebo	100 ± 19	137 ± 18		X	
(%)	Experimental	87.0 ± 8.2	141 ± 2 1 #			
Nitrate	Placebo	1.03 ± 0.15	0.401 ± 0.096 #		X	
(umol/10^{3}cells)	Experimental	1.10 ± 0.15	0.700 ± 0.236			
Comet assay						
% Tail	Placebo	4.17 ± 0.39	5.90 ± 0.64 #		X	
	Experimental	3.94 ± 0.39	5.48 ± 0.59 #			
Tail moment	Placebo	0.34 ± 0.06	0.44 ± 0.07			
	Experimental	0.36 ± 0.07	0.42 ± 0.06			

(A) The initial and final data studies, in basal conditions, (B) Final study data, in basal and post-exercise conditions. Statistical analysis: Two-way ANOVA, $p<0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between supplementation and time, (E) Significant effect of exercise, (SxE) Significant interaction between supplementation and exercise. One-way ANOVA, $p<0.05$. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period or significant differences between basal and post-exercise.

Table 5. Antioxidant enzyme activities, protein levels and gene expressions on PBMC

A		Initial	Final	ANOVA S	T	SxT
Enzyme activity						
Catalase	Placebo	27.5 ± 8.14	8.06 ± 3.94#		X	
(K/10⁹cells)	Experimental	16.7 ± 3.03	7.24 ± 4.04			
GRd	Placebo	71.3 ± 16.7	81.5 ± 27.6			
(nkat/10⁹cells)	Experimental	80.2 ± 17.9	57.7 ± 16.7			
GPx	Placebo	74.8 ± 13.2	101 ± 5.13 #	X	X	
(nkat/10⁹cells)	Experimental	52.1 ± 6.74	88.5 ± 8.97*,#			
Protein levels						
Catalase	Placebo	100 ± 17	96.1 ± 25.4			
(%)	Experimental	99.3 ± 19.5	92.8 ± 16.7			
Cu/Zn-SOD	Placebo	100 ± 29	79.4 ± 14.7			
(%)	Experimental	82.9 ± 14.9	123 ± 20			
Mn-SOD	Placebo	100 ± 32	92.3 ± 47.3			
(%)	Experimental	78.3 ± 25.9	88.5 ± 39.6			
GRd	Placebo	100 ± 31	99.5 ± 6.9			
(%)	Experimental	113 ± 22	138 ± 28			
GPx	Placebo	100 ± 28	132 ± 22			
(%)	Experimental	97.2 ± 38.5	157 ± 31			
TrxRl	Placebo	100 ± 27	83.7 ± 23.6			
(%)	Experimental	130 ± 39	79.8 ± 16.8			
UCP2	Placebo	100 ± 22	362 ± 98#		X	
(%)	Experimental	126 ± 23	331 ± 53#			
UCP3	Placebo	100 ± 18	464 ± 71#	X	X	
(%)	Experimental	179 ± 39	684 ± 98 *,#			
iNOS	Placebo	100 ± 14	166 ± 26		X	
(%)	Experimental	90.1 ± 20.6	170 ± 17 #			

B		Basal	Post-exercise	S	E	SxE
Enzyme activity						
Catalase	Placebo	8.06 ± 3.94	16.8 ± 3.88#		X	
(K/10^3 cells)	Experimental	7.24 ± 4.04	33.5 ± 9.29#			
GRd	Placebo	81.5 ± 27.6	218 ± 69.2 #		X	
(nkat/10^3 cells)	Experimental	57.7 ± 16.7	118 ± 40.2			
GPx	Placebo	101 ± 5.13	120 ± 6.77		X	
(nkat/10^3 cells)	Experimental	88.5 ± 8.97	153 ± 20.4 #			
Protein levels						
Catalase	Placebo	100 ± 26	143 ± 17		X	
(%)	Experimental	96.6 ± 17.5	190 ± 30 #			
Cu/Zn-SOD	Placebo	100 ± 18	184 ± 43	X	X	
(%)	Experimental	155 ± 25	276 ± 40 #			
Mn-SOD	Placebo	100 ± 51	160 ± 74			
(%)	Experimental	95.2 ± 42.6	156 ± 54			
GRd	Placebo	100 ± 28	158 ± 32			
(%)	Experimental	178 ± 47	166 ± 41			
GPx	Placebo	100 ± 17	165 ± 25		X	
(%)	Experimental	119 ± 24	213 ± 40 #			
TrxR1	Placebo	100 ± 28	146 ± 27		X	
(%)	Experimental	95.3 ± 20	151 ± 23			
UCP-2	Placebo	100 ± 27	158 ± 46		X	
(%)	Experimental	91.4 ± 14.6	184 ± 31			
UCP-3	Placebo	100 ± 15	114 ± 12			
(%)	Experimental	147 ± 21	128 ± 20			
iNOS	Placebo	100 ± 12	172 ± 27		X	
(%)	Experimental	107 ± 11	138 ± 28			
Gene expresion						
Catalase	Placebo	1.00 ± 0.25	1.18 ± 0.75			
	Experimental	0.83 ± 0.39	1.01 ± 0.36			
Cu/Zn-SOD	Placebo	1.00 ± 0.37	3.37 ± 2.70			
	Experimental	1.55 ± 0.90	1.22 ± 0.35			
Mn-SOD	Placebo	1.00 ± 0.42	1.07 ± 0.46			
	Experimental	1.67 ± 0.71	0.99 ± 0.54			
GPx	Placebo	1.00 ± 0.28	1.7 ± 1.17			
	Experimental	1.49 ± 0.50	0.80 ± 0.19			
UCP-3	Placebo	1.00 ± 0.33	1.26 ± 0.7			
	Experimental	2.08 ± 1.39	1.04 ± 0.37			

(A) The initial and final data studies, in basal conditions, (B) Final study data, in basal and post-exercise conditions. Statistical analysis: Two-way ANOVA, $p<0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between supplementation and time, (E) Significant effect of exercise, (SxE) Significant interaction between supplementation and exercise. One-way ANOVA, $p<0.05$. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period or significant differences between basal and post-exercise.

Figure 1A

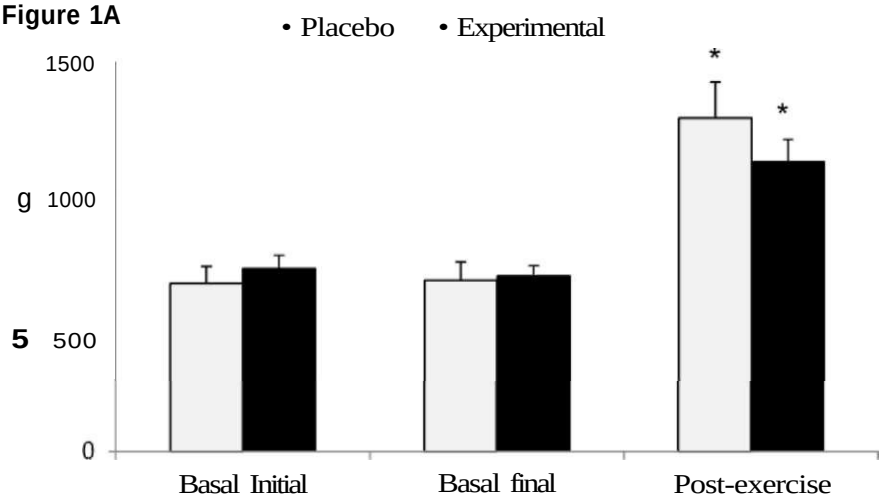

Figure 1B

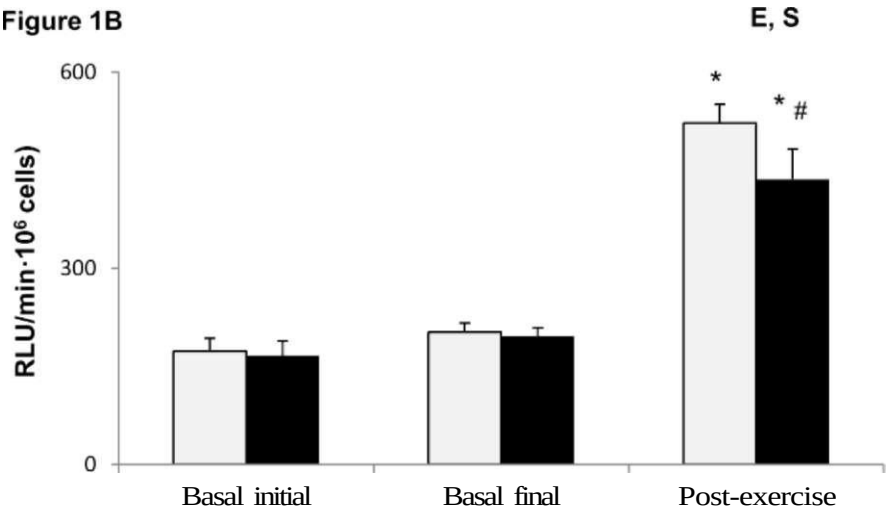

Figure 1A represents ROS production by PBMCs stimulated with PMA. Figure 1B represents ROS production by PBMCs stimulated with LPS. Statistical analysis: Two-way ANOVA, $p<0.05$. (S) Significant effect of supplementation, (T) Significant effect of time, (SxT) Significant interaction between supplementation and time, (E) Significant effect of exercise, (SxE) Significant interaction between supplementation and exercise. One-way ANOVA, $p<0.05$. (*) Significant differences between placebo and experimental, (#) Significant differences between initial and final training period or significant differences between basal and post-exercise.

MANUSCRIPT VI

Sodium nitrate supplementation does not enhance performance of endurance athletes

ABSTRACT

Purpose: Supplementation with inorganic nitrate has been suggested to be an ergogenic aid for athletes as nitric oxide donor. The purpose of this study was to determine whether ingestion of inorganic sodium nitrate benefits well-trained athletes performing a 40-minute exercise test in laboratory conditions. Additionally, we investigated the effect of this supplement on plasma levels of endothelin-1 and in nitrated-proteins.

Methods: Thirteen trained athletes participated in this randomised, double-blind, crossover study. They performed a 40-minute cycle ergometer distance-trial test after two 3-day periods of dietary supplementation with sodium nitrate (10 mg-kg^{-1} of body mass) or placebo.

Results: Concentration of plasma nitrate (256 ± 35 u.M) and nitrite (334 ± 86 nM) increased significantly ($P < 0.05$) after nitrate supplementation compared with placebo (nitrate: 44 ± 11 u.M; nitrite: 187 ± 43 nM). In terms of exercise performance, there were no differences in either the mean distance (nitrate: 26.4 ± 1.1 km; placebo: 26.3 ± 1.2 km; $P = 0.61$) or mean power output (nitrate: 258 ± 28 W; placebo: 257 ± 28 W; $P = 0.89$) between treatments. Plasma endothelin-1 increased significantly ($P < 0.05$) just after exercise in nitrate (4.0 ± 0.8 pg-ml^{-1}) and placebo (2.4 ± 0.4 pg-ml^{-1}) conditions. This increase was significantly greater ($P < 0.05$) in the nitrate group. Levels of nitrated-proteins did not differ between treatments (nitrate: pre-exercise: 91 ± 23%; post-exercise: 81 ± 23 %; placebo: pre-exercise: 95 ± 20%; post-exercise: 99 ± 19 %).

Conclusion: Sodium nitrate supplementation did not improve a 40-minute distance-trial performance in endurance athletes. In addition, concentration of plasma endothelin-1 increased significantly after exercise following supplementation with sodium nitrate.

Keywords: nitric oxide, nitrate, nitrite, exercise performance, endothelin-1.

INTRODUCTION

Inorganic nitrate has been identified as an important precursor for nitric oxide (NO) syntheses that complements the classical L-Arginine NO synthesis pathway. This alternative NO pathway has been suggested to play an important role in the regulation of blood pressure and blood flow, gastric integrity and tissue protection against ischemic injury (26). Consequently, interest in inorganic dietary nitrate has increased substantially in pharmacology and physiology research during the last decade (26).

Inorganic nitrate supplementation has also been suggested to be a potential ergogenic aid for athletes (7, 19). Recent studies have reported that supplementation with beetroot juice rich in nitrate improves exercise performance measured as time-to-exhaustion during a fixed workload and during an incremental exercise test in healthy humans (2, 20, 39). Furthermore, three recent studies, using supplementation with beetroot have also shown benefits in exercise performance measured in a treadmill (31) and cycle ergometer (7, 19) time trial tests, respectively. The mechanism(s) behind this intriguing effect remains uncertain, although, two candidates have been proposed to explain it. The first includes a NO-mediated improvement in muscle contractile efficiency (1), while the second relates to an improvement in mitochondrial respiration efficiency (21).

In contrast to previous findings a recent study demonstrated no increase in exercise performance with well-trained cyclists during a 50 mile time-trial test after an acute supplementation of beetroot juice (41). Three subsequent studies using pharmacological sodium nitrate also showed no increase in exercise capacity during an incremental exercise test (3, 22-23). However, it should be noted that, perhaps, the incremental protocols until exhaustion performed in these studies were not the best method in assessing exercise performance. These protocols are commonly shorter and less sensitive to possible improvements in performance compared with endurance events lasting longer than 30 min (10). For this reason, time-trial protocols are used as a better method to assess changes in endurance performance (10). For instance, there is evidence that an

enhancement of only 1% of exercise efficiency may improve 40 km cycle time trial performance by ~ 60 seconds (30). Therefore, if inorganic nitrate supplementation is able to enhance energy efficiency, it may be expected that this supplement could also promote an increase in exercise performance during a long time-trial test.

On the other hand, a sustained increase in NO synthesis in healthy humans may not always be beneficial. It is known that an excess of NO production may also lead to a reaction with superoxide radical and produce highly reactive peroxynitrite. The latter causes the nitration of tyrosine residues of proteins to form 3-nitrotyrosine (3-NT) and thereby irreversibly alters the biological function of proteins (33). Prolonged, exhaustive endurance exercise may also favor these reactions and increase oxidative stress in athletes (32). In addition, the reduction of nitrite to NO in hypoxic and/or acidic conditions may form nitrogen oxides and raise plasma concentration of 3-NT and nitrated-proteins (33). To avoid these harmful effects, an adequate balance between pro-oxidants and antioxidants is needed. Although exercise training is known to enhance endogenous antioxidant systems (6), there is a lack of studies assessing whether dietary nitrate supplementation affects the level of nitrogen oxides with nitrating properties in athletes after exhaustive exercise. Interestingly, Lundberg et al. (25) have recently launched advice to refrain from the uncontrolled use of nitrate and nitrite supplementation in pharmacological form since there is evidence from internet forums, articles and discussions within the sports community, that the use of these products are spreading rapidly among athletes. However, supplementation with natural sources of nitrate such as whole vegetables or vegetables juices in moderate amounts seems to be safe of any acute risk (16).

It is also known that there are other important factors independent of NO which contribute to the regulation of vascular tone in humans. One of the most important is endothelin-1 (ET-1). This molecule is a potent vasoconstrictor peptide produced by vascular endothelial cells which interacts with other vascular mediators, most notably NO (36). Very low concentrations of NO (as

low as 20 ppm) effectively suppress release and physiological action of ET-1. Moreover, NO can also nitrosylate endothelin receptors and reduce affinity for ET-1(11). Thus, in addition to its direct vasodilator effects, NO induces vasodilation indirectly by limiting the release of ET-1. Since dietary nitrate is claimed to be a NO donor in humans, its impact on plasma concentration of ET-1 at rest and after exhaustive exercise would extend knowledge of the role of dietary nitrate supplementation on vascular tone.

Accordingly, the first aim of this study was to assess the ergogenic effect of dietary sodium nitrate supplementation in endurance-trained subjects using a 40-minute distance-trial test. Additionally, we evaluated plasma concentration of nitrated-proteins and ET-1 before and after exercise in two experimental groups, one receiving inorganic nitrate supplementation and the other a placebo. We hypothesized that if inorganic nitrate supplementation can enhance energy efficiency of exercise it may also benefit overall exercise performance during a long distance-trial test. Moreover, we hypothesized that the ingestion of a moderate amount of inorganic nitrate cannot alter plasma concentration of 3-NT after high intensity exercise test in well-trained subjects. Since the potential for an increase in free radicals derived from nitrate-nitrite-NO pathway these molecules can be effectively reduced by endogenous antioxidant systems of athletes. Lastly, it was anticipated that an increase in NO synthesis following sodium nitrate ingestion may reduce plasma concentration of ET-1 compared with placebo after exercise.

METHODS

Subjects

Thirteen non-professional male cyclists and triathletes (age 32.6 ± 5.6 yrs; body weight 72.4 ± 9.7 kg^{-1}; body mass index 23.4 ± 2.0 $kg\text{-}m^{-2}$; body fat: 9.6 ± 3.3 %) volunteered to participate in this study. Athletes were members of competitive cycling or triathlon squads and none of them reported any medical conditions at the time of the study. They had 8 ± 5 years of experience in endurance events, and

their average weekly training volume was 15.7 ± 5.0 hours per week. None of the subjects smoked tobacco. The procedures employed in this study were approved by the Ethics Committee of the Catalonian Sports Council. All subjects gave their written informed consent after an explanation of the experimental procedures and before commencement of the study.

Nitrate supplementation

Subjects were randomly assigned in a double-blind, crossover design to follow 3-days of supplementation with either sodium nitrate (10 mg-kg^{-1} of body mass; Acofarma, code 18211, Spain) or the placebo (sodium chloride; 10 mg-kg^{-1} of body mass) dissolved in water. Supplementation was ingested each morning before breakfast. On the last day, subjects ingested the supplement or placebo 3 hours before the exercise test. A diet with low levels of moderate or high nitrate content foods (green vegetables, beetroot, strawberries, grapes and tea) was followed two days prior to the tests. During this time, athletes received nutritional guidelines and were encouraged to follow a high carbohydrate diet to optimize glycogen deposition. In addition, they were told to avoid alcohol, caffeine products and dietary supplements 24 h prior to the exercise test. A 4-day washout separated the supplementation periods.

Ergometry test

Subjects were required to report to the laboratory on four occasions, each separated by one week. The first week, subjects performed an anthropometric assessment and an incremental exercise test under laboratory controlled conditions to determine maximal oxygen uptake (FO_{2max}) maximal power output (W_{max}), ventilatory threshold (VT) and respiratory compensation point (RCP). The exercise protocol started at 50 W and increased 25 W every minute until voluntary exhaustion. An electronically braked cycle ergometer (Schoberer Rad Messtechnik, SRM, Germany) was used for all tests. The configuration of the ergometer - crank length, pedals, saddle and handlebar position - was adapted to the measurements of subjects' own road bicycles. Before each test the cycle ergometer was

calibrated following the manufacturer's instructions. Pedaling cadence was individually chosen within the range of 70 - 100 rpm. In the next three weeks subjects performed three distance-trials in the laboratory with controlled environmental conditions (23.8 ± 1.0 °C). The first was carried out to familiarize subjects with the bicycle ergometer, gas analyzer and the testing procedure. The following two distance-trials were carried out in both conditions (placebo and nitrate) at the same time of day (± 1 h). They were asked to cover as much distance as possible over the 40 min. We chose this duration because it was related to the distance (22 km) and time (~ 35 - 40 min) of the regional and national time-trial championships. Some athletes in the current study were training for these championships and accepted to participate in this study as part of their training for these events. Before the test, athletes performed 15 min warm up at 60% of FO_{2max} followed by 5-10 min passive recovery before starting the distance-trial. The ergometer (Schoberer Rad Messtechnik, SRM, Germany) was programmed in the mode "open end test". Subjects started the test in "gear 9" and were allowed to change gear. In this mode, power output varies with pedal rate and/or a gear change. For each distance-trial, time, distance, power and torque was recorded every second by SRM software. To avoid any experimental bias, the only feedback available to cyclists during the distance-trial was time elapsed. In addition, they were strongly encouraged verbally during both distance-trials. Food and fluid ingestion was forbidden during the test.

Gas analysis

During the incremental exercise test, oxygen uptake (FO_2), minute ventilation (FE), carbon dioxide production (FCO_2) and respiratory exchange ratio (RER) were measured continuously breath-by-breath by a computerized gas analyzer (Jaeger Oxycon Mobile, Germany). FO_{2peak} was determined as the mean FO_2 measured over the final 60 s of exercise. The criteria for a true FO_{2peak} were the attainment of a plateau in oxygen uptake (increase less than 150 ml/min during the last minute of exercise) despite an increase in workload. Wmax and HRmax were defined as the HR and W at the point of exhaustion during the test. To determine ventilatory threshold (VT)

and respiratory compensation point (RCP), data were averaged over 30 s intervals and analyzed by two independent reviewers as described previously (3). During the 40-minute distance-trials the respiratory response was not measured continuously. Three samples of respiratory gas exchange were taken during the test: 1) between 12 and 15 min; 2) between 22 and 25 min, and 3) between 32 and 35 min. Data regarding FO_2, FE, FCO_2 and RER were recorded breath-by-breath and values of the last minute were averaged and used to assess the respiratory response during exercise (Jaeger Oxycon Mobile, Germany). In addition, HR was continuously recorded (beat-by-beat) with a portable heart rate monitor (Polar RS800 SD, Finland).

Blood sampling

Two blood samples were collected from the antecubital vein to analyze nitrate and nitrite: 1) after three days of nitrate supplementation or placebo in resting conditions before the exercise test; 2) during the first three minutes after distance-trials (placebo and nitrate). Venous blood was drawn with a 5-mL syringe EDTA and was immediately centrifuged at 1,000 *g* for 20 min to separate plasma from blood cells. Plasma samples were then centrifuged for 30 min at 14,000 g in 10K filters (Amicon Ultra, Millipore, USA) to remove proteins. The supernatant was recovered and used to measure nitrite and nitrate concentration by detecting liberated NO in a gas-phase chemiluminescence reaction with ozone using a nitric oxide analyzer (NOA 280i, Sievers, USA) as described previously (3).

Additionally, from the same blood samples, a determination of plasma endothelin-1 concentration was made using commercially available immunoassay kits (Assay Designs, Inc., USA) following the manufacturer's instructions. Assays were performed in duplicate and optical density was determined using a microplate reader set to 450 nm. In addition, plasma 3-nitrotyrosine concentration in proteins were analyzed using commercially available immunoassay kits (OxiSelect™ Nitrotyrosine Immunoblot, Cell Biolabs, Inc., USA).

Lastly, during the distance-trials, four samples of capillary blood (10 u±) were collected from the ear lobe to analyze lactate

([Hla]) using a Lange Miniphotometer LP2 (Germany) system: 1-3) in minute 10, 20 and 30 of the test; and 4) three minutes after the maximal test.

Urine sampling

To analyze nitrate - nitrite urine concentration, two samples were collected: 1) in resting conditions before the exercise test after three days of nitrate supplementation or placebo ingestion; 2) during the first hour post-exercise (placebo and nitrate). The same method used for blood samples was applied to analyze nitrate and nitrite concentration in urine. % urinary nitrate excretion with respect to supplemented dietary nitrate was estimated based on nitrate excreted in the urine before and after the exercise test (~ 45 minutes each) for each treatment using the following formula:

$$NO_3^- \text{ losses } (\%) = [US_{NO_3^-} - UP_{NO_3^-}] \times 100 / DS_{NO_3^-}$$

where $US_{NO_3^-}$ represents urinary nitrate excretion (mg) after dietary nitrate supplementation; $UP_{NO_3^-}$ represents urinary nitrate excretion (mg) after placebo treatment; and $DS_{NO_3^-}$ represents the amount of dietary nitrate supplementation per day (mg).

Statistics

Results are expressed as means ± standard deviation of the mean. The coefficient of variation (CV) for distance and power output between the third and fourth distance-trial was calculated by dividing each subject's standard deviation (SD) by his mean. A spreadsheet proposed by Hopkins that analyzes validity by linear regression was used for calculations (14). In addition, an intraclass correlation coefficient (ICC) was also computed for the same variables. To investigate the influence of treatment (S) and time (T), and interaction between both these variables (S*T) the data were treated with two-way repeated measures analysis of variance (ANOVA). The sets of data in which there was significant *S*T* interaction were tested by one-way ANOVA test. When significant effects of *S* or *T* were found, a Student's *t*-test for paired data was used to determine differences between the groups (nitrate and placebo) involved. Additionally,

Pearson correlation coefficient was used to assess the relationships between variables. Differences between two independent groups (low-responders and high-responders) were assessed using a Student's t-test for unpaired data. All data were analyzed to determine the normal distribution, and post-hoc analyses were performed using Tukey's HSD. Significance level was set at $P < 0.05$, while a trend was noted when $P < 0.10$.

RESULTS

Performance during the VO_{2max} test

Mean results of FO_{2max}, HR_{max} and W_{max} during the incremental exercise test were 4.3 ± 0.3 L-min^{-1} (60 ± 7 mL-kg^{-1}-min^{-1}), 180 ± 11 beats-min^{-1} and 378 ± 30 W (5.3 ± 0.8 W-kg^{-1}), respectively. Ventilatory threshold (VT) was determined at a mean intensity of 215 ± 38 W, 144 ± 12 beats-min^{-1} (80 ± 4 % of HRmax) and 38.6 ± 7.7 mL-kg^{-1}-min^{-1} of (64.3 ± 8.3 % of FO_{2max}), whereas respiratory compensation point (RCP) was estimated at an average intensity of 301 ± 37 W, 166 ± 12 beats-min^{-1} (92 ± 3 % of HRmax) and 50.8 ± 7.8 mL-kg^{-1}-min^{-1} of VT^ (85.1 ± 8.1 % of FO2max).

Performance during 40-minute distance-trial

The mean coefficient of variation (CV %) for distance and power output between the third and fourth distance-trial independent of treatment was 1.1% (95% CI = 0.9 - 1.8) and 2.2% (95% CI = 1.7 - 3.5), respectively. These differences were not statistically significant (P > 0.05). Additionally, the intraclass correlation coefficient ICC for distance was 0.98 (95% CI = 0.95 - 1.00) and 0.99 (95% CI = 0.96 - 1.00) for power output.

Average distance and power output profiles are shown in **Figure 1.** There were no significant differences between nitrate and placebo groups in overall distance (nitrate: 26.4 ± 1.1 km; placebo: 26.3 ± 1.2 km; $P = 0.61$) or mean power output (nitrate: 258 ± 28 W and 3.6 ± 0.6 W-kg^{-1}; placebo: 257 ± 28 W and 3.6 ± 0.6 W-kg^{-1}; $P = 0.89$) achieved during the 40-minute distance-trial. Athletes performed the distance-trial at a mean intensity of 91.1 ± 3.3 (85.1 ± 5.0 %

^O_{2max}) and 91.3 ± 3.0 % of HR_{max} (85.1 ± 6.3 % FO_{2max}) in placebo and nitrate conditions respectively. Mean cadence during distance-trials was 93 ± 7 and 93 ± 6 revolutions per minute (rpm) for placebo and nitrate.

Cardiorespiratory and metabolic response during the 40-minute distance-trial

Main respiratory variables (FO_2, FCO_2, *VE* and RER) were unaffected after nitrate supplementation compared with placebo **(Table 1).** HR increased significantly during the test in both treatments (P< 0.001) **(Table 1).** There were no differences in average blood lactate concentration at any point of the test between placebo and nitrate groups **(Table 1).**

Plasma and urinary concentration of nitrate and nitrite

Concentration of plasma nitrate (256 ± 35 u.M; *P* < 0.001) and nitrite (334 ± 86 nM; *P* = 0.008) increased significantly following sodium nitrate supplementation compared with placebo (nitrate: 44 ± 11 uMl; nitrite: 187 ± 43 nM) **(Figure 3).** After exercise, plasma nitrate concentration were higher in nitrate condition compared with placebo (nitrate: 272 ± 54 u.M; placebo: 52 ± 8 u.M *P* < 0.001). Otherwise, physical exercise did not significantly alter plasma nitrate just after a 40-minute distance-trial in either group (placebo or nitrate). Although concentration of plasma nitrite showed a reduction after the exercise test (plasma: 248 ± 72 nM) compared with values in resting conditions (plasma: 334 ± 86 nM), these differences were not statistically significant (plasma: *P* = 0.107) **(Figure 3).**

We performed analyses of correlations between data of the incremental test (FO_{2max} and W_{max}), without supplementation, with basal plasma concentration of nitrate and nitrite, as well as with plasma values of both anions after dietary nitrate supplementation. We did the same analysis of correlation between distance, power and speed performed during the 40-min distance trial tests in both conditions (placebo and nitrate) and plasma concentration of nitrate and nitrite. These analyses were performed to assess whether subjects with high performance parameters were more or less sensitive to

changes in plasma concentration of nitrate and nitrite after dietary nitrate supplementation. We found that subjects with high FO_{2max} (r = 0.56; *P* = 0.048) and W_{max} (r = 0.59; *P* = 0.032) in the incremental test (without supplementation) showed a greater increase in plasma nitrate concentration after nitrate treatment. In addition, there was found to be a positive trend between the increase in plasma nitrate concentration after dietary consumption of nitrate and increase in distance (r = 0.53; *P* = 0.062), speed (r = 0.52; *P* = 0.066) and power (r = 0.54; *P* = 0.055) in the 40-min distance time trial. However, no effect was found between performance parameters and changes in plasma nitrite (P > 0.05).

Furthermore, it was found that seven subjects showed a small increase (< 30 %) in plasma nitrite after dietary supplementation with nitrate compared with placebo. For this reason, these subjects were classified as low-responders **(Table 2).** The remaining 6 subjects were classified as high-responders due to a greater increase in plasma nitrite concentration (> 50 %) after nitrate treatment. However, despite of this fact no statistical differences were found in exercise performance (distance and power output) parameters between both groups after nitrate and placebo treatments **(Table 2).** A small reduction, but not statistically significant, was found in FO_2 (~ 1.7 %), FCO_2 (~ 3.4 %) and ratio between oxygen consumption and power (~ 1.1 %) in the high-responder group during the 40-min distance-trial test after nitrate supplementation compared with placebo. In addition, blood lactate concentration showed a trend to decrease at three minutes post¬ exercise after nitrate treatment in high-responders *(P* = 0.076) **(Table 2).**

Urinary nitrate (7,624 ± 795 u.M; *P* = 0.004) and nitrite (283 ± 122 nM; *P* = 0.004) excretion rose significantly after dietary nitrate supplementation compared with placebo in resting conditions (nitrate: 1,299 ± 121 u.M; nitrite: 111± 40 nM) **(Figure 3).** After exercise, urinary nitrate excretion was also higher in nitrate condition (7,046 ± 1,354 umol) compared with placebo (705 ± 165 umol; *P* < 0.001). % urinary nitrate losses with respect to supplemented dietary nitrate were 4.8 ± 1.9 % and 3.7 ± 1.5 % before and after exercise. Like plasma concentration, urinary nitrite showed a decrease after

exercise test (188 ± 85 nM) compared with peak values at resting conditions (283 ± 102 nM) in nitrate treatment **(Figure 3).** However this decrease was not statistically significant (P = 0.174).

Plasma concentration of endothelin-1 (ET-1) and 3-nitrotyrosine (3-NT)

In resting conditions, plasma ET-1 concentration did not differ between nitrate and placebo conditions **(Figure 4).** However, a significant increase was shown just after exercise in placebo *(P* = 0.030) and nitrate (P < 0.001) groups compared with resting values. In addition, this effect was significantly *(P* = 0.010) greater in the nitrate group compared with placebo. On the other hand, plasma concentration of nitrated-proteins was not affected by dietary nitrate supplementation in resting or post-exercise conditions **(Figure 5).** There were no differences in plasma concentration of ET-1 and 3-NT between high and low-responder groups **(Table 2).**

DISCUSSION

The main finding of this study is that supplementation with sodium nitrate did not enhance the performance of endurance-trained athletes during a 40-min test. These results are contrary to the first hypothesis of this study. However, we confirmed the second hypothesis by showing that the ingestion of a moderate amount of sodium nitrate did not induce an increase of plasma concentration of nitrated-protein just after exhaustive exercise in well-trained athletes. Additionally, and in contrast to the third hypothesis we found that the concentration of plasma ET-1 was significantly higher just after the exercise test in the nitrate condition compared with placebo.

Effects of dietary inorganic nitrate supplementation on endurance performance

Several recent studies have reported that supplementation with beetroot can enhance exercise performance in healthy subjects (2, 5, 7, 19-20, 31, 39-40) or in patients with cardiovascular disease (17) throughout different form of protocols such as time-to-exhaustion or time-trials. However, the ergogenic effect of nitrate

supplementation in well-trained endurance athletes remains uncertain (4). For instance, several studies supplementing endurance athletes for at least four days with pharmacological sodium nitrate have not reported an increase in exercise performance during incremental protocols until exhaustion (3, 22-23). Furthermore, a recent study by Wilkerson et al. (41) showed that an acute dose of beetroot juice was not effective to enhance exercise performance during a 50 mile time-trial test in well-trained cyclists. The present results are in agreement with this study indicating that the ingestion of sodium nitrate over 3 days did not show an ergogenic effect in a 40-min distance-trial test. However, in contrast with these results, another recent study by Cermak et al. (7) showed that 6 days of supplementation with beetroot juice was able to reduce the time required for cyclists to complete a time-trial test of 10 km.

In an attempt to explain the above discrepancies between studies there are at least three factors which need to be discussed. The first is related to the duration of the nitrate supplementation. While Cermak el al. (7) analyzed 6 days of supplementation with beetroot juice and found a significant increase in exercise performance, Wilkerson et al. (41) and the present study did not find such benefits after an acute dose of beetroot juice rich in nitrate and 3 days of sodium nitrate ingestion, respectively. Therefore, it could be suggested that trained subjects may need at least one week of nitrate supplementation to induce some benefit in exercise performance.

The second factor is associated with the duration and intensity of the exercise test. These parameters are also an important key point since it is suggested that the nitrate-nitrite-NO pathway is mainly activated under anaerobic and acidic conditions (26). A recent study in mice has shown that dietary nitrate supplementation increased force production and calcium handling in fast-twitch muscles with a larger capacity to use anaerobic metabolism (13). In contrast, no effects were found in slow-twitch muscles with high aerobic capacity. Consistent with this fact, we found previously that in well-trained athletes inorganic nitrate supplementation significantly decreased the ratio between oxygen consumption and power output only at maximal loads of exercise above their respiratory compensation point

(RCP) (3). From this viewpoint, Cermak et al. (7) and Lansley et al. (19) found that a supplementation of beetroot juice rich in nitrate improved performance in a time-trial tests shorter than 30 minutes. In addition, another recent study by Bond et al. (5) has indicated that beetroot juice supplementation improved performance of rowers during a high-intensity intermittent test. On the contrary, the study by Wilkerson et al. (41) and the current study evaluated the effect of nitrate supplementation over a longer duration (> 30 minutes). Accordingly, perhaps, one confounding variable of these last studies might be the longer duration of the test. At exercise intensities below the RCP the energy to sustain exercise is mainly provided by the aerobic system and, therefore, the nitrate-nitrite-NO pathway might not have been fully elicited. In addition, this fact could be more pronounced in well-trained endurance subjects compared with normal population because the increase in capillary density in skeletal muscle resulting from endurance training are likely to reduce the likelihood of developing hypoxic and/or acidic environment in the active muscle (15).

The third factor is related with the response to increase plasma nitrite after nitrate ingestion. This is an important issue since nitrite is a more sensitive marker of NO synthesis and provides a NOS independent source for NO generation (26). A recent study by Totzeck et al. (38) has shown that plasma nitrite concentration is related with exercise capacity in moderate trained subjects. This finding could also be related with an increase of nitrite throughout NOS activity since there is evidence that endurance exercise stimulates skeletal muscle NOS function in humans during exercise (24). Regarding the above studies with nitrate supplementation, study by Cermak et al. (7) did not analyze plasma levels of nitrite and, consequently, it is not possible to know whether all subjects responded equally to nitrate treatment. Interestingly, in this study we found that 7 subjects showed a low response (< 30 %) to increase plasma nitrite after nitrate load. Wilkerson et al. (41) also found that three of their eight subjects had a low response to increase plasma nitrite after nitrate supplementation. Perhaps, this lower capacity to increase plasma nitrite concentration after nitrate ingestion could be related with

some modification in the microflora of the oral cavity (12). Although the use of antibacterial mouthwash was discarded in this study since no subjects reported a regular use of these products, we found that all low-responders were triathletes performing a considerable amount of training in the pool, while all high-responders, except one, were cyclists whom did not swim regularly. This fact suggests to us that a possible explanation for the low response showed by triathletes could be related with the presence of disinfectants such as chlorine in the water pool. As water commonly enters the mouth during swimming there is the possibility that the chlorine may interfere with the oral bacteria in a similar manner shown with antibacterial mouthwash (12). However, this is only a speculation and further studies are needed to analyze if regular training in chlorinated swimming pools can or not attenuate the conversion from nitrate to nitrite after the ingestion of supplement or food rich in nitrate.

Effects of dietary inorganic nitrate supplementation on oxygen consumption during exercise

Different studies have reported that inorganic nitrate supplementation in form of beetroot juice, as well as sodium nitrate supplementation, reduces the oxygen demands of moderately-trained subjects during exercise at several intensities (2, 20, 22-23, 39). Although the mechanism(s) behind this intriguing effect remains to be elucidated, two possible explanations have been reported, recently, linking the dietary nitrate consumption with an improvement in muscle contractile efficiency (1) and in mitochondrial respiration (21). In well-trained athletes, we found previously that sodium nitrate supplementation significantly reduced oxygen consumption only at maximal loads of exercise (FO_{2peak}) without significant changes at intensities below the RCP. Interestingly, the results of the current study were in agreement with our previous data since athletes performed exercise at an average intensity equivalent to RCP (~ 85.1 % of FO_{2peak}) and no differences in oxygen consumption were found between treatments. In addition, although there was a trend, not statistically significant, towards a decrease in FO_2, FCO_2 and ratio between FO_2 and power output after nitrate supplementation in the high-respond group, this effect was not associated with an increase in

exercise performance **(Table 2).** These results were in agreement with the recent study by Wilkerson et al. (41) indicating no substantial change in the respiratory response of well-trained endurance athletes after nitrate treatment during a long time-trial test.

Effects of dietary inorganic nitrate ingestion on blood concentration of 3-nitrotyrosine (3-NT)

In accordance with the second hypothesis of this study, a modest amount of dietary nitrate ingestion which could also be achieved by ingestion of several nitrate-rich foods such as green leafy vegetables and beetroot juice did not raise plasma levels of nitrated-proteins at rest or after exhaustive exercise. Similar results were reported by two previous studies assessing the effect of dietary nitrate consumption on plasma nitrated-proteins levels in resting conditions (32, 34). In addition, a recent study in mice has reported that an increase in nitrite bioavailability was associated with lower production of nitrotyrosine, superoxide production and expression of NADPH oxidase (37). However, no previous studies analyzed the effect of dietary nitrate ingestion on the formation of reactive nitrogen species (RNS) after exhaustive endurance exercise. This fact could be important because high-intensity endurance exercise, which induces an increase in muscle acidosis and nitrite, has been demonstrated to be involved in the formation of RNS under acidic conditions *in vitro* (33). In addition, there is also evidence that exhaustive exercise itself may increase the formation of RNS in athletes (35). Nevertheless, although we could not corroborate data in mice (37) because no differences were found between the low and high-responder groups in both conditions, pre and post-exercise **(Table 2),** we found that the ingestion of moderate amounts of dietary inorganic nitrate did not alter plasma concentration of nitrated-proteins in resting conditions and immediately after an intense exercise.

Effects of dietary inorganic nitrate ingestion on blood concentration of endothelin-1 (ET-1)

In contrast to our third hypothesis, plasma ET-1 levels rose significantly after exercise in the group supplemented with nitrate compared to the placebo group. There is evidence that acute exercise causes a tissue-specific change in the release of ET-1 (28). Studies by Maeda et al. (27, 29) in humans exercising one leg showed that the concentration of ET-1 increased in the venous blood of the non-exercising leg, whereas it remaining unchanged in the exercising one. Two endogenous mechanisms have been put forward to explain this response. The first is associated with the stimulus of shear stress induced by physical exercise. The release of ET-1 in cultured vascular endothelial cells is linked to low levels of shear stress while higher levels of stress depress the release of this molecule (18, 29). In the present study, athletes performed a cycle ergometer test exercising leg muscles, however, blood samples were taken from the antecubital vein. Thus, on the basis of studies by Maeda et al. (27, 29), an increase in plasma ET-1 would be expected in the forearms just after exercise in both treatments (nitrate and placebo).

The second mechanism of ET-1 release is associated to neurohumoral factors, such as NO, prostacyclin, and arginine vasopressin, which may be released during exercise (36). From this viewpoint, there is evidence that nitrite induces vasodilation and increases the blood flow in humans (8). This response is mediated by hemoglobin in red blood cells which sense hypoxia (9). Interestingly, using NIRS device Bailey et al. (2) showed that nitrate supplementation with beetroot juice was able to increase blood volume of active tissues during exercise linking this fact with an increase of vasodilation via nitrite reduction to NO. Considering this data and the present results, we hypothesize that an increase in plasma ET-1 in non-active tissues, derived from dietary nitrate ingestion, causes enhanced vascular tone and the consequent decrease in blood flow in these tissues, which could contribute to increasing blood flow in exercising muscles or in the lungs (8). Future studies are needed to corroborate or discard this hypothesis analyzing

the arterial-venous gradient of ET-1 between the main exercising and the non-exercising muscles after ingestion of inorganic nitrate.

In conclusion, three days of dietary inorganic nitrate supplementation (10 mg-kg^{-1} of body mass) did not enhance performance during a 40-minute distance-trial test in well-trained endurance athletes. Perhaps, the duration of supplementation, as well as the duration of the exercise test performed in this study might partially explain the more limited effect of sodium nitrate. Interestingly, this study confirmed that a moderate amount of inorganic nitrate ingestion did not stimulate an increase in plasma concentration of nitrated-proteins after exhaustive exercise indicating that this dose is safe. Furthermore, we found that nitrate supplementation induced a significant increase in plasma ET-1 concentration in forearms just after exercise. However, the mechanism behind this intriguing response remains to be elucidated.

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TABLE 1. Cardiorespiratory and metabolic response at three different points during 40-min time trials after dietary inorganic nitrate and placebo supplementation (n = 13).

Time	15 min		25 min		35 min		Average				
Treatment	Placebo	Nitrate	Placebo	Nitrate	Placebo	Nitrate	Placebo	Nitrate	*S*	*T*	*SxT*
VO.											
L-min	3-64 - 0.31	3,70 ± 0.32	3.64 ± 0.28	3.57 ± 0 32	3.62 ± 0.31	3.63 ± 0,42	3.63 - 026	3.63 ± 0.33			
mLmin⁻¹ kg⁻¹	51.1 = 7.6	51.9 ± 7.9	50.9 ± 6.6	50.2 ± 7.9	50.6 ± 6.4	50.9 ± 8.7	50.9 = 6.6	51.0 ± 7.9			
VCOÜ (L-min ⁻¹)	352 • 0.30	3.53 ± 0.29	3.50 ± 0.36	3.41 – 0.33	3.44 ± O.30	3.48 ± 0.45	3.49 • 0.27	3.47 ± 0.32			
14 (L-min-1)	110 = 13	112 ± 13	109 ± 12	110 – 13	110 • 13	116 ± 18	110 ± 11	113 ± 14			
RER	0.97 - 0.04	0.96 ± 0.05	0.96 ± 0.05	0.96 ± 0.05	0.95 ± 0.04	0.96 ± 0.05	0.96 *i* 0.04	0.96 ± 0.04			
HR (beats-min~¹)	160 11	160 ± 11	166 ± 12"	165 11*	167 • 10"	167 ± 12ˆ-ˆ	164 ± 11	165 ± 11			
Lactate (mmolmin⁻¹)	7.3 ± 4.0	6.9 ± 3.0	7.6 ± 4.0	7.9 ± 3.6	7.4 ± 3.4	7.8 ± 3.7	7.4 ± 3.7	7.5 ± 3.3			

Values are means ± SD.

¹ Significant difference with respect to 15-min values.

"Significant difference with respect to 25-rnin values.

VC-2, oxygen uptake; VCO2, expired carbon dioxide; V_e minute ventilation; X, significant effects (S or *J)* or significant interaction (SX 7) of two-way repeated-measures ANOVA *(P<* 0.05)

Table 2. Differences between low-responder (<30%) and high-responder (>30%) subjects to increase plasma nitrite concentration after dietary nitrate supplementation.

	Law Responders (*n* = 7)			High Responders In = 6)		
Treatment	Placebo	Nitrate	Difference	Placebo	Nitrate	Difference
Plasma nitrate (jimol L~[1])	33 ± 5	242 + 24	209 + 25*	29 ±5	272 + 47	243 -43*
Plasma nitrite (nmol-L [1])	191 ±31	217 ± 47	26 ± 21	183 ±7	471 ± 70	288 ± 70*'*
Urinary nitrate (n,molL[H])	755 ± 214	7447 ±817	6692 ± 588*	№ ± 122	7921 ± 646	7487 ± 641*
Urinary nitrite (nrnolL[-1])	138 = 90	252 101	114 50'	80 ±43	320 ±151	240 ± 115*
Distance (km[-1])	26.2 0.7	26.3 ± 0.7	0.1 ± 0.2	26.5 ±0.5	26.5 ± 0.3	0.0 0.5
Power (W)	256 · 17	258 18	2.0 ± 5.0	258 · 11	258 ± 8	0.0 · 0.5
VO, (Lmin ')	3.58 - 0.11	3.63 ±0.19	0.05 ± 0.16	370 + 0.18	3.63 ±0.15	-0.07 - 0.16
Ratio WVOÎ	71.7 8.2	71.2 ± 8.2	-0.5 + 7.1	69.8 + 2.9	71.0 i 7.6	1.2 + 6.7
VCO? (L-mlrr[-1])	3.45 ±0.14	3.54 ±0.16	0.90 ± 0.02	3.53 ±0,13	3 40 ± 0.34	-0.13 ± 0.20
Vi (Lmin[-1])	111 + 7	117 ± 8	6.0 ± 5.0	108 + 5	108 1 5	0.0 + 0.5
RER	0.97 _ 0.04	0.98 ± 0.05	0.01 ± 0.03	0.95 ±0.04	0.94 t 0.04	-0.01 _ 0.04
HR (beats min"')	166 ±5	168 ±5	2 ± 2	163 ±7	162 ± 6	-1 ±2
Lactate (mmol L [1])	/ 8 4.3	8.5 2.8	07 ±1.8	6.9 ±3.1	6.5 ± 3.8	-0.4 1.7
Lactate 3 (mmol L"[1])	8.9 ± 2.8	10.0 ±3.0	1.1 ±1.9	8.4 ±4.1	7.6 ±4.7	-0.8 = 0.9
ET-1 Pre (pg-mL [1])	0.8 + 0.4	10 + 0.3	0.2 0.5	0.9 + 0.2	1.4 + 0.5	0.5 0.5
ET-1 Post (pgmL [1])	2.0 0.6	4.1 ± 1.1	2.1 ±1.3*	2.7 ±0.7	4,0 i 1.1	1.3 ± 0.6*
3-NT Pre (%)	10? ± 15	90 ±14	1? 10	***6»*** 24	93 42	4 - 21
3-NT Post (%)	99 ± 19	78 ±37	-21 15	100 ± 18	91 ±23	-9 ± 16

Plasma and urinary concentration of nitrate and nitrite are mean ± SD values in basal conditions. Data of respiratory parameters and blood lactate concentration are mean ± SD o1 three measures throughout the test. Heart rate data are mean ± SD) values during the whole test.

* Statistical significance between nitrate and placebo ***{P<*** 0.05).

Statistical significance between low- and high-responder groups (P < 0.05).

$V0_{2}$, oxygen uptake; ratio VO_{2}/W, ratio between oxygen uptake and power output: VCO?, expired carbon dioxide; t/„ minute ventilation; lactate 3, mean concentration ot blood lactate 3 min after the exercise test; ET-1 Pre, mean plasma concentration of ET-1 preexercise; ET-1 Post, mean plasma concentration of ET-1 postexercise; 3-NT Pre. mean plasma concentration of nitrated proteins preexercise; 3-NT Post, mean plasma concentration of nitrated proteins postexercise.

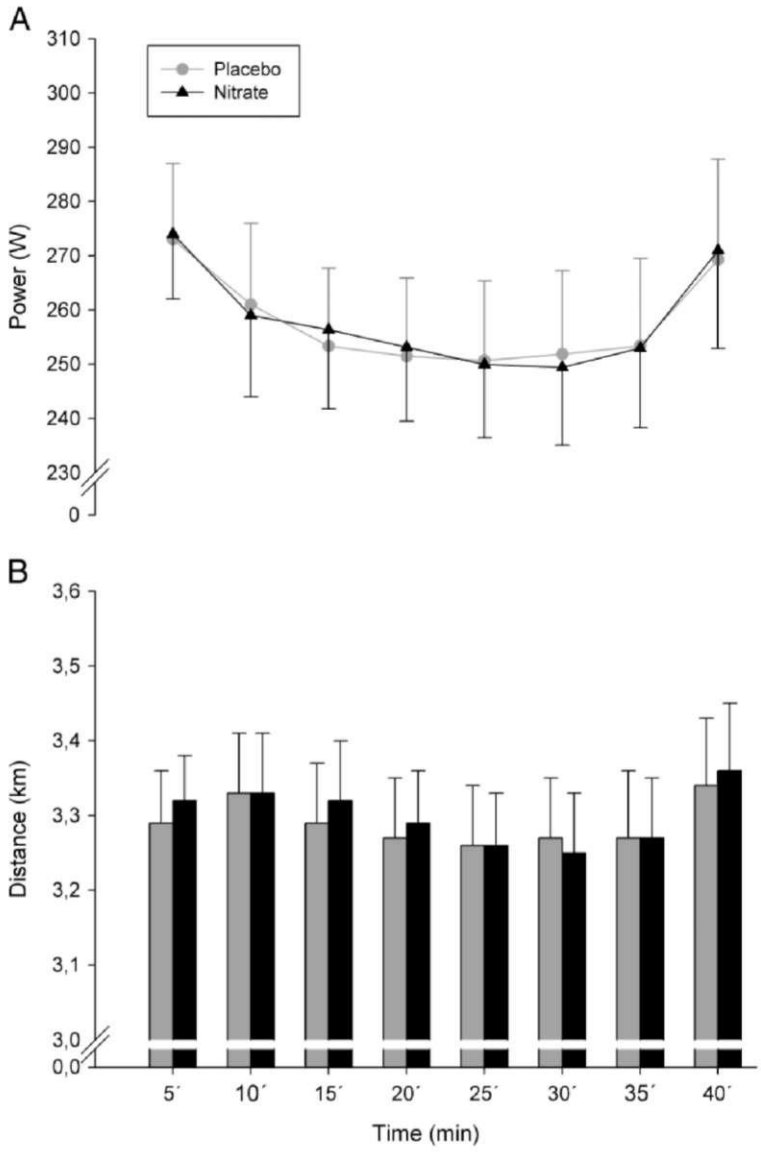

FIGURE 1—Profile of distance (A) and power output (B) performed by subjects during the 40-min distance trial after dietary inorganic nitrate and placebo supplementation (n = 13).

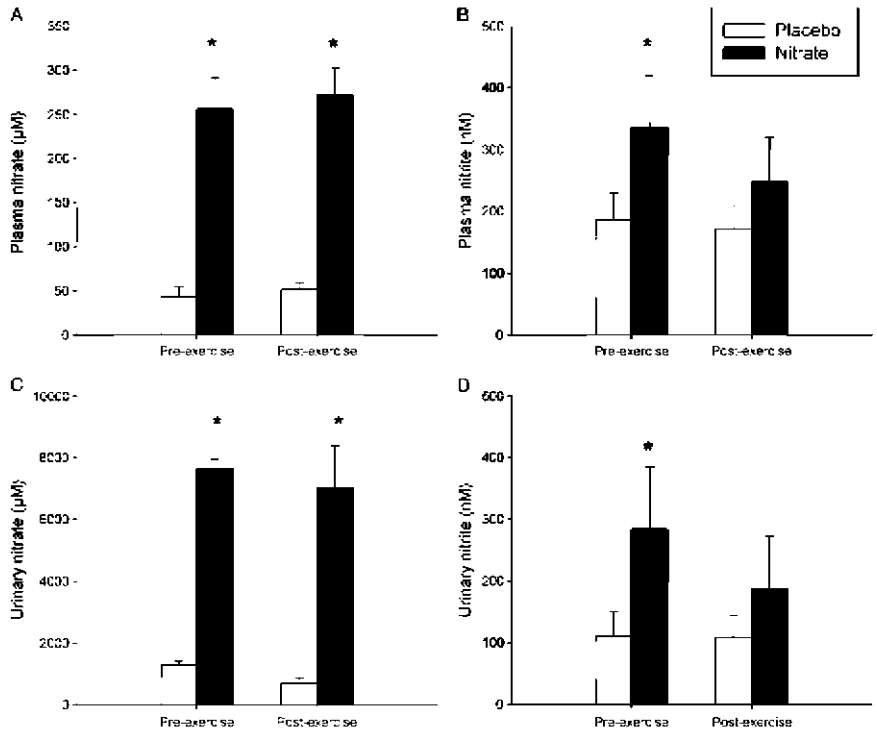

FIGURE 2.—Plasma and urinary levels of nitrate (A, C) and nitrite (B, D) in resting conditions and just after the 40-min distance trial after 3 d of supplementation with inorganic dietary nitrate or placebo ($n = 13$). *Statistical significance between nitrate and placebo. Two-way repeated-measures ANOVA ($P < 0.05$).

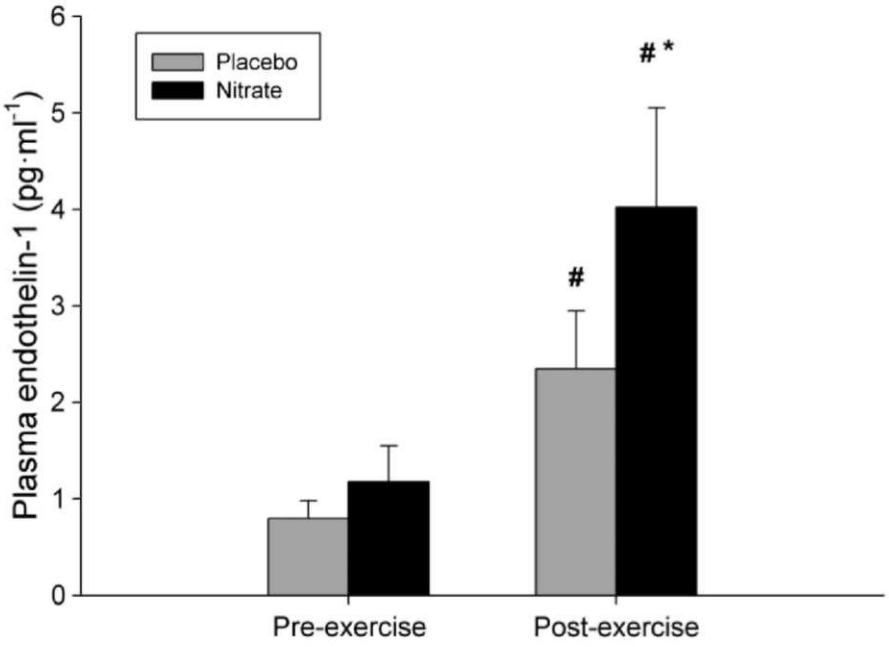

FIGURE 3—Plasma levels of ET-1 before and after the 40-min distance trial in both conditions (nitrate and placebo) (n = 13). *Statistical significance between nitrate and placebo. Two-way repeated-measures ANOVA ($P < 0.05$). #Statistical significance between preexercise and postexercise. Two-way repeated-measures ANOVA ($P < 0.05$).

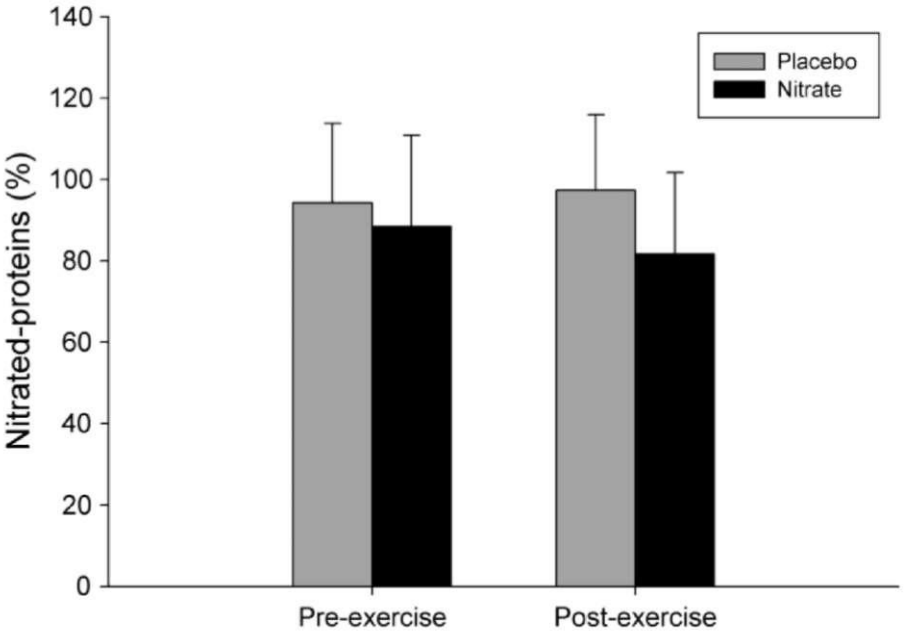

FIGURE 4—Plasma levels of nitrated proteins before and after the 40-min distance trial in both conditions (nitrate and placebo) (n = 13).

RECAPITULACIÓN

Efectos del DHA sobre el daño oxidativo

La suplementación con omega-3 administrada mediante una bebida funcional rica en DHA ha aumentado los niveles circulantes de estos AG, demostrándose efectiva para incrementar las disponibilidades de estos ácidos grasos para la asimilación tisular. Estos resultados son similares a los obtenidos en diferentes estudios, en los que los ácidos grasos omega-3 se administraban como suplemento mediante margarinas o cápsulas de aceites de pescado (Egert, et al., 2012, Ottestad, et al., 2012, Sanz París, et al., 2012). En nuestro estudio se ha observado un incremento plasmático y eritrocitario selectivo de DHA en el grupo experimental. El aumento de DHA en las membranas eritrocitarias se ha utilizado como reflejo de posibles aumentos de DHA en la composición de otras membranas celulares del organismo como las de los neutrófilos y las células mononucleares de la sangre periférica (PBMCs). De hecho, la suplementación de la dieta con aceites de pescado durante un periodo inferior a las 8 semanas de este estudio se ha revelado como adecuada para incrementar la proporción de DHA y EPA en membranas linfocitarias (Toft, et al., 2000). El aumento observado de DHA en plasma tanto en los NEFAs como en los TGFAs indica que el DHA no sólo se incorpora en las membranas como un AG estructural sino que también está disponible para una utilización energética. Por tanto, podemos afirmar que la suplementación con DHA mediante una bebida funcional con un 0.13% de DHA ha sido efectiva y selectiva para incorporar el DHA en las estructuras celulares y en las lipoproteínas plasmáticas, además de estar disponible en forma libre en plasma.

Diferentes estudios han puesto de manifiesto efectos funcionales de los omega-3 que influencian de forma positiva el estado de salud e incluso incrementan el rendimiento deportivo (Mickleborough, 2013, Swanson, et al., 2012, Tur, et al., 2012). En la actualidad no se atribuye una toxicidad intrínseca a la suplementación de la dieta mediante AGs insaturados (Tur, et al., 2012), pero debido a su susceptibilidad de oxidación se suele advertir de la posible oxidación de las membranas celulares y una mayor posibilidad de lisis.

La dosis suplementada en la intervención nutricional mediante la bebida funcional (1.14g/día de DHA) no ha sido muy alta, siendo inferior a la que se ha utilizado en otros estudios (Bloomer, et al., 2009, Egert, et al., 2012, Mehra, et al., 2006, Peoples, et al., 2008, Toft, et al., 2000), intentando aproximarse a dosis moderadas administradas mediante un alimento funcional de forma similar a la que se han llevado a cabo en otros estudios con otros alimentos funcionales (Barceló-Coblijn, et al., 2008, Din, et al., 2008). La bebida experimental diseñada permite que con la cantidad y frecuencia de consumo realizada en el estudio se aporte a la dieta 1.14 g de DHA diariamente. La cantidad de DHA que aporta la dieta de los deportistas no ha sido evaluada; sin embargo, teniendo en cuenta el consumo de pescado de la población adolescente de las Islas Baleares (Salas, et al., 2013) y el contenido medio de DHA que aportan los diferentes tipos de pescados (HHS and USDA, 2005) se estima que la ingesta diaria de DHA podría situarse entre 171 y 376 mg. De esta manera el aporte de DHA de la bebida experimental casi triplica o cuadriplica el que proviene de la dieta. Adicionalmente, tanto la bebida placebo como la experimental utilizadas en el estudio contienen los antioxidantes naturales propios de las almendras (vitamina E y fenoles) utilizadas para su elaboración y la vitamina E (acetato de alfa-tocoferol) añadida para prevenir una posible oxidación de los AGs. La cantidad y frecuencia de consumo de la bebida funcional experimental y placebo aporta diariamente una cantidad de vitamina E equivalente a 2.6 veces la dosis diaria recomendada para la población en general (Ortega RM, 2004). La actividad física incrementa la producción de RONS por lo que se podrían favorecer los procesos de oxidación de los PUFA. Los deportistas participantes en el estudio, al estar sujetos a un entrenamiento regular, presentan mayor producción de RONS y por ello mayor susceptibilidad a padecer un daño oxidativo en las diferentes fracciones sanguíneas. El daño oxidativo podría aumentar con la suplementación en el grupo experimental si los omega-3 suplementados en la dieta inician procesos de peroxidación lipídica. Los resultados obtenidos no muestran un mayor daño oxidativo en el grupo experimental tras el periodo de estudio en condiciones basales, ni tras el ejercicio físico agudo e intenso realizado a las 8 semanas de

suplementación. No hay ningún aumento de daño oxidativo o nitrosativo plasmático, ni en ningún tipo celular, asociado a la suplementación con omega-3. Por tanto, podemos concluir que la dosis crónica de omega-3 utilizada en el estudio no afecta a los marcadores de daño oxidativo en deportistas durante un periodo de entrenamiento regular y competitivo.

Dinámica del daño oxidativo y nitrosativo asociado a la actividad física aguda y al entrenamiento

La realización de una actividad física aumenta el consumo de O_2 y las demandas energéticas, aumentando la producción de RONS que si no es contrarrestada por los diferentes antioxidantes puede llevar a producir estrés oxidativo generando una situación que puede llegar a ser patológica (Radak, et al., 2013). El daño oxidativo que se produce en diferentes moléculas diana debe ser reparado, o bien las moléculas renovadas, para poder seguir manteniendo su función. Hemos evaluado la dinámica del daño oxidativo en lípidos mediante el seguimiento de marcadores de oxidación en plasma de ciclistas que participaron en una competición ciclista de cuatro etapas. La realización de esta actividad física intensa y repetida, con periodos de reposo largos entre etapas, induce una situación en la que algunos marcadores de daño oxidativo tienden a acumularse en el plasma, a pesar de que durante los periodos de reposo se observa una tendencia a revertir el daño oxidativo que aparece como resultado de la intensa actividad física. Se observa un aumento de los marcadores de daño oxidativo plasmático después de cada etapa de actividad física intensa, pero al día siguiente estos marcadores vuelven a recuperar los mismos niveles basales del día anterior. Sin embargo, algunos marcadores (MDA y CK) no llegan a recuperar los niveles del día anterior, sino que permanecen ligeramente elevados, indicando una acumulación de daño oxidativo. La capacidad de reducir el nivel de los marcadores de oxidación lipídica durante los periodos de reposo pone de manifiesto la existencia de mecanismos que desactivan y/o capturan los radicales peróxido, o directamente los hidroperóxidos de los ácidos grasos insaturados, que permitan parar las reacciones en cadena de peroxidación lipídica iniciadas durante la

realización de la actividad física. A la vez, deben existir vías de eliminación del MDA que se ha generado. Antioxidantes como la vitamina E podrían participar en la captura de los radicales peróxido de los ácidos grasos insaturados, mientras que enzimas como la GPx podrían desactivar los hidroperóxidos de estos ácidos grasos, transformándolos en alcoholes de estos ácidos grasos, aunque la funcionalidad de este enzima en el plasma es dudosa. Hay evidencias de que un ejercicio exhaustivo, como la realización de un duathlon, induce una activación de la GPx eritrocitaria (Tauler, et al., 2003) que está relacionada con una posible reparación de los hidroperóxidos eritrocitarios durante el periodo de reposo y recuperación post¬ ejercicio.

El plasma recoge la producción de especies reactivas de diferentes tejidos y células durante el ejercicio, pero además, presenta enzimas y metabolitos antioxidantes secretados por diversas células (neutrófilos, células endoteliales) o tejidos (como transferrina, ceruloplasmina, vitamina C, bilirrrubina y ácido úrico), de manera que aunque en el plasma no se sintetizan enzimas antioxidantes los niveles y actividad de estos pueden ser sensibles a situaciones de mayor producción de RONS. Además, en plasma se observa un aumento de los niveles de ácido úrico típico tras un ejercicio intenso (Hellsten Y In: Sen CK, 2000, Sureda, et al., 2007, Tauler, et al., 2003, Tauler, et al., 2006), reflejando una mayor actividad de la XO que va acompañada de una mayor producción de $O_2^{\cdot}$. La actividad física aumenta los niveles de RONS debido a diferentes fuentes de producción, como la cadena de transporte mitocondrial, la XO y la NOX. Los deportistas que han participado en los diferentes estudios entrenaban regularmente, de manera que podrían presentar una mayor adaptación para hacer frente al estrés oxidativo (Radak, et al., 2008). Las posibles adaptaciones antioxidantes plasmáticas parecen ser suficientes, en el caso de los futbolistas, para evitar el daño oxidativo en plasma tras el ejercicio agudo e intenso, pero no son suficientes para evitar el daño oxidativo plasmático en una competición ciclista como la Vuelta al Bidasoa.

La realización de una actividad física intensa incrementa la producción de NO y de otras especies reactivas de oxígeno como el

anión superóxido (Nikolaidis and Jamurtas, 2009, Sureda, et al., 2009, Totzeck, et al., 2012). La síntesis de NO durante la realización de actividad física se incrementa si se consume nitrato (Bescós, et al., 2012), con lo que juntamente con una mayor producción de anión superóxido podría producir mayores niveles de peroxinitrito e incrementar el daño nitrosativo con aumentos de los niveles de nitrotirosina. En una suplementación con nitrato existe el riesgo de provocar un aumento del daño nitrosativo, sobretodo en condiciones de ejercicio intenso donde se producen altas cantidades de O_2" que puede reaccionar con NO para dar lugar a NOO-, capaz de provocar la nitración de proteínas (Pacher, et al., 2007). La actividad física intensa y aguda en individuos bien entrenados no afecta de forma significativa el nivel de daño nitrosativo en proteínas plasmáticas. La prueba física realizada en cicloergómetro durante 40 minutos con una VO_2max entre 50.2 y 51.9 mL-min^-kg"[1], en la que se pretendía valorar la máxima distancia recorrida en bicicleta durante este tiempo y la influencia del consumo de nitrato sobre esta marca, no afecta al daño nitrosativo de las proteínas del plasma. De manera similar, la sesión de entrenamiento como actividad física aguda realizada por los futbolistas, tampoco afecta el nivel de daño nitrosativo que presentan las proteínas del plasma. Probablemente debido a la participación de los sistemas plasmáticos antioxidantes de los deportistas. El ejercicio físico intenso realizado por los futbolistas, aunque menos intenso que el realizado por los ciclistas, no induce daños nitrosativos ni tampoco oxidativos en las proteínas del plasma, en cambio, sí que se observan efectos en los compartimentos celulares sanguíneos.

La realización de la actividad física aguda e intensa realizada por los futbolistas tras dos meses de entrenamiento produce cambios en los marcadores de daño oxidativo y nitrosativo de diferentes fracciones celulares sanguíneas. Los eritrocitos durante el ejercicio transportan mayor contenido de O2 lo que aumenta el riesgo de producción de anión superóxido y de daño oxidativo, a la vez se ha evidenciado un incremento de los niveles eritrocitarios de nitrito (indicador de producción de NO) tras ejercicios intensos (Sureda, et al., 2006), lo cual puede inducir una mayor reacción entre NO y O_2", con un incremento de la nitración de las proteínas eritrocitarias. La

maquinaria antioxidante eritrocitaria parece no ser suficiente para prevenir el daño oxidativo en lípidos y proteínas en ciclistas durante la práctica deportiva (Muñoz Marín, et al., 2010, Sureda, et al., 2005). Las PBMCs presentan una producción mitocondrial de ROS importante, la cual puede incrementarse durante una actividad física. El índice de nitración de proteínas en las PBMCs aumenta como en la fracción eritrocitaria tras la realización de la actividad física aguda e intensa, además de aumentar la peroxidación lipídica sin modificar el índice de proteínas carboniladas y proteger la carga génica. La carbonilación de las proteínas se produce como resultado de la reacción de moléculas dicarbonílicas como el MDA con grupos amino libres de las proteínas, aunque también existen otros mecanismos como la oxidación directa de algunos aminoácidos, la deshidratación rehidratación de aminoácidos alcohol u otros (Rao and M0ller, 2011). La ausencia de cambios en la carbonilación de las proteínas de las PBMCs podría indicar que o bien no se ha dado el tiempo necesario para que se formen los grupos carbonilos o bien el proceso de adaptación debido al entrenamiento aumenta la protección de proteínas y ADN. Los neutrófilos a diferencia a las PBMCs al tener menor número de mitocondrias tienen una menor capacidad de producción mitocondrial de ROS pero presentan una capacidad de producción de ROS lisosomal importante. La actividad física aguda e intensa realizada por los futbolistas no refleja un daño oxidativo atribuible a un aumento en su producción de ROS. Contrariamente a los otros tipos celulares los neutrófilos no presentan cambios inducidos por la actividad física intensa en el índice de nitración de proteínas, presentan una disminución de MDA y de carbonilación de proteínas, indicando que no hay una mayor producción de $O2^{\cdot}$ y de otras ROS capaz de reflejarse en aumentos de estos marcadores de oxidación o bien los neutrófilos presentan una mejor adaptación frente a la generación de daño oxidativo inducida por la realización de una actividad física regular durante las sesiones de entrenamiento respecto a los demás tipos celulares. La realización de una actividad física intensa y aguda induce una mayor capacidad de producción de ROS frente a estímulos como LPS, zymosan o PMA tanto en PBMCs como en neutrófilos. Este tipo de activaciones en neutrófilos ponen de manifiesto que tras una actividad física intensa estas células están

más preparadas para la explosión oxidativa desencadenada por el acoplamiento de la NADPH oxidasa (NOX), responsable de la generación masiva de $O2^{\cdot}$ y el consecuente aumento de HOCl y H2O2 (Paletta-Silva, et al., 2013).

El seguimiento de los marcadores de daño oxidativo en condiciones basales a lo largo de una temporada de entrenamiento puede poner de manifiesto la acción de estos mecanismos de hormesis que podrían reforzar el nivel de defensas antioxidantes de estos deportistas. Los diferentes compartimentos sanguíneos pueden responder de forma diferente a estos mecanismos de hormesis y al daño oxidativo. El ejercicio intenso puede conllevar a una situación de estrés oxidativo provocando un daño oxidativo y nitrosativo, en los diferentes compartimentos sanguíneos, en cambio, la práctica regular de una actividad física moderada parece potenciar las defensas antioxidantes endógenas disminuyendo la posibilidad de aparición de estrés oxidativo (Tian, et al., 2010). El mecanismo que actualmente tiene más peso para explicar esta adaptación es conocido como hormesis (Fisher-Wellman and Bloomer, 2009), aunque puede tener diferente importancia en los diferentes compartimentos sanguíneos y no ser lo suficientemente efectivo para evitar la aparición de daño oxidativo y/o nitrosativo. Diferentes estudios ponen de manifiesto un aumento de la peroxidación lipídica en la fracción eritrocitaria durante el periodo de entrenamiento (Carrera-Quintanar, et al., 2012, Miyazaki, et al., 2001, Shin, et al., 2008) y coinciden con los resultados obtenidos al finalizar el periodo de entrenamiento en el experimento realizado con futbolistas. El aumento de MDA eritrocitario observado al finalizar el periodo de entrenamiento no va acompañado con un aumento en las proteínas carboniladas, lo que indica que la producción de ROS no es suficiente para superar a las defensas antioxidantes y dañar a las proteínas o que el MDA no llega a reaccionar con las proteínas eritrocitarias. Además, el índice de nitración de proteínas disminuye tras 8 semanas de entrenamiento, indicando que no hay suficiente producción de $O2^{\cdot}$ para reaccionar con NO y formar el NOO-. Que solo se dañen los lípidos y no las proteínas puede ser reflejo de un aumento de las defensas antioxidantes en los eritrocitos debido al entrenamiento, como el

observado en el aumento de la actividad de la enzima SOD y, también podría reflejar que los lípidos eritrocitarios son más susceptibles de dañarse al finalizar la fase de entrenamiento al tener una mayor proporción de ácidos grasos poliinsaturados, debido a su mayor susceptibilidad a la oxidación. En las PBMCs encontramos una situación totalmente diferente al daño oxidativo presente en eritrocitos. Se observa que el entrenamiento produce una protección frente a la peroxidación lipídica, disminuyendo los valores de MDA, con concordancia a otros estudios (Ferrer, et al., 2009), pero se observa un incremento sobre el daño en las proteínas, en la carbonilación y nitración. Esto sugiere que el ejercicio regular realizado ha ido provocando una producción de ROS mitocondrial importante en las PBMCs y que sus defensas antioxidantes no han evitado el daño en proteínas pero si en lípidos y en ADN. En cambio, los neutrófilos sí que presentan una disminución de los marcadores de peroxidación lipídica, carbonilación y nitración de proteínas, indicando una mayor respuesta adaptativa en comparación a las PBMCs, frente a la generación regular de ROS. En el plasma, que es reflejo de la producción de ROS y antioxidantes por parte de los diferentes tejidos, presenta al cabo del periodo de entrenamiento unos niveles de LDH aumentados, indicando un daño celular, y unos niveles de proteínas carboniladas también aumentados. Aun así presenta una disminución de ácido úrico, reflejando una menor actividad XO. Se pone de manifiesto que un periodo de entrenamiento provoca daño oxidativo en los diferentes tejidos que va variando en función de su capacidad de adaptación. El periodo de entrenamiento induce peroxidación lipídica en eritrocitos pero la reduce en PBMCs y en neutrófilos, produce carbonilación de proteínas en plasma y en PBMCs pero la reduce en neutrófilos, y aumenta el índice de nitración de proteínas en PBMCs pero lo reduce en eritrocitos y neutrófilos.

Efectos del ejercicio sobre la nitración de proteínas

La nitración de las proteínas es resultado de la reacción entre el peroxinitrito y los grupos aromáticos de las cadenas laterales de tirosina y fenilalanina (Pietraforte, et al., 2003). El peroxinitrito es el

producto de la reacción equimolecular entre en anión superóxido y el óxido nítrico, de manera que los niveles de nitración de proteínas son un índice de la producción de peroxinitrito y, a la vez un indicador de la producción simultánea de anión superóxido y de óxido nítrico. Los distintos tipos celulares sanguíneos tienen diferentes dinámicas de producción y eliminación de $O_2^{\cdot}$ y de NO y de comportamiento frente a una actividad física intensa (Sureda, et al., 2009, Sureda, et al., 2006). Las PBMCs tienen una producción básicamente mitocondrial de $O_2^{\cdot}$ (Li and Verma, 2002), mientras que los neutrófilos si bien también tienen mitocondrias, producen masivamente el $O_2^{\cdot}$ a nivel lisosomal por acción de la NADPH oxidasa (Abramson and Wheeler, 1993). Los eritrocitos, al carecer de mitocondrias producen el anión superóxido por la reacción entre la hemoglobina y el oxígeno que transporta (Perrone, et al., 2012). El óxido nítrico es producido por los macrófagos como mecanismo de eliminación de microorganismos (Segal, 2005). El NO se produce básicamente por acción de la NO sintetasa, flavoproteína que tiene como sustrato la L-arginina y rinde como productos la L-citrulina y el NO, utilizando NADPH como cosustrato (Weissman and Gross, 2001). Se ha indicado que como subproducto de la actividad de la NOS, al igual que en otras flavoproteínas, se puede producir anión superóxido (Xia, et al., 1998). Existen diferentes formas isoenzimáticas de la NO sintetasa, algunas de ellas presentes en PBMCs y en neutrofilos como la iNOS. Existe una vía alternativa de producción de NO a partir de nitrato (Lundberg, et al., 2008). Esta vía alternativa necesita del concurso de las bacterias anaerobias de la cavidad bucal para transformar el nitrato en nitrito, el cual es fácilmente asimilado y transformado en NO en el plasma o en células por acción de enzimas como la xantina oxidasa (Cantu-Medellin and Kelley, 2013, Lundberg, et al., 2008). Por otro lado, el NO es un activador de la biogénesis mitocondrial (Piantadosi and Suliman, 2012). La administración de nitrato en la dieta incrementa realmente la producción de NO, describiéndose efectos importantes del consumo de nitrato sobre la tensión sanguínea (Vanhatalo, et al., 2010) o incluso sobre el rendimiento deportivo (Bescós, et al., 2011, Jones, et al., 2012, Jones, et al., 2013). El incremento simultáneo en la tasa de producción de anión superóxido y de NO puede producir un aumento de la tasa de producción de peroxinitrito y de la nitración de

las proteínas. La eliminación de estas especies reactivas es pues un requisito para evitar daños en las proteínas. El anión superóxido se elimina por acción de la SOD y ulterior dismutación del peróxido de hidrogeno por acción de la catalasa o GPx. El NO se oxida espontáneamente a nitrito y, éste a nitrato (Guzik, et al., 2003). La producción de NO suele monitorizarse mediante la determinación de los niveles de nitrito y de nitrato (Bescós, et al., 2012, Ghasemi, et al., 2010, Lansley, et al., 2011, Totzeck, et al., 2012). En las PBMCs se ha observado que el periodo de entrenamiento disminuye el nitrato intracelular y aumenta los niveles proteicos de la iNOS, lo que sería indicativo de una mayor capacidad de producción de NO, lo que puede ser causante del aumento del grado de nitración de las proteínas observado. La actividad física aguda realizada por los jugadores de fútbol durante el periodo de entrenamiento provoca una disminución del nitrato intracelular en las PBMCs y los neutrófilos, con un aumento paralelo de nitrito y nitrato en suero. La realización del ejercicio agudo intenso genera el mismo patrón de cambio en estos tres parámetros que siguen disminuyendo (nitrato) o aumentando (iNOS y nitración de proteínas) tras el ejercicio. Este comportamiento es compatible con un incremento en la producción de peroxinitrito en PBMCs debido a la realización de una actividad física intensa. El NOO- si no reacciona con sus dianas (lípidos o proteínas) se descompone en nitrato (70 %) y $OH^{\cdot}$ (30 %) (Beckman, et al., 1990), un no aumento de los niveles de nitrato, en condiciones de generación de NO, indica que el NOO- ha reaccionado con alguna de sus dianas, las cuales parecen ser las proteínas por el aumento de su índice de nitración. En neutrófilos no se observa un cambio en los niveles de nitrato intracelular provocado por el entrenamiento, en cambio sí que se observa una disminución provocada por el ejercicio intenso que es paralela a un aumento de nitrato y nitrito en suero. Un aumento de nitrito en plasma es un marcador de la síntesis de NO por parte de la NOS (Bescós, et al., 2012, Lundberg, et al., 2008). En la situación observada en el ejercicio de los futbolistas tras un ejercicio intenso, donde disminuye el nitrato intracelular de las PBMCs y los neutrófilos, indica que hay una producción importante de NO el cual traspasa las membranas y se oxida provocando un aumento de nitrito y nitrato plasmático. Hay estudios donde se ha evidenciado una

producción de nitrito intracelular en respuesta a una actividad física intensa o una sesión de buceo (Sureda, et al., 2004, Sureda, et al., 2006, Sureda, et al., 2009), de manera que el nitrito puede atravesar la membrana a favor de gradiente y acumularse en el plasma.

La simultaneidad en la producción de anión superóxido y NO es importante de cara a la producción de peroxinitrito y de la nitración de proteínas. Hemos explorado los efectos de la potenciación de la vía alternativa a la iNOS de síntesis de óxido nítrico sobre la nitración de las proteínas plasmáticas. Se ha administrado una dosis suficiente de nitrato (10 mg de nitrato de sodio por kg del sujeto) o de placebo durante tres días consecutivos a deportistas bien entrenados que luego han realizado una actividad física extenuante, simulando una carrera ciclista de contrarreloj. Se trata de un estudio a doble ciego cruzado en el que cada deportista es control de sí mismo. La suplementación con nitrato no afecta el tiempo de llegar a la extenuación, ni los kilómetros recorridos ni la velocidad alcanzada en las pruebas; en cambio su ingesta sí que aumenta los niveles en plasma y orina. En comparación, tras el ejercicio intenso realizado en la simulación de la contrarreloj en sujetos entrenados no observamos un aumento ni de nitrito ni de nitrato en el plasma, y tampoco en el índice plasmático de nitración de proteínas. El diferente momento de toma de muestras tras el ejercicio, 2 horas en los futbolistas y 3 minutos en la prueba ciclista, podría dar resultados diferentes en los niveles circulantes de nitrato y nitrito (Sureda, et al., 2012, Sureda, et al., 2006).

Cambios en los marcadores antioxidantes asociados a una actividad física moderada y regular, e intensa

La presencia continuada y prolongada de estimulantes fisiológicos oxidantes durante el entrenamiento provoca una adaptación frente al estrés oxidativo dando como resultado una mayor protección antioxidante (Fisher-Wellman and Bloomer, 2009, Radak, et al., 2008, Radak, et al., 2008), mediante diferentes mecanismos como un incremento de las actividades enzimáticas y de sus niveles proteicos (Ferrer, et al., 2010, Knez, et al., 2007, Pikosky,

et al., 2006) o regulando la calidad mitocondrial (Yan, et al., 2012). Los eritrocitos al ser células anucleadas no tienen capacidad de síntesis proteica pero son capaces de adaptar las defensas antioxidantes mediante mecanismos de regulación post-traduccional. Mediante estos mecanismos las actividades de las enzimas antioxidantes (catalasa, SOD, GPx y GRd) de los eritrocitos aumentan, tras el periodo de entrenamiento realizado por los futbolistas, sin verse modificado sus niveles proteicos, con la excepción de la proteína GRd que puede haber aumentado sus niveles, probablemente debido a un cambio en el proceso de la maduración de los reticulocitos (Sailaja, et al., 2003). La alta actividad de SOD al final del estudio indica una alta capacidad de eliminación de $O2^{\cdot}$, previniendo la formación peroxinitrito y la consecuente menor nitración de proteínas (como hemos discutido anteriormente). El H2O2 producido por la actividad SOD se destoxifica por la alta actividad de catalasa y GPx, la cual se puede ir regenerado por el sistema GSSG/GSH y la acción de la GRd. Por otra parte, las PBMCs y neutrófilos, al tener núcleo, pueden responder de una manera más adaptativa frente un periodo de entrenamiento, aunque no se han observado ningún cambio sobre la expresión de ningún gen antioxidante. El entrenamiento provoca en las PBMCs una disminución de la actividad enzimática de la catalasa y un aumento de la GPx, sin cambios en los niveles proteicos. La GPx es la primera barrera contra el H2O2 a bajas concentraciones, en cambio, a altas concentraciones la catalasa, con mayor constante de Michaelis-Menten, adquiere un papel predominante (Sasaki, et al., 1998, Sureda, et al., 2005). Esto parece indicar que durante la temporada de entrenamiento se produce un cambio en la estrategia de eliminación de ROS en las PBMCs, favoreciéndose la eliminación de bajas producciones características del entrenamiento mediante la potenciación del sistema antioxidante basado en el glutatión. Por otro lado, los niveles proteicos de UCPs, proteínas que evitan la producción mitocondrial de ROS, se ven aumentados tras el periodo de entrenamiento, indicando una menor capacidad de las PBMCs de producción mitocondrial de ROS. Aun así, y como ya hemos comentado, este aumento de la capacidad antioxidante evita el daño en lípidos y ADN pero no evita un incremento en la carbonilación de las proteínas de las PBMCs. El incremento de las actividades

antioxidantes (catalasa, GPx y GRd) en neutrófilos tras el periodo de entrenamiento tampoco va acompañado de un aumento en sus niveles de proteicos, sugiriendo una activación post-traduccional de estos enzimas antioxidantes (Hollander, et al., 2000, OH-ISHI, 1996). En el caso de la GPx de neutrófilos esta activación debe ser más acentuada debido a que el nivel de proteína disminuye tras el entrenamiento, mientras que su actividad enzimática se incrementa. El entrenamiento también aumenta el nivel de TrxR1 en neutrófilos, la cual tiene un papel importante en la regeneración de puentes disulfuros de proteínas oxidadas (Wang, et al., 2013). Además, las UCPs también aumenta sus niveles proteicos, como en las PBMCs, previniendo una excesiva producción mitocondrial de radicales libres (Figueira, et al., 2013). La adaptación antioxidante inducida por el entrenamiento en los neutrófilos disminuye el nivel de los marcadores de peroxidación lipídica, carbonilación y nitración de proteínas, indicando una mayor respuesta adaptativa y eficaz que los PBMCs frente a la generación regular de ROS.

La efectividad de algunas de las defensas antioxidantes se reduce en el plasma dado que algunos sustratos enzimáticos no están presentes en concentraciones significativas como es el caso del glutatión, mayoritariamente intracelular en sangre (Rose, et al., 2012); además, la posibilidad de regulación de la síntesis de enzimas está ausente en plasma, aunque es posible la secreción de diferentes enzimas antioxidantes. Sin embargo, la SOD, con una forma enzimática característica del plasma, y la catalasa plasmática, que no necesita otros sustratos para eliminar el peróxido de hidrogeno, pueden tener un papel en la dinámica de eliminación de las ROS en plasma. La producción plasmática de ROS puede atribuirse a la acción de enzimas como la XO plasmática (Viña, et al., 2000) y la NADPH oxidasa vascular (Hamanaka and Chandel, 2010), el primero con una especial importancia en relación a la actividad física ya que se aprecia un aumento de la disponibilidad de sustrato (xantina e hipoxantina) tras la realización de una actividad física intensa (Sanchis-Gomar, et al., 2013). Adicionalmente, el metabolismo celular que genera ROS puede verter al plasma parte de esta producción, principalmente en forma de peróxido de hidrógeno. Aunque en el plasma no haya

posibilidad de síntesis proteica, hemos observado un aumento de la capacidad antioxidante y prooxidante en respuesta al periodo de entrenamiento, reflejada en un aumento de las actividades enzimáticas catalasa y SOD y de los niveles de ácido úrico, producto de la actividad XO. El aumento de las actividades antioxidantes en estos enzimas podría ser un efecto activador de las ROS y de las moléculas antioxidantes de bajo peso molecular que provocarían una activación post-traduccional (Carrera-Quintanar, et al., 2012, Mestre-Alfaro, et al., 2011) de estos enzimas, aunque también podría ser reflejo de una secreción de estos enzimas procedente de tejidos y/o de células sanguíneas hacia el plasma (Sureda, et al., 2007). Aun así, el aumento de la capacidad antioxidante observado en plasma no es suficiente para evitar un incremento de la carbonilación de proteínas plasmáticas durante el entrenamiento en futbolistas, pero si para evitar su nitración y el daño en lípidos.

En resumen, hemos observado diferentes comportamientos adaptativos frente a un periodo de entrenamiento en los diferentes compartimentos sanguíneos. Por una parte el plasma, reflejo de la secreción de antioxidantes por parte de los diferentes tejidos y células sanguíneas, con una mayor producción en estos. En eritrocitos, PBMCs y neutrófilos el entrenamiento aumenta la defensa antioxidante y en las PBMCs se potencia la eliminación de bajas concentraciones de ROS aumentando el papel del sistema GPx frente al sistema catalasa. El periodo de entrenamiento induce una mayor actividad enzimática de catalasa, SOD, GPx y GRd en eritrocitos y de catalasa, GPx y GRd en neutrófilos, en cambio en PBMCs hay un aumento de la actividad GPx en detrimento de la actividad catalasa. Además de cambios en las actividades de enzimas antioxidantes, el entrenamiento también provoca cambios en los niveles proteicos, como un aumento de GRd en eritrocito, un aumento de TxR1 y disminución de GPx en neutrófilos y un aumento de UCPs en PBMCs y neutrófilos

La práctica de una actividad física regular, representada por las sesiones de entrenamiento que los futbolistas han realizado desde el inicio de la temporada durante 8 semanas, ha inducido cambios notables en el nivel de defensas antioxidantes y en el daño oxidativo

de plasma y de diferentes tipos de células sanguíneas. Sin embargo, no se conocen suficientemente los efectos que la realización de una actividad física intensa y de forma aguda pueden producirse en estos deportistas mejor adaptados frente al estrés oxidativo. En eritrocitos, las actividades de las enzimas antioxidantes han incrementado debido a la temporada de entrenamiento y han mantenido sus actividades y niveles de proteínas tras la realización de un ejercicio agudo e intenso, con la excepción de una disminución de la actividad GPx eritrocitaria, indicando una cierta inactivación enzimática, al igual que se observa tras una etapa ciclista de montaña (Aguiló, et al., 2005). Esta disminución de la actividad GPx eritrocitaria tras un ejercicio intenso también se observa en los neutrófilos, acompañada de un aumento proteico de Mn-SOD y GRd. Se ha indicado que el H2O2, producido en la destoxificicación de O_2^-, es un activador de la GPx (Montgomery, 1999, Mutze, et al., 2003), por lo que se sugiere que esta enzima es activada tras la destoxificación de $O2^-$, cuando baja la concentración de H2O2 como consecuencia de su eliminación. La desactivación de la GPx tras el ejercicio agudo podría reflejar una producción masiva de $O2^-$, eliminado por las mayores actividades de SOD, y el H2O2 en exceso es eliminado preferentemente por la catalasa a la vez que produce una desactivación de la GPx (Mueller, et al., 1997). Por tanto, observamos que aunque los eritrocitos tengan las defensas antioxidantes elevadas antes del ejercicio, estas no evitan la desactivación de enzimas como la GPx ni la nitración proteica, iniciada por la reacción entre NO y $O2^-$, posiblemente porque la producción de NO es elevada y la destoxificación de $O2^-$ no es suficiente. En cambio, la mayor adaptación de los neutrófilos tras la temporada de entrenamiento observada consigue evitar el acumulamiento excesivo de $O2^-$ producido en el ejercicio intenso, protegiendo lípidos y proteínas de la producción de daño oxidativo. Las PBMCs presentan un mayor aumento de las actividades de enzimas antioxidantes (catalasa, GRd y GPx) y un aumento en los niveles proteicos de catalasa, Cu/Zn-SOD, GPx y TrxR1 tras la realización de la actividad física aguda e intensa. Aun así, de forma paralela aumentan el índice de nitración de proteínas y el MDA, indicando que las PBMCs responden a los altos niveles de ROS producidos al realizar una actividad física intensa incrementado el

nivel de defensas antioxidantes, la respuesta no es suficiente o es paralela a la producción de daño oxidativo por los mismos efectores que inducen la respuesta antioxidante. Hay que tener presente que la temporada de entrenamiento ha inducido un cambio de estrategia en la eliminación de peróxido de hidrogeno en las PBMCs, favoreciendo la eliminación de bajas producciones de ROS (aumentando la actividad GPx) y desfavoreciendo la respuesta frente altas producciones (disminuyendo la actividad catalasa). Un aumento agudo e intenso de la producción de ROS podría inducir un incremento en los niveles de peróxido de hidrogeno en PBMCs, que no podrían eliminarse al disminuir la capacidad de la catalasa. El peróxido de hidrogeno es un buen inductor de la expresión de los genes antioxidantes a la vez que incrementa la producción de daño oxidativo (Ferrer, et al., 2010).

El ejercicio físico agudo e intenso no altera ni la maquinaria antioxidante ni los marcadores de daño oxidativo en plasma. El único cambio que detectamos en plasma como consecuencia del ejercicio agudo e intenso es un aumento de los niveles de ácido úrico, probablemente reflejo de una mayor actividad de la XO y/o de una mayor disponibilidad de su sustrato, la xantina y la hipoxantina. Paralelamente a la transformación de xantina/hipoxantina en urato por acción de la XO se produce anión superóxido. El aumento de la concentración de urato plasmático tras el ejercicio agudo e intenso podría reflejar un incremento en la producción plasmática de anión superóxido, sin más consecuencias en la generación de daño oxidativo en los diferentes componentes del plasma. Hemos de suponer que la mayor producción de anión superóxido en plasma se compensaría con la presencia suficiente de SOD y catalasa plasmáticas. Por otro lado, la intensidad y duración del ejercicio realizado por los futbolistas durante su sesión de entrenamiento es menor que la que se realiza en una etapa ciclista de montaña o en una contrarreloj. El daño oxidativo plasmático se pone de manifiesto en las diferentes etapas ciclistas de la Vuelta al Bidasoa, especialmente a partir de la segunda etapa, indicando que si bien los marcadores de daño oxidativo se recuperan parcialmente tras el periodo de descanso diario, también se incrementan más tras una nueva etapa ciclista poniendo de

manifiesto alteraciones en las capacidades de producción/eliminación de las ROS en plasma tras la repetición de ejercicios de larga duración e intensos. El daño oxidativo que se produce en las diferentes etapas ciclistas se puede traducir en daño celular como queda reflejado en el incremento de la actividad LDH circulante, marcador de daño celular, que se mantiene inalterado después de la sesión de entrenamiento en el caso de los futbolistas.

La suplementación con omega-3 aumenta su biodisponibilidad afectando al estrés oxidativo y a la función mitocondrial

La suplementación realizada con omega-3 durante 8 semanas en futbolistas no afecta al estrés oxidativo tan notoriamente como el entrenamiento y el ejercicio agudo. En términos generales la suplementación utilizada ni mejora ni empeora la situación de estrés oxidativo en los deportistas. No obstante sí que se observan cambios atribuibles al incremento del consumo de DHA en la biodisponibilidad de los omega-3 en plasma, en algún marcador de función mitocondrial, en la producción de H2O2 por parte de las PBMCs y los neutrófilos, y en la protección antioxidante y nitrosativa de las proteínas en eritrocitos.

El incremento dietario del consumo de DHA proporciona una mayor defensa antioxidante y un menor daño nitrosativo en los eritrocitos. Tras el periodo de entrenamiento y suplementación con DHA los deportistas presentan mayor actividad SOD eritrocitaria que evita una mayor nitración de proteínas debido a una actividad física intensa. Una mayor actividad eritrocitaria SOD implica una mayor capacidad de destoxificación de $O2^{\cdot}$, lo que podría repercutir en una menor tasa de formación de NOO-, resultado de la reacción entre $O2^{\cdot}$ y NO. El DHA también tiene un efecto en los niveles de la proteína eritrocitaria GRd, probablemente potencia el incremento de la tasa de síntesis de GRd eritrocitaria inducido por el estímulo de la actividad física regular durante el periodo de entrenamiento, y que continua con niveles elevados tras un ejercicio agudo e intenso. Este cambio en los niveles de la proteína eritrocitaria podría atribuirse a un efecto del DHA sobre la maduración de reticulocitos a eritrocitos; la GRd

disminuye sus niveles durante la maduración de reticulocitos a eritrocitos (Sailaja, et al., 2003), lo que se retrasaría debido al entrenamiento, potenciado por el consumo de DHA. Por otra parte, el incremento de las actividades eritrocitarias de catalasa y GPx provocado por la actividad física regular durante el periodo de entrenamiento parece atenuarse por el incremento del consumo DHA y podría estar relacionado con una mayor actividad eritrocitaria de la SOD (Tauler, et al., 2002, Tauler, et al., 1999). Similarmente, el incremento de la actividad enzimática de GPx inducido por el entrenamiento no es tan elevado en las PBMCs con la suplementación con DHA. El DHA parece tener un efecto sobre el estrés oxidativo mitocondrial. El incremento en el consumo de DHA durante la temporada de entrenamiento induce una disminución de la capacidad mitocondrial de producir ROS, aumentando los niveles de la UCP3, proteína desacopladora de la respiración mitocondrial con actividad reguladora de la producción mitocondrial de $O_2^{\cdot}$ (Figueira, et al., 2013, Sluse, et al., 2006), y aumentar la capacidad de destoxificar el $O_2^{\cdot}$ producido en la actividad física, aumentando los niveles de Cu/Zn-SOD. Estas dos mejoras antioxidantes por parte del DHA también se ven reflejada en una menor tasa de producción de ROS después del ejercicio, por parte de las PBMCs activadas con LPS. Estas adaptaciones que se observan en las PBMCs atribuibles al consumo de DHA no parecen presentarse en los neutrófilos. Contrariamente a lo observado en las PBMCs, el DHA provoca una atenuación del aumento de los niveles proteicos de Mn-SOD y UCP3 en respuesta a la actividad física. La activación de los neutrófilos con zymosan incrementa la producción de ROS; la actividad física aguda también incrementa la tasa basal de producción de ROS tras la activación de los neutrófilos; sin embargo este incremento no se ve afectado por el incremento en el consumo de DHA, aunque sí que se reduce el tiempo de respuesta en la producción de ROS ya que el grupo que ha consumido más DHA acelera el tiempo necesario para producir la explosión oxidativa de producción de ROS. La activación de PBMCs y neutrófilos se produce por mecanismos diferentes con diferentes responsables en la producción masiva de ROS. En linfocitos las principales fuentes de ROS tras activación de los receptores de células T son el metabolismo del ácido araquidónico (Los, et al., 1995), la

mitocondria (Schulze-Osthoff, et al., 1993) y la NOX (Jackson, et al., 2004). En cambio, en los neutrófilos, al poseer menor cantidad de mitocondrias, tiene un mayor papel el acoplamiento de la NOX lisosomal. Por tanto, el efecto de un aumento proteico de UCP3 y Cu/Zn-SOD en PBMCs puede ser más notorio en la producción de ROS que no la disminución o atenuación de los cambios de UCP3 y Mn-SOD en neutrófilos.

Estas modulaciones del DHA en las células sanguíneas no se ha observado en el plasma, ni en la capacidad antioxidante, ni en el daño oxidativo, ni en el estrés oxidativo. No obstante sí que se ha observado un aumento de los omega-3 de los NEFAs y en los TGFAs circulantes, mejorando la disponibilidad de estos como fuente energética o de síntesis de estructuras celulares. Tras un ejercicio físico intenso los NEFAs plasmáticos presentan un mayor grado movilización e intercambio que los TGFAs plasmáticos.

CONCLUSIONES / CONCLUSIONS

1. La realización de un entrenamiento regular aumenta el daño oxidativo y nitrosativo en diferentes fracciones sanguíneas, plasma, eritrocitos y PBMCs, con la excepción de los neutrófilos donde se observa una disminución de los marcadores de daño oxidativo en lípidos y proteínas.

2. El entrenamiento deportivo mejora la maquinaria antioxidante endógena de diferentes fracciones sanguíneas, plasma, eritrocitos, neutrófilos y PBMCs, mediante mecanismos de activación de enzimas y de incremento de niveles de proteínas antioxidantes. Además, el entrenamiento provoca un cambio de estrategia en las defensas antioxidantes de las PBMCs, mediante un aumento del sistema antioxidante basado en la actividad GPx en detrimento del basado en la actividad catalasa, favoreciendo la eliminación de bajas concentraciones de H_2O_2 frente a las altas.

3. La mayor capacidad antioxidante inducida por el entrenamiento regular protege a las diferentes fracciones sanguíneas frente a la alta tasa de producción de RONS que conlleva un ejercicio físico intenso. Las PBMCs son las células sanguíneas peor adaptadas, debido a un entrenamiento regular, frente a una alta producción de RONS, la cual provoca peroxidación lipídica y nitración de proteínas.

4. La realización de una competición ciclista por etapas provoca un perfil acumulativo de los marcadores de daño oxidativo y una respuesta inflamatoria sistémica.

5. La suplementación de la dieta mediante 1.14 g DHA/día aumenta los niveles plasmáticos y eritrocitarios de este ácido graso poliinsaturado, aumentando su disponibilidad como fuente energética para el mantenimiento del ejercicio y como parte estructural de membrana, y sin aumentar los marcadores de daño oxidativo.

6. La combinación de técnicas enzimáticas de hidrólisis de triglicéridos, de técnicas de cromatografía en columna de purificación de ácidos grasos libres y de técnicas de cromatografía de gases para la separación y cuantificación de ácidos grasos individuales consigue cuantificar el nivel de ácidos grasos individuales en forma libre y en forma de triglicéridos circulantes en plasma. Los niveles de ácidos grasos esterificados de los triglicéridos son unas seis (5.7) veces superiores a los que circulan en forma libre, siendo diferente la composición de ácidos grasos individuales en las dos fracciones circulantes. Los ácidos grasos esterificados son más ricos en ácidos grasos poliinsaturados que los que circulan en forma libre, siendo los triglicéridos circulantes una importante reserva de PUFAs.

7. La suplementación realizada con DHA no afecta al balance oxidativo tan notoriamente como el entrenamiento y el ejercicio agudo. Sin embargo, la suplementación con DHA, durante un periodo de entrenamiento, proporciona una mayor capacidad antioxidante que protege frente al daño nitrosativo tras un ejercicio agudo en eritrocitos, en las PBMCs disminuye la capacidad mitocondrial de formación de ROS y en los neutrófilos disminuye el tiempo para producir la explosión oxidativa después del ejercicio.

8. Un ejercicio intenso realizado por deportistas bien entrenados induce la producción de óxido nítrico por las células mononucleares de la sangre periférica y por los neutrófilos, que se refleja en un aumento de nitrato intracelular y un aumento paralelo en suero de nitrito y nitrato. Esta mayor producción de óxido nítrico asociada a la realización de ejercicios de alta intensidad y duración, también aumenta la nitración de proteínas de las células mononucleares de la sangre periférica y de eritrocitos. La suplementación mediante 10 mg de nitrato de sodio por kg de masa corporal durante tres días no mejora el rendimiento deportivo en deportistas bien entrenados y no conlleva asociado daño nitrosativo proteico.

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ANEXO

PUESTA A PUNTO Y MEJORA DE UN MÉTODO ENZIMÁTICO Y CROMATOGRÁFICO PARA LA DETERMINACIÓN PLASMÁTICA DE NEFAS Y TGFAS

INTRODUCCIÓN

La determinación de ácidos grasos plasmáticos se ha realizado en diferentes estudios (Barceló-Coblijn, et al., 2008, Bloomer, et al., 2009, Gray, et al., 2012, McAnulty, et al., 2010), pero en estos no se diferencia los ácidos grasos no esterificados (NEFAs) de los ácidos grasos de los triglicéridos (TGFAs) o fosfolípidos, aunque la fracción de ácidos grasos libres en plasma ha tenido interés para valorar la biodisponibilidad plasmática de estos ácidos grasos, diferenciándose de la disponibilidad que representa la fracción de ácidos grasos esterificada (Ottestad, et al., 2012, Sabaté, et al., 2003). En el músculo, los ácidos grasos provenientes de la lipolisis de los triglicéridos almacenados son un combustible principal para la contracción muscular y el mantenimiento de la actividad física (Jeppesen and Kiens, 2012).

Existen pocos métodos específicos, sencillos y rápidos de determinación de los ácidos grasos libres y esterificados en plasma. Uno de los métodos más específicos y rápidos de realizar contempla la determinación simultánea de los NEFA y TGFA combinando la acción específica de la lipoproteína lipasa (LPL), la purificación de los ácidos grasos libres y su cuantificación mediante cromatografía de gases posterior a la metilación de los ácidos grasos (Lladó, et al., 1993). La purificación de los NEFAs se lleva a cabo mediante cromatografía en columna de carbón activo (Borra, et al., 1984). Se purifican los ácidos grasos libres presentes en plasma tratado o no con LPL. Los ácidos grasos procedentes de la muestra no tratada con LPL proporciona los NEFA circulantes en plasma; los ácidos grasos procedentes de la muestra tratada con LPL proporciona la suma de los ácidos grasos libres en plasma y de los esterificados. La derivatización de los ácidos grasos libres, necesaria para su cuantificación por cromatografía de gases se realiza con diazometano, reactivo de difícil

preparación, muy inestable y de uso inmediato tras su obtención; además el procedimiento de derivatización precisa de la obtención del residuo seco de los ácidos grasos dos veces. Las condiciones cromatográficas de separación de los metil derivados de los ácidos grasos proporcionan un perfil de separación de un número escaso de ácidos graso. El cálculo de los factores de recuperación de los ácidos grasos respecto del patrón interno depende de la reacción de derivatización y de las condiciones cromatográficas.

En este trabajo se propone un método para determinar una amplia variedad de NEFAs y TGFAs en plasma. Inicialmente los ácidos grasos libres esterificados a triglicéridos se liberan mediante una digestión enzimática, obteniendo una mezcla de TGFA y NEFAs. Tanto los NEFAs, como la mezcla de TGFA más NEFAs, se purifican mediante una cromatografía de adsorción específica en carbón activo. Seguidamente los ácidos grasos se concentran y se esterifican mediante un proceso cuantitativo, seguro y rápido para su posterior determinación mediante cromatografía de gases.

MATERIALES Y MÉTODOS

Obtención del plasma

Las determinaciones plasmáticas de NEFAs y TGFAs se realizaron en 14 varones deportistas en condiciones basales. Previamente se realizó una valoración nutricional y antropométrica de los participantes para comprobar que los participantes presentaban unas dietas y características antropométricas similares. Todos los participantes accedieron voluntariamente a la participación del estudio tras haber sido debidamente informados del objetivo del estudio y de los posibles riesgos. Se cumplió con los requisitos indicados en la Declaración de Helsinki, y todos los protocolos fueron previamente aprobados por la comisión de ética local. Ninguno de los participantes fumaba, consumía habitualmente alcohol o tomaba medicación que pudiera alterar la respuesta hormonal.

Las muestras sanguíneas fueron obtenidas de las venas antecubitales mediante vacutainers con EDTA (ácido etilendiaminotetraacético) como anticoagulante. El plasma fue obtenido en frio mediante centrifugación (900 x g, 30 min, 4°C). Se centrifugaron dos muestras de sangre para obtener el plasma, a una de ellas, antes de la centrifugación, se le incorporó 2 ul 50 mM de fluoruro de p-toluensulfonilo (TSF) como inhibidor de LPL, siendo ésta la muestra destinada a la cuantificación de los NEFA circulantes.

Determinación de NEFAs y TGFAs de muestras plasmáticas

Para la determinación de los NEFAs se diluían dentro de un tubo pírex previamente lavado con acetona, 100 ul de plasma en 5 mL de metanol:agua (1:1, v:v) que contenían 0.01 % de BHA (hidroxibutilanisol) como antioxidante y 2 uL de C17:0 15 mM como patrón interno disuelto en metanol. Para la determinación de los TGFA se ponían 200 ul de Triglyceride Reagent (SIGMA), que contiene la LPL, dentro de un tubo pírex, previamente lavado con acetona, y se adicionaban 10 uL de plasma (sin inhibidor de la lipasa), se vorteaba y se ponía a 37°C durante 5 minutos para que actuara la lipasa. A continuación se añadían 4 mL de metanol:agua (5:3, v:v) que contenían 0.01% de BHA y 2 uL de C17:0 15 mM. Esta muestra contiene los NEFA y los ácidos grasos que se han hidrolizado de los TGFA.

A continuación los NEFAs y los ácidos grasos TGFA se purificaban mediante una cromatografía por gravedad con 0.25 g de carbón activo, Carbopack™ 60/80 (Supelco), como fase estacionaria dentro de una columna para cromatografía de 15 mL de capacidad y placa filtrante de 10 mm de diámetro. La columna se limpiaba inicialmente pasando consecutivamente 5 mL de cloroformo, 3 mL de metanol y 3 mL de agua destilada. A continuación se pasaba la muestra lentamente para retener los ácidos grasos y demás moléculas apolares. Seguidamente se limpiaba el tubo que había contenido la muestra con 5 mL de HCl 3 mM y se pasaba el volumen por la columna de cromatografía. Consecutivamente se limpiaba la columna con 1.5 mL de metanol. Inmediatamente se eluyeron los ácidos grasos dentro de un tubo pírex previamente limpiado con

acetona, haciendo pasar 10 mL de cloroformo:metanol (1:1, v:v). Finalmente se renovaba la columna pasando consecutivamente 5 mL de cloroformo, 3 mL de metanol y 3 mL de agua destilada. La eficiencia de la purificación de los ácidos grasos libres y las posibles interferencias de ácidos graso de otras fuentes está validada (Lladó, et al., 1993) y corregida por el uso del patrón interno C17:0 desde el inicio del proceso; una posible pérdida se corrige mediante el patrón interno.

La fase orgánica obtenida de la columna de carbón activo se evaporó con una corriente de nitrógeno a 55°C. El residuo seco se resuspendió con 75 uL de n-hexano y 25 uL de reactivo de derivatización Meth-Prep™ II (GRACE), y a continuación se traspasó el volumen dentro de un vial de cromatografía donde se dejó reaccionar a temperatura ambiente un mínimo de 30 minutos antes de su inyección al cromatógrafo de gases.

La cromatografía de gases se realizó inyectando una alícuota de 1 ul y usando un flujo de 2.17 mL/min de helio como fase móvil, medido a 150°C en cabeza de columna. El cromatógrafo de gases fue del modelo Agilent 5890 con un detector de ionización de llama (FID) y una columna Supelcowax® 10 Capillary GC column, 30 m x 0.53 mm, d_f 0.50 um. La curva de temperatura empezaba a 150°C con un gradiente de 4°C/min hasta alcanzar los 260°C para seguidamente mantener una isoterma durante 15 minutos. El inyector estaba a una temperatura de 280°C y el FID a 300°C. Para la identificación de los diferentes picos cromatográficos se utilizaron ácidos grasos individuales, que se derivatizaron siguiendo el mismo procedimiento que las muestras, y mezclas comerciales de esteres metílicos de los ácidos grasos (Supelco). La cuantificación se realizó a partir del patrón interno y la diferente respuesta a la separación cromatográfica y detección en el FID de los diferentes ácidos grasos se corrigió mediante un factor de respuesta. Este factor de respuesta fue calculado a partir de las áreas de los distintos ácidos grasos estándar de diferentes cromatogramas de diferente concentración. Finalmente los TGFAs fueron calculados a partir de la resta entre los valores obtenidos en las dos muestras procesadas: las que llevaban inhibidor de la LPL y las que no lo llevaban y fueron tratadas con LPL.

Cuantificación de los ácidos grasos

La cuantificación de los ácidos grasos se realizó aplicando la siguiente formula:

$$AG_x\ (/M) = \text{Área}\ AG_x \times \frac{Y\ \text{¡}JM}{\text{Área}\ \text{C17:0}}$$

donde AG_x es el ácido graso a cuantificar, Y viene de los 2 uL de C17:0 15 mM y de su relación con el volumen de plasma (para la cuantificación de los NEFAs tiene un valor de 300 y para el de los ácidos grasos totales de 3000) y F_x es el factor de corrección. F_x se calculó mediante el uso de diferentes cromatogramas de gases obtenidos de diferentes concentraciones de una mezcla de ácidos grasos metil éster comercial, que incluían el metil éster del C17:0, con la siguiente formula:

$$\frac{\text{Área}\ \text{C17:0} \times Mw\ AG_x \times \%\ AG_r}{\text{Área}\ AG_x \times Mw\ \text{C17:0} \times \%\text{C17:0}}$$

donde Mw es el peso molecular de los ácidos grasos y el $\%AG_x$ es el porcentaje del ácido graso a cuantificar dentro de la mezcla de ácidos grasos metil ester comercial.

Los valores de F_x se recopilan en la Tabla 1. En la tabla se recogen los F_x que se utilizaron en la determinación plasmática realizada.

Análisis estadístico

El análisis estadístico fue realizado mediante el *Statistical Package for Social Sciences* (SPSS v.15.0 for Windows). Los resultados son expresados como la media ± SEM. La estadística se realizó mediante t-student para comparar las diferencias entre NEFAs y TGFAs, $p < 0.05$ fue considerado estadísticamente significativo. Se comprobó que los datos seguían una distribución normal.

Tabla 1. Factores de corrección.

ᶠC14:0	0.344
ᶠC16:0	0.303
Fci6:1	0.873
ᶠC18:0	0.576
Fci8:1	0.349
Fci8:2	0.568
ᶠC18:3n6	1.157
Fc18:3n3	1.350
ᶠC20:0	0.764
ᶠc20:1	1.20
Fc20:3	0.573
ᶠC20:4n6	1.15
Fc22:0	0.551
Fc22:1	1.38
ᶠC22:6n3	1.90

RESULTADOS

La puesta a punto de la cromatografía de gases se realizó mediante la punción de ácidos grasos individuales metilados con el reactivo Meth-Prep™ II, con mezclas de estos y mediante la utilización de mezclas comerciales de ésteres metílicos de los ácidos grasos. De esta manera se obtuvo un cromatograma en el que por el tiempo de retención se identificaron todos los picos presentes (Figura 1).

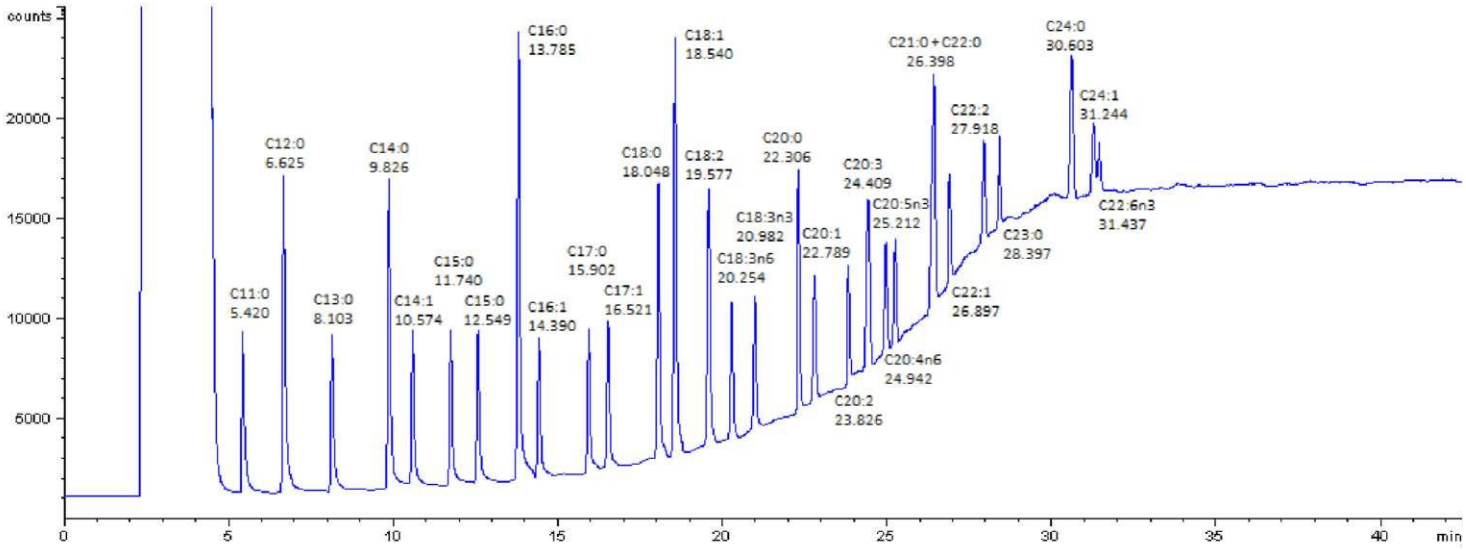

Figura 1. Cromatograma dilución 1:20 (v:v) SUPELCO 37 COMPONENT FAME MIX (10 mg/mL).

En la Tabla 2 se recogen los valores plasmáticos de los NEFAs y TGFAs individuales. Los ácidos grasos circulan en plasma mayoritariamente en forma esterificada, siendo en C18:2 el ácidos graso mayoritario en ambas fracciones; sin embargo se aprecian diferencias en la proporción de algunos ácidos grasos dependiendo de si están en la fracción esterificada o en la libre. El C18:2 es el ácido graso mayoritario en los NEFAs y en los TGFA, le sigue el C18:1, el C16:0, el C18:0, el C20:4n6, el C18:3n6 y el C18:3n3 como mayoritarios en los TGFA mientras que en los NEFA le sigue el 16:0, el C18:0, el C18:1, el C20:4n6, el C18:3n6, siendo el resto minoritarios. La fracción esterificada contiene unas 5.7 veces más ácidos grasos que la fracción libre.

Tabla 2. Composición plasmática de NEFAs y TGFAs en u.M.

	NEFAs (uM)	**TGFAs** (uM)
C14:0	5.98 ± 0.57	19.4 ± 1.8 *
C16:0	137 ± 16	467 ± 30 *
C16:1	3.52 ± 0.41	18.6 ± 1.3 *
C18:0	92.4 ± 5.2	347 ± 26 *
C18:1	66.4 ± 5.8	500 ± 43 *
C18:2	128 ± 12	1,215 ± 87 *
C18:3n6	45.2 ± 8.6	212 ± 78
C18:3n3	3.54 ± 0.43	177 ± 18 *
C20:0	4.85 ± 0.31	3.75 ± 0.90
C20:1	3.73 ± 0.29	6.20 ± 2.05
C20:3	8.59 ± 1.05	25.0 ± 3.1 *
C20:4n6	48.2 ± 5.1	278 ± 31 *
C22:0	1.38 ± 0.23	47.7 ± 5.7 *
C22:1	9.99 ± 0.67	62.7 ± 4.5 *
C22:6n3	11.8 ± 1.5	33.2 ± 8.2
SFA	241 ± 20	883 ± 51 *
MUFA	83.4 ± 6.5	583 ± 45 *
PUFA	245 ± 22	1,755 ± 95 *
Total	570 ± 45	3,221 ± 178 *

SFA ácidos grasos saturados, MUFA ácidos grasos monoinsaturados, PUFA ácidos grasos poliinsaturados. Análisis estadístico: t-student para datos desapareados. (*) Diferencias significativas entre NEFAs y TGFAs, $p < 0.05$.

En la Tabla 3 se observa que los NEFAs tienen un porcentaje similar de SFAs y PUFAs y que estas son 2.8 veces superiores a los MUFAs. En cambio, los TGFA mantienen el porcentaje de MUFAs pero presentan el doble de PUFAs que de SFAs. Los ácidos grasos que no presentan diferencias porcentuales entre NEFAs y TGFAs son el C16:1, el C18:1, el C18:3n6 y el C20:4n6.

Tabla 3. Composición plasmática de NEFAs y TGFAs en porcentaje.

	NEFAs (%)	**TGFAs** (%)
C14:0	1.05 ± 0.05	0.613 ± 0.056 *
C16:0	23.3 ± 1.3	14.5 ± 0.6 *
C16:1	0.613 ± 0.057	0.610 ± 0.035
C18:0	16.9 ± 1.0	10.8 ± 0.6 *
C18:1	11.9 ± 1.0	15.3 ± 0.6
C18:2	22.5 ± 1.2	37.5 ± 1.3 *
C18:3n6	7.56 ± 1.11	7.06 ± 2.70
C18:3n3	0.623 ± 0.070	5.38 ± 0.60 *
C20:0	0.983 ± 0.108	0.113 ± 0.020 *
C20:1	0.683 ± 0.070	0.184 ± 0.061 *
C20:3	1.53 ± 0.16	0.771 ± 0.067 *
C20:4n6	8.38 ± 0.50	8.67 ± 0.69
C22:0	0.238 ± 0.032	1.48 ± 0.16 *
C22:1	1.83 ± 0.11	1.98 ± 0.14
C22:6n3	2.05 ± 0.21	1.00 ± 0.22 *
SFA	42.4 ± 0.8	27.5 ± 0.7 *
MUFA	15.0 ± 1.1	17.9 ± 0.6
PUFA	42.6 ± 1.0	54.6 ± 1.0 *

SFA ácidos grasos saturados, MUFA ácidos grasos monoinsaturados, PUFA ácidos grasos poliinsaturados. Análisis estadístico: t-student para datos desapareados. (*) Diferencias significativas entre NEFAs y TGFAs, $p < 0.05$.

DISCUSIÓN

En el cromatograma obtenido se observa que los ácidos grasos con menor afinidad a la columna cromatográfica utilizada son los de menor tamaño y a medida que aumenta el número de insaturaciones de los diferentes ácidos grasos provoca que estos tengan mayor tiempo de retención. Hay una serie de ácidos grasos que presentan picos en el cromatograma pero que no aparecen en los resultados presentados. Los ácidos grasos de cadena impar no son sintetizados por el organismo, aunque se pueden metabolizar, por tanto difícilmente se pueden encontrar en el plasma y por eso se utiliza el C17:0 como patrón interno. Los otros ácidos grasos que se podrían identificar y no se han cuantificado debido a que no aparecían en el cromatograma realizado con las condiciones utilizadas son: C12:0, C14:1, C20:2, C20:5, C22:2, C24:0 y C24:1. Si es necesaria su cuantificación se puede partir de mayor cantidad de plasma, disminuir el volumen de resuspensión del residuo seco de ácidos grasos y/o aumentar el tamaño de alícuota pinchada en el cromatógrafo de gases, intentando evitar afectar a la vida útil de la columna cromatográfica y al FID.

Los resultados obtenidos son difícilmente comparables a la bibliografía, ya que la mayoría da los resultados se dan en forma de porcentajes y estos varían dependiendo de la cantidad de ácidos grasos determinados (Barceló-Coblijn, et al., 2008, Bloomer, et al., 2009, Gray, et al., 2012, McAnulty, et al., 2010, Ottestad, et al., 2012, Sabaté, et al., 2003). Gray y colaboradores indican un contenido plasmático de DHA como NEFA entre un 1 y un 2% (Gray, et al., 2012), similar al valor obtenido (2.05 ± 0.21 %). En cambio, Sabaté y colaboradores indican un contenido plasmático de TGFA de 33.2 ± 0.7 % SFA, 32.7 ± 0.4 % MUFA, y los PUFAs están representados por dos ácidos grasos, 21.7 ± 0.1 % C18:2 y 1.63 ± 0.04 % C18:3n3 (Sabaté, et al., 2003). Estos valores son diferentes a los obtenidos, la fracción PUFA es mayoritaria en nuestros datos, pero hay que incidir que nuestra metodología determina 4 PUFAs adicionales, entre ellos el C20:4n6 como PUFA mayoritario. Por otra parte, la composición plasmática de los ácidos grasos depende de la dieta, la ingesta de alimentos ricos en un ácido graso concreto, como el DHA, aumenta

los niveles sanguíneos de este (Bloomer, et al., 2009, Ottestad, et al., 2012, Tur, et al., 2012).

Los valores de los factores de corrección de los diferentes ácidos grasos indican que estos tienen diferente señal según su naturaleza química, un mol de un ácido graso da diferente valor de área cromatográfica que un mol de otro ácido graso. Se observa que los valores de F_x tienden a aumentar al presentar más insaturaciones, indicando que a más insaturaciones el FID detecta menos señal. El método puesto a punto por Lladó y colaboradores tiene en cuenta este factor de corrección. En cambio, el uso de este factor de corrección no se realiza en otras metodologías (Barceló-Coblijn, et al., 2008, Bloomer, et al., 2009, Gray, et al., 2012, Sabaté, et al., 2003), lo que indica que podrían infravalorar la cantidad de PUFAs que realmente hay en las muestras.

En comparación al método de referencia (Lladó, et al., 1993) nuestro método puede diferenciar los omega-3 y omega-6 del ácido graso C18:3, además de poder cuantificar adicionalmente los ácidos grasos C20:1, C22:0 y C22:6n3. Además, el método de derivatización presenta mejoras respecto al uso de diazometano (Lladó, et al., 1993). Es más rápido, de un solo paso, se realiza a temperatura ambiente y no requiere de una extracción anterior a la inyección al cromatógrafo de gases.

Los triglicéridos son una fuente importante de ácidos grasos (Hakimi, et al., 2005, Xie, et al., 2010). Los valores plasmáticos obtenidos de los TGFAs son unas 5.7 veces superiores a los NEFAs. Los triglicéridos son una importante reserva energética (Lagarde, 2003) y hemos observado que los ácidos grasos esterificados a triglicéridos son una importante reserva de PUFAs ya que más de la mitad de TGFAs son poliinsaturados. En cambio, en los NEFAs plasmáticos la relación entre SFA y PUFAs es similar.

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(Human being to the mob
(What's a mob to a king?
(What's a king to a god?
(What's a god to a non-believer?
(Who don t believe in anything?

Kanye West & Jay-Z (feat Frank Ocean)
Watch the Throne
No Church in the Wild