



UNIVERSITAT<sub>DE</sub>  
BARCELONA

## Efectes immunomoduladors de l'exercici físic intens i extenuant i de l'hesperidina en rates

Seila Estruel Amades



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**Facultat de Farmàcia i Ciències de l'Alimentació**

**EFFECTES IMMUNOMODULADORS DE  
L'EXERCICI FÍSIC INTENS I EXTENUANT  
I DE L'HESPERIDINA EN RATES**

**Seila Estruel Amades**  
Barcelona, 2019





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Facultat de Farmàcia i Ciències de l'Alimentació  
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## EFFECTES IMMUNOMODULADORS DE L'EXERCICI FÍSIC INTENS I EXTENUANT I DE L'HESPERIDINA EN RATES

Memòria presentada per **SEILA ESTRUEL AMADES** per optar al títol de doctor per la Universitat de Barcelona.

Aquesta tesi ha estat realitzada al grup d'*Autoimmunitat i Tolerància* sota la supervisió de la Dra. **Margarida Castell Escuer** i la Dra. **Mariona Camps i Bossacoma**. La doctoranda ha realitzat una estada de recerca a l'Escola d'Esport, Exercici i Ciències de la Salut (Dra. Nicolette Bishop) de la Universitat de Loughborough.

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#### INFORMEN

Que la memòria titulada ***Efectes immunomoduladors de l'exercici físic intens i extenuant i de l'hesperidina en rates*** presentada per **SEILA ESTRUEL AMADES** per optar al Títol de Doctor per la Universitat de Barcelona, ha estat realitzada sota la nostra direcció a la Secció de Fisiologia, i considerant-la conclosa, autoritzem la seva presentació per ser jutjada pel tribunal corresponent.

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**Dra. Mariona Camps i Bossacoma**



***“The more I learn, the more I realize how much I don't know”***

Albert Einstein





ÍNDEx





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## ABREVIATURES



|                                   |                                                                      |
|-----------------------------------|----------------------------------------------------------------------|
| <b>ACTH</b>                       | Hormona adrenocorticotròpica                                         |
| <b>ADP</b>                        | Adenosina difosfat                                                   |
| <b>APC</b>                        | Cèl·lules presentadores d'antigen                                    |
| <b>ATP</b>                        | Adenosina trifosfat                                                  |
| <b>BCR</b>                        | Receptor de cèl·lules B                                              |
| <b>ConA</b>                       | Concanavalina A                                                      |
| <b>CRH</b>                        | Hormona alliberadora de corticotropina                               |
| <b>DN</b>                         | <i>Double negative</i>                                               |
| <b>DP</b>                         | <i>Double positive</i>                                               |
| <b>GH</b>                         | Hormona del creixement                                               |
| <b>GPx</b>                        | Glutatió peroxidasa                                                  |
| <b>GRd</b>                        | Glutatió reductasa                                                   |
| <b>GSH</b>                        | Glutatió reduït                                                      |
| <b>GSSH</b>                       | Glutatió oxidat                                                      |
| <b>IFN</b>                        | Interferó                                                            |
| <b>Ig</b>                         | Immunoglobulina                                                      |
| <b>IL</b>                         | Interleucina                                                         |
| <b>LPS</b>                        | Lipopolisacàrid                                                      |
| <b>MAPK</b>                       | Proteïna quinasa activada per mitogens                               |
| <b>MCP</b>                        | Proteïna quimiotàctica de monòcits                                   |
| <b>MHC</b>                        | Complex major d'histocompatibilitat                                  |
| <b>NADPH</b>                      | Nicotinamida Adenina Dinucleòtid Fosfat                              |
| <b>NFκB</b>                       | Factor nuclear κ B                                                   |
| <b>NK</b>                         | <i>Natural killer</i>                                                |
| <b>NKT</b>                        | Cèl·lules T <i>natural killer</i>                                    |
| <b>NOS</b>                        | Òxid nítric sintasa                                                  |
| <b>O<sub>2</sub><sup>·-</sup></b> | Radical superòxid                                                    |
| <b>OH<sup>·</sup></b>             | Radical hidroxil                                                     |
| <b>OMS</b>                        | Organització Mundial de la Salut                                     |
| <b>OTS</b>                        | <i>Overtraining syndrome</i>                                         |
| <b>OVA</b>                        | Ovoalbúmina                                                          |
| <b>PCR</b>                        | Proteïna C Reactiva                                                  |
| <b>PGC-1α</b>                     | <i>Peroxisome proliferator-activated receptor γ co-activator 1 α</i> |

|                          |                                                 |
|--------------------------|-------------------------------------------------|
| <b>PWM</b>               | Mitogen pokeweed                                |
| <b>ROS</b>               | Espècies reactives d'oxigen                     |
| <b>SOD</b>               | Superòxid dismutasa                             |
| <b>SP</b>                | <i>Single positive</i>                          |
| <b>Tc</b>                | Limfòcit T citotòxic                            |
| <b>TCR</b>               | Receptor de cèl·lules T                         |
| <b>TGF</b>               | <i>Transforming growth factor</i>               |
| <b>Th</b>                | Limfòcit T helper                               |
| <b>TLR</b>               | <i>Toll-like receptor</i>                       |
| <b>TNF</b>               | <i>Tumour necrosis factor</i>                   |
| <b>Treg</b>              | Cèl·lules T reguladores                         |
| <b>URTI</b>              | Infeccions del tracte respiratori superior      |
| <b>VO<sub>2</sub></b>    | Consum d'oxigen                                 |
| <b>VO<sub>2</sub>màx</b> | Consum d'oxigen màxim                           |
| <b>PAMP</b>              | <i>Pathogen-associated molecular patterns</i>   |
| <b>KIR</b>               | <i>Killer-cell immunoglobulin-like receptor</i> |



ABSTRACT





The influence of intensive and exhausting exercise on the innate and adaptive immune system is gaining attention due to its association with harmful effects on the functionality of the immune system and the high number of people participating in intense sport competitions. Nevertheless, diet containing bioactive compounds such as polyphenols can play a key role in preventing or delaying these negative effects.

Based on these factors, the aim of this thesis was to study in depth the influence of both the intensive and exhausting exercise and the polyphenol hesperidin on the rat immune system.

In order to achieve the initial part of the study's goal, four-week-old Wistar rats underwent a short (two weeks, which included two daily trainings plus a final exhaustion test) or a longer (five weeks, which included two exhaustion tests and three regular trainings per week plus a final exhaustion test) intensive exercise training. Afterwards, different variables of innate and adaptive immune response were determined.

With regard to the innate immune system, results show that blood monocytes collected after the five-week intensive training exhibited a lower phagocytic activity, and the ability of peritoneal macrophages to secrete pro-inflammatory cytokines decreased. These changes were overcome in the recovery period. In addition, the longer training reduced the spleen NK cell proportion but increased their cytotoxic activity. Considering the adaptive immune system, the longer training decreased T-cell proliferative capacity and the secretion of interferon  $\gamma$ . However, the longer training increased the ability to synthesize immunoglobulin G, which was not observed after the final exhaustion exercise.

To accomplish the second part of the objective, three-week-old Lewis rats were orally administrated 100 or 200 mg/kg hesperidin three times per week for four weeks, and then caecal microbiota as well as indicators of intestinal immune system were analysed. Results show that 200 mg/kg of hesperidin increased the total bacteria number in the caecum, due to, among others, an increase in the *Lactobacillus* group. Hesperidin also enhanced the number of bacteria coated with IgA in the caecum content and the amount of intestinal IgA.

Finally, the impact of hesperidin on the oxidative stress induced by the longer exercise training was assessed. For that purpose, the rats trained

for five weeks were orally administrated with 200 mg/kg of hesperidin (three days/week). Hesperidin improved exercise performance during the training and prevented the increase in the reactive oxygen species induced by the final exhaustion test. It also inhibited the decrease in superoxide dismutase and catalase activities in the thymus and spleen derived from the final exhaustion test.

In conclusion, the applied longer exercise training in rats induced alterations in the innate and acquired immune system. Moreover, hesperidin was able to prevent oxidative stress induced by exercise, while improving exercise performance.



## INTRODUCCIÓ



## 1. Exercici físic

Segons l'Organització Mundial de la Salut (OMS), l'activitat física és qualsevol moviment corporal produït pels músculs esquelètics i que requereix despesa d'energia [1]. Quan l'activitat física és planificada, estructurada i repetitiva amb l'objectiu de millorar o mantenir la forma física es parla d'exercici físic. La pràctica regular d'exercici físic és molt important per al manteniment de la salut i s'ha establert que la inactivitat física és el quart factor de risc de mortalitat a nivell mundial [1]. L'exercici físic redueix el risc a patir diverses malalties, com per exemple el càncer de mama i de còlon (entre un 20-40%) i la diabetis (entre un 35 i 50%) (revisat a [2,3]). Per això, l'OMS ha elaborat una sèrie de recomanacions on proporciona orientació sobre la freqüència, durada i intensitat, així com el tipus i quantitat d'exercici segons l'edat de la persona [1].

### 1.1 Múscul esquelètic i contracció muscular

Entre les funcions del múscul esquelètic hi ha el manteniment de la postura i la generació del moviment, funcions que realitza gràcies a la seva capacitat de contracció i la transformació d'energia química en energia mecànica [4]. A més, el múscul esquelètic contribueix al metabolisme energètic. En resposta a l'activitat física i a l'exercici, tant moderat com intens, el múscul esquelètic produeix i allibera interleucina (IL) 6, també anomenada miocina pel seu origen muscular. La formació d'IL-6 s'ha associat amb l'estimulació del creixement muscular hipertròfic i la miogènesi, així com amb la regulació del metabolisme energètic [5].

El múscul està format per centenars o milers de fibres musculars o miòcits, disposades en paral·lel entre sí i organitzades en feixos. Cada fibra muscular està envoltada per l'endomisi, una formació de teixit conjuntiu, i a sota hi ha la membrana plasmàtica o sarcolemma i el citoplasma o sarcoplasma. A l'interior de la fibra muscular hi ha milers de miofibril·les

formades per filaments d'actina i miosina juntament amb troponina i tropomiosina, proteïnes que permeten la contracció muscular. El conjunt ordenat dels filaments d'actina i miosina conformen el sarcòmer [6,7].

La contracció muscular s'inicia quan un impuls nerviós arriba a les terminacions de la neurona motora i provoca l'alliberament d'acetilcolina que s'uneix a receptors del sarcolemma. Quan la quantitat d'acetilcolina és suficient, es produeix l'entrada de sodi a la fibra muscular i, en conseqüència, la seva despolarització. L'impuls viatja pels túbuls T de la fibra i provoca l'alliberament de grans quantitats de calci des del reticle sarcoplasmàtic [7,8].

La contracció muscular es produeix quan els filaments d'actina i de miosina es desplacen un al llarg de l'altre. Quan el múscul està en repòs, la concentració de calci en el sarcoplasma és baixa i la tropomiosina de les miofibril·les bloqueja els punts actius dels filaments d'actina i impedeix la seva unió amb la miosina. Quan s'allibera calci, aquest s'uneix a la troponina, i la tropomiosina es desplaça exposant els llocs actius d'unió de l'actina, el que permet la interacció dels caps de la miosina amb els filaments d'actina. Així es forma un pont creuat entre actina i miosina i, immediatament, ambdues proteïnes experimenten un canvi conformacional, el que fa que el cap de la miosina s'inclini cap al braç de l'actina, es desplaci cap a la seva posició original i s'uneixi al següent punt actiu del filament d'actina [8].

El procés de contracció muscular requereix energia que s'obté a través de la hidròlisi de l'adenosina trifosfat (ATP). Concretament, el cap de la miosina, amb activitat ATPasa, degrada l'ATP en adenosina difosfat (ADP) i fosfat i allibera energia que s'utilitza per a la unió del cap de la miosina amb el filament d'actina. La contracció muscular continua fins que el calci s'esgota. En aquest moment, el calci és bombejat cap al reticle sarcoplasmàtic, mitjançant un procés que requereix energia, on s'emmagatzema fins que arriba un nou impuls nerviós a la fibra muscular. Quan el calci s'elimina, la troponina i la tropomiosina es desactiven, es bloquegen els enllaços creuats de la miosina i els filaments d'actina, i el múscul es relaxa [8].

## 1.2 Exercici aeròbic i anaeròbic

L'energia necessària per a la contracció muscular es pot obtenir de forma aeròbica o anaeròbica. Els exercicis aeròbics permeten una activitat d'intensitat baixa o moderada i de llarga durada, com per exemple caminar, córrer, ballar o anar en bicicleta. Per altra banda, l'exercici anaeròbic permet realitzar exercicis d'elevada intensitat i de curta durada, com són les curses de velocitat [6].

Per a la contracció muscular, el múscul ha de disposar d'una quantitat suficient d'ATP que s'obté a partir del metabolisme de la glucosa. En exercicis de baixa intensitat, s'utilitza el metabolisme aeròbic de la glucosa. A mesura que la intensitat de l'exercici incrementa, l'aportació d'oxigen als músculs resulta insuficient, es passa a metabolisme anaeròbic i això genera àcid làctic i, conseqüentment, acidosi metabòlica [9,10]. El moment en què la concentració d'àcid làctic es dispara degut al canvi del metabolisme aeròbic a anaeròbic s'anomena llindar anaeròbic. L'increment de la concentració sanguínia d'àcid làctic comporta la disminució del pH intramuscular, factor que provoca un augment de la fatiga i disminució del rendiment esportiu. Per això, el llindar anaeròbic d'un atleta permet avaluar la seva condició física, dissenyar un programa d'entrenament adequat i avaluar la seva resistència [11–13].

## 1.3 Canvis fisiològics en resposta a l'exercici físic

La realització d'exercici físic comporta canvis no només al sistema muscular, sinó també canvis hormonals, cardiovasculars, hematològics i respiratoris. A més a més, la realització d'exercici físic també influeix sobre la funcionalitat del sistema immunitari (detallat a l'apartat 3).

### 1.3.1 Canvis al múscul esquelètic

La composició de les fibres musculars s'adapta al tipus d'entrenament físic que realitza l'esportista. Un atleta entrenat mitjançant proves de velocitat presenta una gran proporció de fibres ràpides o de tipus II, i un atleta



preparat per proves de resistència presenta un percentatge superior de fibres lentes o de tipus I. Els exercicis de resistència també indueixen un augment de l'activitat d'enzims de la cadena de transport d'electrons mitocondrials i de la concentració de proteïnes mitocondrials, fet que incrementa la capacitat oxidativa de les fibres i genera un múscul més resistent a la fatiga [6].

A més a més, el múscul esquelètic pot experimentar altres modificacions per tal d'incrementar o millorar la generació de força, velocitat i resistència a la fatiga. En aquest sentit, es pot desenvolupar hipertròfia muscular en resposta a l'entrenament, amb un increment del nombre de miofibril·les [14]. A més, la irrigació capil·lar al voltant del múscul entrenat pot incrementar un 20-30%, per afavorir l'intercanvi de gasos i nutrients entre la sang i el múscul actiu (revisat a [15]).

### 1.3.2 Canvis hormonal

La pràctica d'exercici físic comporta una sèrie de canvis i adaptacions hormonals que afecten, sobretot, la secreció de catecolamines i cortisol (**Figura 1**), i també de l'hormona de creixement i la testosterona.

Les hormones secretades per la glàndula adrenal, tant per part de l'escorça com de la medul·la, tenen una gran importància en el procés d'adaptació a l'exercici. L'escorça adrenal conté una zona glomerulosa (més externa), on se sintetitza aldosterona, una zona fascicular (més extensa), on se sintetitza cortisol, i una zona reticular (més interna), on se sintetitzen les hormones sexuals suprarenals [16]. La medul·la adrenal, constituïda per cèl·lules cromafines, és la responsable de la secreció de les catecolamines adrenalina i noradrenalina. L'exercici físic estimula el sistema nerviós simpàtic i l'estimulació simpàtica activa la secreció d'adrenalina de la medul·la adrenal [7]. Les catecolamines alliberades actuen mitjançant la interacció amb els receptors adrenèrgics, els quals poden ser  $\alpha_1$  (amb diferents subtipus),  $\alpha_2$  (amb diferents subtipus),  $\beta_1$ ,  $\beta_2$  o  $\beta_3$  [17].

En funció del receptor amb el que interaccionin, la funció de les catecolamines varia (**Figura 1**). La unió a receptors  $\alpha_1$  i  $\alpha_2$  indueix, entre d'altres efectes, vasoconstricció. La unió amb receptors  $\beta_1$  és important sobre el cor, on augmenta la força i freqüència de contracció. La interacció de catecolamines sobre receptors  $\beta_2$  indueix vasodilatació del múscul esquelètic, entre d'altres efectes i sobre receptors  $\beta_3$  situats únicament al teixit adipós, potencia la lipòlisi [18]. Les catecolamines, a través sobretot de receptors  $\beta_2$ -adrenèrgics, regulen moltes funcions de la melsa, com la producció de citocines [19–21], canvis en la seva composició [22,23] i l'apoptosi [24] i la fagocitosi [25] de les cèl·lules que la conformen.

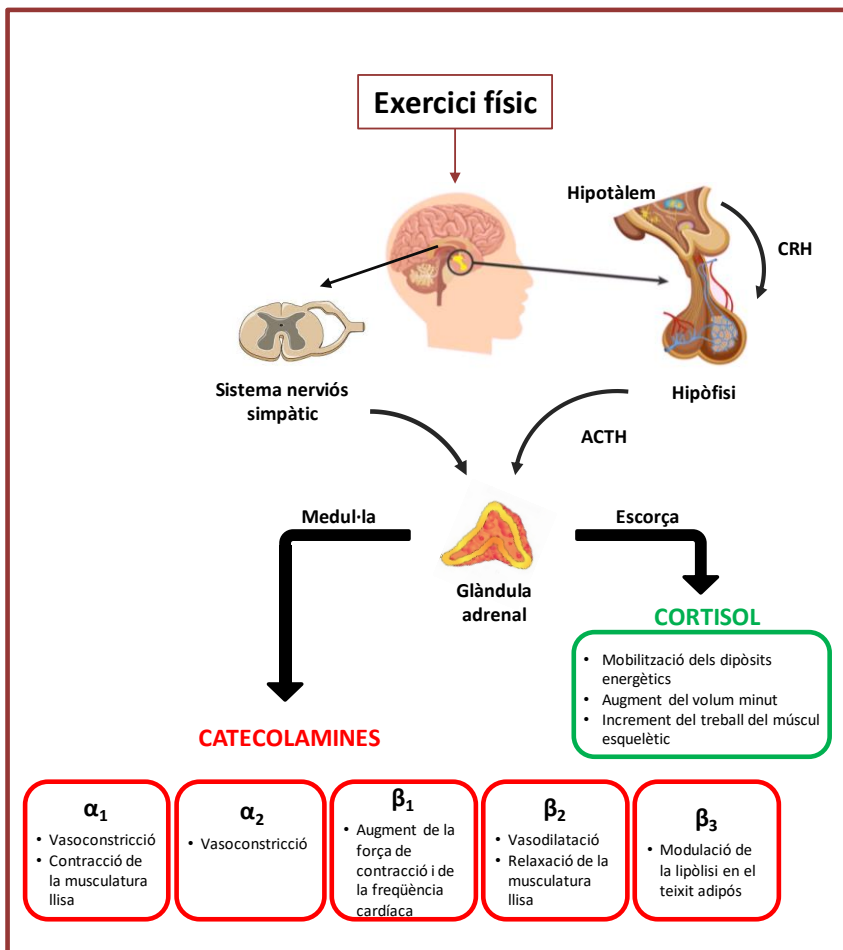
*Figura 1. Respostes hormonals a l'exercici físic*

ACTH: hormona adrenocorticotròpica, CRH: hormona alliberadora de corticotropina

Imatges extretes de:

<https://www.meditip.lat/el-cuerpo-humano/funcion-de-las-hormonas/>

<https://www.pictoeduca.com/leccion/196/el-cerebro/pag/1410>



Els limfòcits presenten receptors adrenèrgics sobretot de tipus  $\beta_2$  [26]. La senyalització  $\beta$ -adrenèrgica en limfòcits pot provocar, entre d'altres efectes, la inhibició de l'activació de les cèl·lules T CD8+ a través de la supressió de l'expressió d'un transportador de glucosa [27]. També s'ha demostrat que l'estimulació  $\beta_2$ -adrenèrgica indueix la mobilització de les cèl·lules T CD8+ i les cèl·lules *natural killer* (NK) [28], i sobre les cèl·lules dendrítiques disminueix l'activitat proliferativa de les cèl·lules T CD4+, però en manté la secreció d'IL-10 [29]. A més, en un model d'asma, l'activació d'aquest subtipus de receptor és capaç de modificar la funció suprimida de les cèl·lules T reguladores (Treg) després d'un exercici aeròbic [30].

Per altra banda, en resposta a una situació d'estrès, com pot ser l'exercici físic, se secreta cortisol. La secreció d'aquesta hormona està regulada per l'eix hipotalàmic – hipofisiari – adrenal. En resposta a una situació d'estrès, l'hipotàlem secreta l'hormona alliberadora de corticotropina (CRH) que desencadena la secreció de l'hormona adrenocorticotròpica (ACTH) per part de l'adenohipòfisi. L'increment d'aquesta hormona activa els receptors adrenocorticotròpics de l'escorça adrenal i estimula la secreció de glucocorticoides com el cortisol [31]. La secreció de cortisol presenta un ritme circadiari, de forma que la seva concentració és més elevada a primera hora del matí. En situacions d'estrès, el cortisol augmenta la disponibilitat de substrats metabòlics i prepara a l'organisme per estar completament actiu [32]. La secreció de glucocorticoides pot incrementar fins a 10 vegades en situacions d'estrès, el que afavoreix la contractilitat cardíaca i, conseqüentment, augmenta el volum minut, la capacitat de mobilitzar depòsits d'energia i la capacitat de treball del múscul esquelètic, entre d'altres (**Figura 1**) [6].

L'hormona de creixement (GH) és secretada per la hipòfisi i, a part de la seva funció en la regulació del creixement, presenta altres funcions fisiològiques, com la regulació de la síntesi proteica i del metabolisme de carbohidrats i lípids [33]. Un dels estímuls de la secreció de GH és l'exercici físic i la seva activitat s'ha associat a hipertròfia muscular. Encara que no es coneix el mecanisme exacte pel qual l'exercici incrementa la producció

de GH, s'ha postulat que intervenen les catecolamines, l'òxid nítric i l'àcid làctic [33].

Una altra hormona alliberada durant l'exercici físic és la testosterona. Aquesta hormona és secretada pels testicles (i, en petita quantitat, pels ovaris) i per les glàndules adrenals. La testosterona és la principal responsable del desenvolupament de les característiques sexuals secundaries masculines i de l'espermatogènesi i, a més, és essencial per al creixement, desenvolupament i maduració de l'esquelet masculí [4]. Durant la pràctica d'exercici físic, la testosterona estimula la retenció de proteïnes musculars i la hipertrofia muscular que es produeix durant l'entrenament de força [4]. Durant la pràctica d'exercici físic, la testosterona estimula la retenció de proteïnes musculars i la hipertrofia muscular que es produeix durant l'entrenament de força [4].

### **1.3.3 Canvis cardiovasculars i sanguinis**

Durant l'exercici, el sistema cardiovascular és important per subministrar nutrients i oxigen a les cèl·lules, eliminar els productes del metabolisme i contribuir en els mecanismes de termoregulació.

Amb la realització l'exercici físic i l'estimulació simpàtica s'incrementa la freqüència i la força de contracció del cor per tal d'augmentar el transport de sang oxigenada cap als teixits [6]. En resposta a l'increment crònic de la força de contracció, el múscul cardíac experimenta hipertròfia, amb un augment del gruix de la paret del ventricle esquerre [4]. A més, l'alliberament de catecolamines durant l'exercici físic provoca vasodilatació dels capil·lars en els músculs esquelètic i muscular i vasoconstricció en la circulació esplàncica [4].

La realització d'exercici físic també comporta canvis a la sang. En resposta a un exercici agut i intens, es produeix una transferència casi immediata de plasma cap a l'espai intersticial, fet que genera hemoconcentració i, per tant, augment de l'hematòcrit i de la concentració d'hemoglobina. Aquest

increment resulta beneficiós ja que augmenta la capacitat de transport d'oxigen cap als teixits [4]. En una segona fase, després de poques hores i en funció de la quantitat i intensitat de l'exercici físic, es produeix hemodilució [34].

#### 1.3.4 Canvis respiratoris

Amb la pràctica d'exercici físic, l'organisme realitza canvis respiratoris per tal de mantenir l'intercanvi de gasos i regular el pH de la sang. L'exercici indueix un increment de la força i velocitat de contracció dels músculs respiratoris, pel que incrementa el volum corrent, la capacitat inspiratòria i la freqüència respiratòria [6].

El consum d'oxigen ( $VO_2$ ) permet mesurar els canvis respiratoris que resulten de la pràctica de l'exercici. El  $VO_2$  és directament proporcional a la intensitat de l'exercici (a més intensitat,  $VO_2$  superior), fins a un cert límit. Davant d'un exercici d'alta intensitat amb una demanda creixent d'energia, l'organisme arriba a un líndar conegut com a volum màxim d'oxigen ( $VO_{2màx}$ ) [6]. El  $VO_{2màx}$  fa referència a la quantitat màxima d'oxigen que l'organisme pot absorbir, transportar i consumir per unitat de temps. En aquest moment, tot i que la intensitat de l'exercici continuï incrementant, el  $VO_2$  es manté constant. Quan un atleta arriba a aquest punt, hi ha pèrdua de l'eficàcia mecànica, augmenta la concentració d'àcid làctic i incrementa la temperatura corporal [6].

### 1.4 **Extralimitació i sobreentrenament**

La pràctica excessiva d'exercici físic pot comportar extralimitació (*overreaching*) i la síndrome del sobreentrenament (*overtraining syndrome*, OTS). Després d'un exercici físic cal un temps de recuperació. L'extralimitació és un estat de l'organisme que es produeix quan la recuperació després d'un exercici físic, sobretot intens, no és suficient [35]. Es considera que hi ha extralimitació funcional quan es produeix durant un període curt de temps on hi ha cert grau de fatiga amb petites

disminucions temporals del rendiment. Es considera que l'extralimitació és no funcional quan hi ha un desequilibri entre l'entrenament i els períodes de recuperació i es produeix una disminució perllongada del rendiment esportiu (setmanes i mesos) [35–37]. L'OTS comprèn un període llarg de temps en el qual hi ha una reducció del rendiment esportiu, juntament amb acumulació d'estrès, molèsties musculars, sensació de dolor o alteracions psicològiques. La OTS requereix mesos de recuperació [35,36].

## 2. El sistema immunitari

El sistema immunitari inclou un conjunt de cèl·lules i teixits responsables de portar a terme processos fisiològics que comporten una resposta defensiva a agents externs o antígens per tal d'eliminar-los i mantenir l'homeòstasi de l'organisme. Aquesta protecció es pot produir de forma inespecífica (immunitat innata) o de forma específica (immunitat adaptativa), si bé ambdues formes de resposta estan estretament relacionades [38].

La immunitat innata inclou mecanismes que actuen immediatament davant un agent estrany, sense implicar el reconeixement específic a l'antigen. Es caracteritza per ser una resposta ràpida, que no té memòria immunològica i que, per tant, no pot reconèixer el mateix antigen en futures exposicions. En canvi, la immunitat adaptativa és un tipus de defensa antigen-dependent i específica, que presenta memòria immunològica i dona lloc a una resposta més eficient enfront a un antigen prèviament exposat [38,39].

### 2.1 Immunitat innata

La funció principal de la resposta immunitària innata és el reclutament de cèl·lules immunitàries en el lloc de l'agressió per tal d'alliberar mediadors que ajudin a eliminar els antígens.

La resposta innata inclou quatre categories de barreres defensives [39]:

- **Anatòmica:** formada per la pell i les membranes mucoses, que conformen la barrera epitelial.
- **Fisiològica:** inclou temperatura (la febre inhibeix el creixement d'alguns patògens), pH àcid i mediadors químics com lisozim, interferó (IFN) i el sistema del complement.



- **Fagocitosi i endocitosi:** monòcits, macròfags i granulòcits fagociten organismes sencers i els destrueixen; altres tipus cel·lulars també poden endocitar molècules externes i digerir-les.
- **Inflamatòria:** una infecció o un dany tissular indueix canvis a la circulació local amb extravasació de proteïnes sèriques, citocines i quimiocines, que comporten el reclutament de cèl·lules fagocítiques al lloc afectat.

Les cèl·lules implicades en la resposta immunitària innata són els monòcits i els macròfags, els granulòcits, les cèl·lules NK, les cèl·lules dendrítiques i els mastòcits.

### 2.1.1 Monòcits, macròfags i fagocitosi

Els monòcits es formen a la medul·la òssia, passen a sang on tenen una vida mitjana d'entre hores i dies, i es distribueixen a diferents teixits, on es diferencien en cèl·lules dendrítiques o macròfags. Els monòcits i els macròfags expressen *toll-like receptors* (TLR), que són glicoproteïnes transmembrana capaces de reconèixer estructures moleculars conservades dels patògens (*pathogen-associated molecular patterns*, PAMP). En funció del seu fenotip, els monòcits es poden classificar en clàssics i no clàssics. Els monòcits clàssics, amb un fenotip CD14+CD16-, representen el 80% dels monòcits i es caracteritzen per secretar més citocines proinflamatòries i per tenir més activitat fagocítica i produir més espècies reactives d'oxigen (ROS) que les formes no clàssiques. Els monòcits no clàssics, amb un fenotip CD14+CD16+, presenten una expressió del complex major d'histocompatibilitat (MHC) de classe II superior a les formes clàssiques [40].

Els macròfags, amb una vida mitjana de mesos o anys, tenen un paper clau en la immunitat innata per la seva capacitat de fagocitar i sintetitzar citocines que activen altres cèl·lules inflamatòries. També intervenen en la immunitat adaptativa ja que actuen com a cèl·lules presentadores d'antigen (APC) [39,41]. Els macròfags activats, segons els estímuls que rebin, poden ser M1 o M2 [42] (**Taula 1**). IFN- $\gamma$  i lipopolisacàrid (LPS) estimulen la diferenciació de macròfags M1, mentre IL-4 i IL-13 promouen l'activació dels macròfags M2 [43]. Els macròfags M1 alliberen citocines

com IL-1 $\beta$ , IL-12, IL-23 i factor de necrosi tumoral (*tumour necrosis factor*, TNF)- $\alpha$ , estimulen la resposta limfocítica de tipus Th1 i presenten propietats proinflamàtòries i microbicides. Les cèl·lules M2, que poden ser de diferents subtipus, produeixen sobretot factor de creixement transformant (*transforming growth factor*, TGF)- $\beta$  i IL-10, el que incrementa la capacitat fagocítica i atenua la resposta inflamatòria, a més d'augmentar la resposta Th2 [43].

*Taula 1. Polarització de macròfags activats, citocines secretades i principals funcions*

IFN: interferó, IL: interleucina, LPS: lipopolisacàrid, ROS: espècies reactives d'oxigen, TLR: *toll-like receptor*, TGF: *transforming growth factor*, Th:

limfòcit T helper, TNF: factor de necrosi tumoral

*Basat en [41]*

| Fenotip    | Estímul              | Citocines secretades                             | Funció                                                                                                                         |
|------------|----------------------|--------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------|
| <b>M1</b>  | IFN- $\gamma$ , LPS  | IL-1 $\beta$ , IL-6, IL-12, IL-23, TNF- $\alpha$ | Activitat antimicrobiana<br>Activitat antitumoral<br>Activitat proinflamatòria<br>Producció de ROS<br>Estimulació resposta Th1 |
| <b>M2a</b> | IL-4, IL-13          | TGF- $\beta$ , IL-10                             | Activitat antiinflamatòria                                                                                                     |
| <b>M2b</b> | IL-1 $\beta$         | TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10       | Estimulació resposta Th2                                                                                                       |
| <b>M2c</b> | TGF- $\beta$ , IL-10 | TGF- $\beta$ , IL-10                             | Activitat fagocítica                                                                                                           |
| <b>M2d</b> | Lligands de TLR      | IL-10                                            | Angiogènesi                                                                                                                    |

Una de les principals funcions efectores de monòcits i macròfags és la fagocitosi, procés que s'origina quan un microorganisme és reconegut pels receptors de membrana de les cèl·lules fagocítiques. Aleshores es produeix la ingestió del patògen i es forma una vesícula intracel·lular, el fagosoma. Un cop el fagosoma ha madurat i té un pH àcid, es fusiona amb lisosomes i forma el fagolisosoma que permet la destrucció de l'antigen a través dels enzims lisosomals i digestius.

L'activació de monòcits i macròfags indueix la producció de ROS amb una gran capacitat oxidativa, el que ajuda a eliminar els antígens fagocitats [38,44]. Els macròfags també produeixen espècies reactives de nitrogen com l'òxid nítric que, en el fagolisosoma, es poden combinar amb ROS i generar radicals peroxinitrit, amb característiques molt reactives i capaçes de destruir patògens [38,45].

### 2.1.2 Granulòcits

Els granulòcits, originats també a la medul·la òssia, inclouen basòfils, eosinòfils i neutròfils. Els granulòcits contenen grànuls amb substàncies químiques capaces d'eliminar agents infecciosos. Els basòfils i els eosinòfils actuen a través de la seva desgranulació i alliberació d'enzims i, en el cas dels basòfils, també d'histamina [39].

Els neutròfils, els granulòcits més abundants en sang, presenten, a més de la capacitat de desgranulació, activitat fagocítica i generen ROS quan s'activen [46].

### 2.1.3 Cèl·lules *natural killer* i citotoxicitat

Les cèl·lules NK s'originen a la medul·la òssia i es troben en melsa i sang, on representen un 5-20% de les cèl·lules mononuclears i tenen una vida mitjana d'uns 10 dies. Contenen abundants grànuls citoplasmàtics i expressen proteïnes de superfície úniques. Tenen propietats citotòxiques i estan implicades en l'eliminació de cèl·lules tumorals i de cèl·lules infectades per virus [38,47].

Les cèl·lules NK no presenten receptor de cèl·lules T (TCR), B (BCR), ni CD3 (molècula pròpia dels limfòcits del sistema immunitari adaptatiu), però presenten diferent grau d'expressió de CD161 (equivalent a NK1.1 en ratolí i rata), CD16 i CD56. A més, presenten una complexa família de receptors polimòrfics (com els *killer-cell immunoglobulin-like receptor* o KIR) implicats en el reconeixement de molècules MHC i capaçes d'inhibir la seva funció citotòxica [47]. A sang perifèrica, la majoria de les cèl·lules NK presenten una elevada expressió de CD16 i una expressió intermèdia de CD56, i tenen una gran activitat citotòxica [48,49]. Les cèl·lules NK

activades són capaces de secretar citocines, com IFN- $\gamma$  i TNF- $\alpha$ , que activen altres components del sistema immunitari [50].

Quan les cèl·lules NK interaccionen amb una cèl·lula diana, la presència de determinades citocines i senyals inhibidors i activadors de les cèl·lules NK, seran determinants per a la seva activació. Els receptors KIR s'uneixen al MHC de classe I i transmeten un senyal inhibitori. En cèl·lules infectades o tumorals, disminueix l'expressió del MHC de classe I i, per tant, es redueix el senyal inhibitori, fet que provoca l'activació de les cèl·lules NK. L'activació d'aquests limfòcits inicia una sèrie de processos cel·lulars que culminen amb l'alliberació del contingut dels grànuls citoplasmàtics sobre la cèl·lula diana [51]. Concretament, les cèl·lules NK alliberen perforines, que penetren la membrana cel·lular, i granzima que causa l'apoptosi de la cèl·lula diana [52].

#### 2.1.4 Cèl·lules T natural killer

Les cèl·lules T *natural killer* (NKT) es formen a la medul·la òssia i maduren al timus. A sang es troben en nombre molt variable, des de quantitats indetectables fins a un 3% dels limfòcits sanguinis. Són un tipus heterogeni de limfòcit T amb la particularitat de que expressen tant receptors típics de limfòcits T (TCR $\alpha\beta$  amb un repertori restringit) com de les cèl·lules NK (CD161). Les cèl·lules NKT reconeixen glicolípid units a molècules similars al complex MHC de classe I, anomenats CD1d [53].

Pel que fa la seva funcionalitat, a diferència dels limfòcits T però de forma similar a les cèl·lules NK, els limfòcits NKT actuen destruint cèl·lules infectades per virus o bacteris i cèl·lules tumorals [53–55]. A més a més, les cèl·lules NKT secreten citocines (IL-4, IFN- $\gamma$ , TNF- $\alpha$ ), incrementen la presentació d'antígens a les cèl·lules T citotòxiques i afavoreixen l'activació de les cèl·lules NK [47,54].

Per a la seva capacitat de destrucció de cèl·lules infectades o tumorals i el seu paper d'intermediari entre la immunitat innata i adaptativa, les cèl·lules NKT resulten de gran importància en certs estats fisiològics i fisiopatològics [56].

### 2.1.5 Cèl·lules dendrítiques

Són cèl·lules derivades de la medul·la òssia presents als teixits epitelials i limfoides. Es caracteritzen per les seves llargues prolongacions membranoses [38]. Són capaces de fagocitar i d'activar macròfags i cèl·lules NK. A més, poden actuar com a APC i iniciar la resposta immunitària adaptativa per tant, presenten una important connexió entre la immunitat innata i l'adaptativa [57]. Com a APC capten antígens, els processen i els transporten fins als ganglis limfàtics més propers per presentar-los als limfòcits T verges [38].

### 2.1.6 Mastòcits

Els mastòcits es troben en teixits i, de la mateixa forma que els basòfils, estan implicats en la resposta inflamatòria i en la resposta al·lèrgica.

Quan els mastòcits s'activen alliberen el contingut dels seus grànuls, que contenen histamina, la qual indueix vasodilatació i augmenta la permeabilitat vascular [38]. A més a més, els mastòcits també representen un dels tipus cel·lulars més primerencs en la producció de citocines en resposta a una infecció [58].

### 2.1.7 Resposta inflamatòria

La resposta inflamatòria consisteix en una reacció de l'organisme davant d'una agressió (infecció o dany tissular) que comporta l'atracció de leucòcits cap al lloc d'agressió (quimiotaxi) i la seva activació, i també indueix l'extravasació de diverses proteïnes plasmàtiques. Tot aquest procés té la finalitat de neutralitzar l'agent causal de la inflamació [38].

En un primer moment, l'estímul inflamatori és reconegut pels macròfags residents (TLR dels macròfags en cas d'infecció) que indueixen la producció de mediadors inflamatoris, com quimiocines i citocines. Al mateix temps es produeix extravasació de proteïnes plasmàtiques i, a més, sortida de leucòcits, principalment de neutròfils, de la circulació sanguínia cap al lloc d'infecció. Aquestes cèl·lules, juntament amb els macròfags residents, actuen eliminant l'agent extern a través de l'alliberament de

ROS, espècies reactives de nitrogen o elastasa, entre d'altres. Si la resposta inflammatòria aguda no aconsegueix eliminar l'agent extern, el procés inflamatori persisteix i involucra els limfòcits T. Si tot i així no s'elimina l'antigen, s'arriba a un estat d'inflamació crònica [59].

## 2.2 Immunitat adaptativa

La resposta immunitària adaptativa es caracteritza pel reconeixement específic d'antígens i el desenvolupament de memòria immunològica. Les cèl·lules implicades en aquesta resposta són les APC i els limfòcits T i B.

### 2.2.1 Cèl·lules presentadores d'antigen

Existeixen diversos tipus cel·lulars que poden actuar com a APC. Les cèl·lules dendrítiques són les APC per excel·lència, però els macròfags i els limfòcits B també poden actuar com APC. La principal funció d'aquestes cèl·lules és la de processar l'antigen i presentar-lo als limfòcits T [60].

### 2.2.2 Limfòcits T

Els limfòcits T s'originen a la medul·la òssia i, quan encara no han acabat de madurar, migren cap al timus (**Figura 2**) [38].

Els limfòcits T immadurs o timòcits arriben a l'escorça tímica i, a mesura que maduren, avancen cap a la medul·la. Quan els timòcits arriben al timus no expressen TCR ni els coreceptors CD4 ni CD8 (*double negative*, DN). En el següent pas de maduració, els timòcits expressen a la seva superfície, simultàniament, tant CD4 com CD8 (*double positive*, DP) i, en una fase més avançada, expressen el receptor TCR [61].

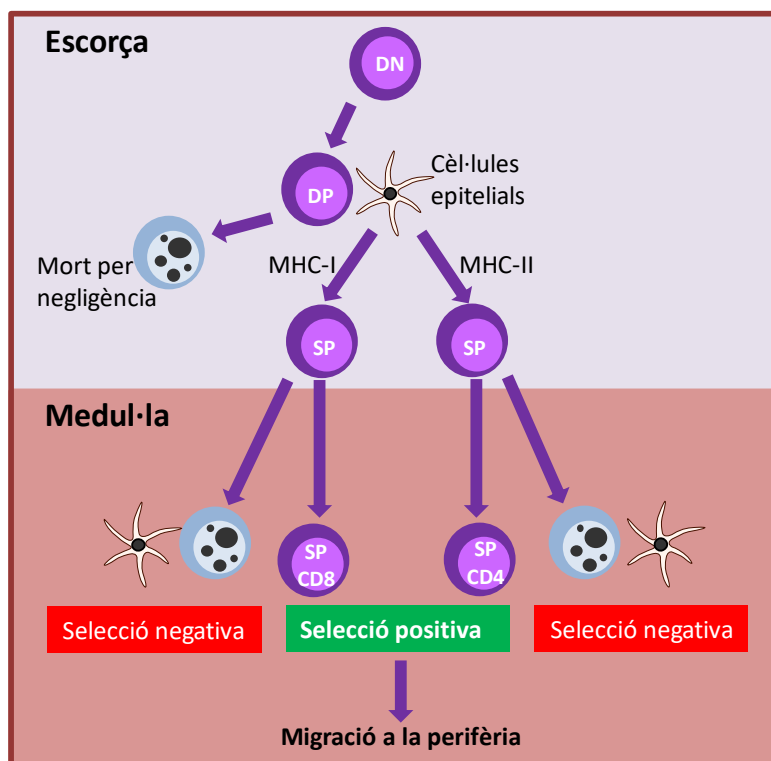
Les cèl·lules DP interaccionen amb les cèl·lules epitelials del timus que expressen MHC de classe I o II. En funció del MHC amb el que interaccionen i el grau de reconeixement amb el complex pèptid presentat - TCR, les cèl·lules experimenten mort per negligència, selecció

positiva o selecció negativa [62]. Si els limfòcits DP interaccionen amb poca afinitat, degut a un mal reordenament del TCR, es produeix apoptosi o l'anomenada mort per negligència. Els limfòcits DP que reaccionen fortament, experimenten selecció negativa, és a dir, es produeix apoptosi de limfòcits autoreactius. En canvi, les cèl·lules DP que reaccionen de forma apropiada amb el complex experimenten la selecció positiva. Els limfòcits més madurs només expressen un dels coreceptors, CD4 o CD8 (*single positive*, SP). Si han interaccionat amb el MHC de classe I, mantindran el CD8 (CD4-, CD8+), i si han interaccionat amb el MHC de classe II mantindran el CD4 (CD4+, CD8-) [61]. Un cop ha acabat el procés de maduració, els limfòcits abandonen el timus i passen a sang [61].

### Figura 2. Maduració fenotípica al timus

DN: *double negative*, DP: *double positive*, MHC: complex major d'histocompatibilitat, SP: *single positive*

Basada en [61]



Els limfòcits T madurs poden ser [38,63–65]:

- **Limfòcits T citotòxics (Tc):** expressen el coreceptor CD8. S'activen quan el seu TCR específic entra en contacte amb l'antigen en el context del complex MHC de classe I. Els limfòcits Tc alliberen granzima i perforina que indueixen l'apoptosi de les cèl·lules diana utilitzant un mecanisme similar al de les cèl·lules NK.
- **Limfòcits T helper (Th):** expressen el coreceptor CD4 i tenen un paper clau en establir i maximitzar la resposta immunitària. Les cèl·lules Th s'activen pel reconeixement del TCR específic amb el pèptid antigènic presentat, en el context de les molècules del complex MHC de classe II, per les APC. Alliberen citocines que permeten l'activació d'altres cèl·lules efectores i la regulació de la resposta immunitària. Els limfòcits Th efectors poden ser de diferents tipus. Entre d'altres, destaquen:
  - **Limfòcits Th1:** es caracteritzen per la producció d'IFN- $\gamma$ , que activa l'activitat microbida dels macròfags. Les citocines produïdes per una resposta Th1 contribueixen a la diferenciació dels limfòcits B en cèl·lules plasmàtiques que produeixen anticossos capaços d'activar el sistema del complement. La resposta Th1 està associada amb la destrucció de patògens intracel·lulars.
  - **Limfòcits Th2:** es caracteritzen per la producció d'IL-4, IL-5 i IL-13, implicades en el desenvolupament de limfòcits B productors d'immunoglobulina (Ig) E, així com el reclutament de mastòcits i eosinòfils. La resposta Th2 està associada amb la destrucció de patògens extracel·lulars i està augmentada en casos d'asma o al·lèrgies.
  - **Limfòcits Treg:** presenten un fenotip CD4+CD25+Foxp3+ i es caracteritzen per regular i limitar la resposta immunitària. Són essencials en el control del desenvolupament de malalties autoimmunitàries.
  - **Limfòcits Th3:** són un tipus de limfòcits reguladors de la resposta immunitària presents sobretot a l'intestí, amb un



fenotip CD4+Foxp3-*latency-associated peptide* (LAP)+ i es caracteritzen per produir elevats nivells de TGF- $\beta$ .

- **Limfòcits Th17:** es caracteritzen per la producció d'IL-17A, IL-17F, IL-21 i IL-22. Estan implicats en la malaltia inflamatòria multi-orgànica.

### 2.2.3 Limfòcits B

Són cèl·lules que es formen i maduren a la medul·la òssia. Expressen el receptor antigènic de cèl·lules B o BCR. Quan els limfòcits B reconeixen un antigen, directament i a través dels limfòcits Th, s'activen, proliferen i es diferencien en cèl·lules plasmàtiques capaces de produir i secretar anticossos [39,66].

Els anticossos poden ser de diferents isotips i presenten diferents característiques [39]:

- **Immunoglobulina M (IgM):** forma estructures pentamèriques. És la primera immunoglobulina que se sintetitza davant d'una infecció i és la principal activadora del sistema del complement.
- **Immunoglobulina G (IgG):** és la més abundant en sang (75% de les immunoglobulines circulants). La seva principal funció és la de combatre agents externs, com virus i bacteris mitjançant la facilitació de la fagocitosi i l'activació del sistema del complement.
- **Immunoglobulina A (IgA):** és la immunoglobulina principal en mucoses, per exemple en el tracte respiratori o el gastrointestinal.
- **Immunoglobulina E (IgE):** és la que es troba en menor concentració en plasma. Està implicada en reaccions al·lèrgiques.
- **Immunoglobulina D (IgD):** aquesta immunoglobulina es troba associada a la membrana dels limfòcits B. Tot i que la seva funció és bastant desconeguda, podria estar implicada en la detecció d'antígens.

### 3. Exercici físic i sistema immunitari

Tal i com s'ha comentat, l'exercici físic comporta una sèrie de canvis fisiològics a l'organisme per tal d'adaptar-se davant d'una situació d'estrès. De forma general, la pràctica d'exercici produeix molts efectes beneficiosos per a la salut, encara que depenen de la intensitat, el volum, la freqüència, la durada i el tipus d'exercici [67–73]. Mentre que un exercici moderat i regular pot reduir el risc a patir infeccions del tracte respiratori superior (URTI), un exercici extenuant o agut indueix una atenuació del sistema immunitari i, conseqüentment, un risc superior a patir URTI [72]. De fet, la relació existent entre la intensitat de l'exercici físic i el risc a desenvolupar URTI es va començar a intuir a principis del segle XX, durant els jocs olímpics d'hivern a Suïssa. Més tard, a l'any 1983, un estudi amb corredors sud-africans que van participar a una ultramarató, va demostrar que un 33,3% dels corredors presentaven símptomes d'URTI dues setmanes després de la competició, mentre que la prevalença en la població sedentària era del 15,3% [74]. Al 1990, Nieman va fer un estudi en corredors de la marató de Los Angeles, i va observar que gairebé un 13% dels participants van presentar alguna simptomatologia d'URTI durant la setmana posterior a la cursa en comparació al 2,2% d'inscrits que no van córrer la cursa [75]. L'any 1994, aquest mateix autor va postular per primera vegada que la relació existent entre la intensitat de l'exercici i el risc a patir URTI genera una corba en forma de J (**Figura 3**) [76]. Segons aquest gràfic, un exercici moderat pot reduir el risc a patir URTI per sota dels nivells de les persones sedentàries, però un excés d'exercici extenuant incrementa el risc per sobre de la mitjana. Aquestes alteracions immunitàries es produeixen principalment durant la recuperació, entre 3 i 72 h després de l'exercici, període en què augmenta la vulnerabilitat enfront a patògens. Aquest període es coneix com a “*open window*” [77].

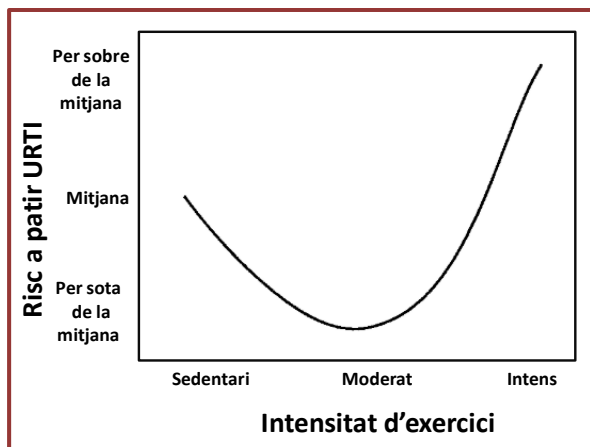
En aquest sentit, Campbell i col·laboradors posen en dubte aquesta relació ja que, segons ells, l'elevada freqüència d'infeccions després de l'exercici intens no s'ha confirmat amb proves de laboratori i, de forma alternativa,

atribueixen la disminució de l'efectivitat del sistema immunitari a altres factors, com pot ser desplaçaments o alteracions del son [78].

*Figura 3. Relació entre la intensitat d'exercici físic i el risc a patir infeccions del tracte respiratori superior*

URTI: Upper respiratory tract infection

Basada en [76]



### 3.1 Exercici físic i sistema immunitari innat

L'impacte de l'exercici realitzat sobre el sistema immunitari innat depèn de la intensitat i la durada de l'exercici i de la condició física de la persona. Per una banda, l'exercici afecta la distribució de leucòcits en els diferents compartiments i, per altra, altera les funcions fagocítiques i inflamatòries dels macròfags, així com la funcionalitat de les cèl·lules NK. La pràctica regular d'un entrenament moderat potencia la immunitat innata, ja que augmenta l'activitat fagocítica [79,80] i la citotoxicitat de les cèl·lules NK, i disminueix la resposta inflamatòria [81,82]. Ara bé, l'exercici intens s'ha associat amb respostes immunitàries alterades com una capacitat fagocítica variable [83–85], un augment de la secreció de citocines proinflamatòries [86] i una alteració en el recompte sanguini de cèl·lules NK [87–90].

### 3.1.1 Exercici físic i leucocitosi

Tot i la gran variabilitat interpersonal existent i les diferents condicions d'exercici físic, de forma general, en resposta a l'exercici físic es produeix una ràpida mobilització de leucòcits des del compartiment marginal i la medul·la òssia, el que indueix una ràpida leucocitosi. Aquesta mobilització és un fenomen bifàsic i transitori [77] i depèn de la durada i intensitat de l'exercici. Un exercici físic de curta durada indueix una menor leucocitosi que un exercici de més durada i de la mateixa intensitat; mentre que un exercici intens indueix més leucocitosi que un de menor intensitat i igual durada [91]. En cas de l'exercici moderat, els nivells de leucòcits augmenten 2-3 vegades i tornen al seu estat basal entre 6 i 24 h després de l'exercici [77,92]. En cas de l'exercici intens, la leucocitosi pot arribar a incrementar fins a 5 vegades [77] i, durant el període de recuperació, es produeix leucopènia, el que s'associa a l'"*open window*" per a patògens [93–95].

No tots els tipus de leucòcits contribueixen amb la mateixa magnitud a la leucocitosi. En general, els principals responsables de la leucocitosi són els limfòcits i els neutròfils, en menor mesura, els monòcits [77]. Els leucòcits afectats per l'exercici comparteixen dues característiques principals:

1. Presenten funcions de tipus citotòxic o efector i un fenotip més madur i diferenciat. L'exercici mobilitza preferentment limfòcits i monòcits amb una elevada expressió de molècules d'adhesió i de fenotip associat a citotoxicitat, activació, inflamació i presentació antigènica, com cèl·lules NK, cèl·lules TCR $\gamma\delta$ , cèl·lules Tc i monòcits de tipus no clàssics [28,96,97].
2. Presenten una elevada quantitat de receptors adrenèrgics i de glucocorticoides i, per tant, són molt sensibles a les catecolamines i al cortisol [98].

Davant d'una situació d'estrès, com pot ser l'exercici físic, es produeix la mobilització quasi instantània de limfòcits a la circulació sanguínia deguda, en part, a l'augment de forces hemodinàmiques, ja que l'increment de la pressió arterial i del flux sanguini, provoca l'alliberament dels leucòcits adherits a l'endoteli vascular, és a dir, del compartiment marginal [99]. De

forma més important, la mobilització del limfòcits és deguda a l'efecte de l'augment de catecolamines circulants sobre els receptors  $\beta$ -adrenèrgics dels limfòcits [77,100]. Als anys 80 es va descriure per primera vegada la presència de receptors adrenèrgics a la superfície dels limfòcits [101] i es va demostrar la innervació simpàtica dels òrgans limfoides primaris i secundaris [102]. Més tard, es va veure que òrgans limfoides com la melsa eren els principals donadors de leucòcits mobilitzats cap a la sang sotmesos al control adrenèrgic [103]. Estudis amb inhibidors selectius dels receptors adrenèrgics han posat de manifest que la senyalització a través dels receptors  $\beta_2$ -adrenèrgics constitueix el mecanisme primari pel qual es mobilitzen els limfòcits i els monòcits durant l'exercici [28]. Tanmateix, alguns canvis en la mobilització dels limfòcits sembla no estar influenciada per les catecolamines [104]. En aquest sentit, s'ha descrit que els glucocorticoides també podrien estar implicats en aquesta mobilització limfocítica en resposta a l'exercici físic [77].

Després de la leucocitosi inicial com a resposta a l'exercici intens, el nombre de leucòcits, sobretot de limfòcits, disminueix a valors inferiors als nivells pre-exercici [91]. Aquesta limfopènia s'ha atribuït a processos d'apoptosi, que depèn del tipus d'exercici físic realitzat, i principalment, a una redistribució dels limfòcits a diferents teixits i òrgans [77]. Concretament, limfòcits amb fenotips compatibles amb la migració de teixits abandonen la sang en resposta als glucocorticoides, per anar a teixits perifèrics com la medul·la òssia, els pulmons o l'intestí [104,105]. Per altra banda s'ha vist que, de forma tardana a un exercici intens, apareix neutrofilia que és deguda a l'alliberament de neutròfils de la medul·la òssia, a causa de l'increment de la concentració plasmàtica del cortisol [72]. A més, s'ha descrit que un exercici agut indueix un retràs en l'apoptosi espontània de neutròfils, fet que podria contribuir al manteniment de la neutrofilia tardana [106].

### 3.1.2 Exercici físic, macròfags i resposta inflamatòria

L'exercici físic modifica la funcionalitat dels macròfags i la resposta inflamatòria. Els macròfags, per la seva capacitat de síntesi i alliberament de citocines, tenen un paper clau en la regulació de la resposta inflamatòria (detallat a l'apartat 2.1.7.).

L'exercici moderat promou el canvi de macròfags de fenotip proinflamatori M1 a macròfags de fenotip antiinflamatori M2 [107]. L'exercici moderat també indueix a què els macròfags augmentin la seva capacitat d'adhesió i quimiotaxi al lloc d'infecció, i també incrementin l'activitat fagocítica i microbicida, l'expressió d'òxid nítric sintasa (NOS) i, conseqüentment la producció d'òxid nítric [85,108]. Concretament s'ha observat un augment de la isoforma NOS-2, que només s'expressa en macròfags activats i que depèn de l'activació del factor nuclear (NF)- $\kappa$ B (revisat a [109]). Per altra banda, un exercici intens o de resistència s'ha relacionat amb una menor expressió del MHC-II, el que redueix la seva capacitat de presentació antigènica als limfòcits T (revisat a [110]).

Pel que fa a la resposta inflamatòria, en general, diversos estudis mostren la relació inversa entre la pràctica d'activitat física i els marcadors sanguinis d'inflamació [111]. Així mateix, s'ha descrit que l'exercici moderat indueix un increment de citocines antiinflamatòries com la IL-10 [106].

Els mecanismes pels quals l'exercici moderat pot reduir la resposta inflamatòria inclouen el paper del cortisol, que té propietats antiinflamatòries, i el de l'adrenalina, capaç d'inhibir la producció de citocines proinflamatòries com IL-1 $\beta$  i TNF- $\alpha$  [112]. A més a més, la IL-6, alliberada principalment pel múscul durant l'exercici, presenta un paper rellevant ja que indueix la producció de cortisol i de citocines antiinflamatòries, com la IL-10 i el receptor antagonista de la IL-1 (IL-1ra) [112].

Per altra banda, l'exercici d'alta intensitat o de resistència s'ha associat a una potenciació de la resposta inflamatòria. Diversos estudis han avaluat l'impacte de l'exercici intens sobre la inflamació. Un estudi realitzat amb atletes que van dur a terme un exercici agut amb bicicleta, mostra un augment de citocines proinflamatòries en plasma, com IL-1 $\beta$  i TNF- $\alpha$ , i de la proteïna quimiotàctica de monòcits (MCP)-1, si bé, de forma tardana, s'observa un increment d'IL-1ra i IL-10 [86]. Un altre estudi amb corredors de maratons descriu resultats similars i complementaris, amb un

increment de marcadors proinflamatoris com TNF- $\alpha$ , IL-6 i de la proteïna C reactiva (PCR) [113].

Globalment, tant l'exercici moderat com intens indueixen l'alliberament de citocines. Algunes de les diferències que poden condicionar el perfil d'aquestes són la intensitat i la condició física de la persona. Tot i això, els dos tipus d'exercici semblen incrementar l'alliberament d'IL-6 i d'IL-10.

### 3.1.3 Exercici físic i cèl·lules NK i NKT

Estudis en humans i en models animals han descrit que un entrenament regular i moderat augmenta la proporció i l'activitat citotòxica de les cèl·lules NK en sang [100,114]. Diversos estudis en pacients amb diferents tipus de càncer que han realitzat activitat física d'intensitat moderada presenten una millora de l'activitat NK durant la rehabilitació i també un augment de la supervivència [115,116]. No obstant, un estudi realitzat en dones amb càncer de pit que van realitzar 6 mesos d'exercici físic aeròbic combinat amb una dieta hipocalòrica, no observa cap efecte respecte l'activitat i proporció de cèl·lules NK [117].

Per altra banda, existeixen evidències de que un exercici crònic intens tendeix a incrementar l'activitat citotòxica de les cèl·lules NK. En aquest sentit, diversos estudis han mostrat una major activitat citotòxica després d'un exercici crònic [114], el que es podria atribuir a modificacions epigenètiques [118]. Altres estudis, però, indiquen que després d'un exercici intens aquesta activitat es redueix al voltant del 40-60%, resultat que s'acompanya d'una disminució del recompte sanguini de cèl·lules NK (revisat a [110]).

La pràctica l'exercici també promou canvis en la proporció i funcionalitat de les cèl·lules NKT. L'exercici moderat, com s'ha demostrat amb la pràctica de Tai Chi durant 6 mesos, incrementa la proporció de cèl·lules NKT i, conseqüentment millora la funcionalitat del sistema immunitari [119]. Per altra banda, s'ha observat que un exercici intens disminueix el percentatge d'aquest tipus cel·lular en melsa, fet que podria induir la immunosupressió típica de l'exercici intens [120].

### 3.2 Exercici físic i sistema immunitari adaptatiu

L'efecte de l'exercici físic sobre el sistema immunitari adaptatiu, de forma similar a la immunitat innata, depèn del grau d'intensitat, durada i tipus d'exercici físic realitzat. De forma global, tal com s'ha esmentat, l'exercici augmenta la mobilització dels limfòcits i això afecta a la seva funcionalitat.

#### 3.2.1 Exercici físic i funcionalitat dels limfòcits T

Tal i com s'ha detallat, després de la leucocitosi inicial observada després de l'exercici físic, s'observa una leucopènia transitòria, caracteritzada per una limfopènia tardana. Aquesta limfopènia es produeix, per la redistribució dels limfòcits T de la sang a diferents teixits i òrgans [104]. La migració pulmonar pot representar un mecanisme adaptatiu per combatre la major exposició a agents externs a causa de l'increment en la ventilació pulmonar, mentre que la migració intestinal també constituïria un mecanisme compensatori per combatre la possible entrada de patògens a causa d'un increment de la permeabilitat intestinal [104].

L'exercici també influeix sobre la funcionalitat limfocitària [121]. Estudis realitzats en persones grans o en pacients amb malaltia pulmonar obstructiva crònica demostren que la pràctica d'exercici moderat augmenta la resposta proliferativa dels limfòcits T [122,123]. Per altra banda, un estudi amb animals també va demostrar l'increment en la activitat proliferativa dels limfòcits T estimulats amb Concanavalina (Con)A, degut a l'exercici moderat. Aquesta resposta també s'ha confirmat en models animals [124].

Pel contrari, la pràctica d'un exercici agut comporta una reducció de la capacitat proliferativa dels limfòcits T, el que s'associa amb un increment de la incidència d'infeccions virals [100,125]. De forma similar, s'ha observat que l'exercici intens o de resistència disminueix la resposta proliferativa dels limfòcits T [126,127], si bé hi ha estudis que no observen canvis significatius [128].



L'exercici sembla afectar de forma diferent a les subpoblacions de limfòcits Th. Hi ha evidències que mostren que la resposta de tipus Th1 està deprimida durant 1-24 h després de l'exercici, mentre que no s'observen canvis en la resposta dels limfòcits Th2 (revisat a [129]). La disminució de la resposta Th1 s'ha associat a l'efecte inhibitori del cortisol sobre la producció d'IL-12, citocina inductora de la resposta Th1 [130]. Ara bé, de vegades no s'ha observat reducció de les citocines Th1 (IFN- $\gamma$  o TNF- $\alpha$ ), ja que altres teixits o cèl·lules, com el múscul, també poden produir citocines proinflamatòries (IL-6 o TNF- $\alpha$ ) (revisat a [129]).

### 3.2.2 Exercici físic i funcionalitat dels limfòcits B

En resposta a l'exercici físic, els limfòcits B segueixen el mateix patró de mobilització i redistribució que els limfòcits T, però amb menys intensitat, ja que presenten menor quantitat de receptors adrenèrgics [100].

Pel que fa a la resposta proliferativa dels limfòcits B davant d'un estímul *in vitro*, hi ha evidències que mostren que l'exercici físic intens no modifica o bé incrementa la seva proliferació [131].

L'exercici físic també modifica la producció d'immunoglobulines. L'exercici moderat indueix una major producció d'immunoglobulines, com per exemple d'IgG o IgA salival [132–134]. En canvi, en un estudi realitzat amb persones sedentàries i obeses on van realitzar tant exercici moderat com intens no es va detectar diferències en la IgA salival [135]. Per altra banda, en el període de recuperació després d'un exercici intens s'observa una inhibició de la producció d'anticossos, el que afavoreix l'"*open window*" [72,106,136].

## 4. Estrès oxidatiu i antioxidants

A continuació es defineix l'estrès oxidatiu i els mecanismes antioxidants endògens i, la segona part d'aquest apartat, se centra en una família d'antioxidants exògens, els polifenols, i particularment en la flavanona emprada en aquesta tesi, l'hesperidina.

### 4.1 Estrès oxidatiu

El metabolisme aeròbic cel·lular està estretament lligat a la producció de ROS, com el radical hidroxil ( $\text{OH}\cdot$ ), el radical superòxid ( $\text{O}_2^{\cdot-}$ ) i el peròxid d'hidrogen, entre d'altres. En concentracions fisiològiques, les ROS tenen efectes beneficiosos a l'organisme, ja que estan implicades en l'extracció d'energia de molècules orgàniques, en la resposta immunitària i en els processos de senyalització [137]. Ara bé, en concentracions elevades causen efectes adversos a components cel·lulars com l'ADN, proteïnes i lípids [137,138]. L'organisme disposa de mecanismes antioxidants per fer front a l'excessiva producció de ROS. Quan l'equilibri entre oxidants i antioxidants es trenca amb domini dels oxidants, es produeix estrès oxidatiu. La inducció d'una elevada producció de ROS pot esdevenir pel tabaquisme, per situacions d'hiperòxia, per una exposició excessiva al sol o a metalls pesants o bé per estrès [138]. L'estrès oxidatiu està implicat en nombrosos processos patològics com malalties cardiovasculars, malaltia crònica renal, malalties neurodegeneratives, degeneració macular, diabetis, malalties pulmonars i càncer [137,138].

Els mecanismes antioxidants que disposa l'organisme es poden classificar en endògens i exògens. Dins dels antioxidants endògens destaquen la superòxid dismutasa (SOD), la catalasa, la glutatíon peroxidasa (GPx) i la glutatíon reductasa (GRd). Els antioxidants exògens inclouen minerals, com zinc i seleni, vitamines, com les vitamines A, C i E, carotenoides i els polifenols [139].

### 4.1.1 Superòxid dismutasa

La superòxid dismutasa (SOD, EC 1.15.1.1) és un enzim que pertany a la família de les metal·loproteïnes, amb una àmplia distribució a l'organisme. La SOD presenta un paper clau contrarestant les ROS (**Figura 4**). Concretament, aquest enzim catalitza la conversió de radical superòxid a oxigen molecular i peròxid d'hidrogen a través de l'oxidació-reducció d'ions metàl·lics que es troben al lloc actiu de l'enzim i que actuen com a cofactors [140]. Existeixen diferents tipus d'ions que poden actuar com a cofactors i donen lloc a les diferents isoformes de SOD: coure-zinc (Cu,Zn-SOD), ferro (Fe-SOD), manganès (Mn-SOD) i níquel (Ni-SOD) [141].

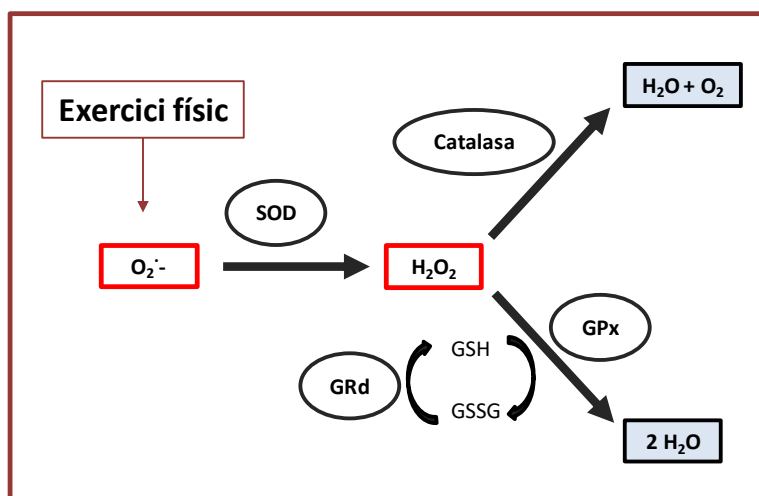
- **Cu,Zn-SOD o SOD1:** es troba al citoplasma, nucli, peroxisomes i membrana externa dels mitocondris dels mamífers. Té estructura homodímera de 32 kDa i conté un àtom de Cu i un de Zn a cada subunitat. Els dos metalls tenen importància en la funcionalitat de l'enzim, mentre que el Cu té importància catalítica, el Zn proporciona estabilitat a l'estructura proteica.
- **Mn-SOD o SOD2:** es troba ubicada a la membrana interna del mitocondri, on resulta de gran importància donat que és l'òrganul més afectat per l'estrès oxidatiu a causa de la seva funció en la respiració cel·lular i on es generen grans quantitats de radicals d'oxigen. L'enzim és un homotetràmer de 89 kDa i conté un àtom de  $Mn^{+3}$  a cada monòmer. La seva expressió està regulada, en gran mesura, per l'estat redox del mitocondri, i per això diverses citocines i factors de creixement de tipus proinflamatori, com per exemple  $TNF-\alpha$ , IL-1 o IL-6, poden induir un augment de l'expressió d'aquest subtipus de SOD.
- **EC-SOD o SOD3:** es localitza a la matriu extracel·lular, majoritàriament unida a heparina i a fibres de col·lagen tipus I. L'enzim presenta una estructura tetramèrica d'uns 135 kDa que conté un àtom de Cu i un de Zn per cada subunitat.

#### 4.1.2 Catalasa

La catalasa (EC 1.11.1.6) és un enzim amb una ampla distribució a l'organisme i, tot i que la seva activitat depèn del teixit on es troba, aquesta resulta més elevada a fetge i ronyons [142]. La catalasa és un metal·loenzim tetramèric, que en el seu lloc actiu conté un àtom de ferro o grup hemo [142]. A més, la catalasa es pot associar a nicotinamida adenina dinucleòtid fosfat reduïda (NADPH), si bé la NADPH no representa un factor clau en l'activitat catalítica de la catalasa, pot actuar com a factor preventiu o en la reversió parcial de la inactivació de la catalasa [138,143].

*Figura 4. Degradació d'espècies reactives d'oxigen per enzims antioxidants endògens*

Requadres vermells: espècies reactives d'oxigen.  $\text{H}_2\text{O}_2$ : peròxid d'hidrogen,  $\text{O}_2^-$ : radical superòxid, GPx: glutatió peroxidasa, GRd: glutatió reductasa, GSH: glutatió reduït, GSSG: glutatió oxidat, SOD: superòxid dismutasa



L'activitat catalítica de la catalasa consisteix en la conversió del peròxid d'hidrogen en aigua i oxigen (**Figura 4**). Tenint en compte la seva estructura i funció, les catalases poden ser de tres tipus [142]:

- **Catalases típiques:** La majoria d'aquestes catalases són homotetràmers, d'entre 200 i 340 kDa, contenen quatre grups prostètics o hemo i es troben àmpliament distribuïdes en organismes aeròbics.
- **Catalases peroxidases:** generalment són homodímers amb un pes molecular d'entre 120 i 340 kDa i també contenen un grup hemo com a cofactor. Aquest tipus es troba en fongs, arqueobacteris i bacteris. Tot i que tenen una major afinitat pel peròxid d'hidrogen, la seva activitat catalítica és menys eficient que la de les catalases típiques.
- **Catalases de manganès:** aquest tipus d'enzim conté dos àtoms de manganès en el seu lloc actiu i es troba exclusivament en bacteris.

### 4.1.3 Glutatió peroxidasa

La glutatió peroxidasa (GPx, EC 1.11.1.9) és un enzim seleno-dependent que es troba àmpliament distribuït en diferents teixits i òrgans. La seva funció biològica és la de catalitzar la reducció del peròxid d'hidrogen o del lipoperòxid, un marcador de dany lipídic, a expenses de l'oxidació del glutatió reduït (GSH) (**Figura 4**). Hi ha tres formes d'aquest enzim (revisat a [144]):

- **GPx intracel·lular o cel·lular:** és un enzim tetramèric amb un pes molecular de 22 kDa. Aquest tipus presenta l'afinitat més gran pel peròxid d'hidrogen.
- **GPx extracel·lular o plasmàtica:** també té una estructura tetramèrica i té un pes molecular de 25 kDa. Mostra una afinitat similar pels dos substrats.
- **GPx de fosfolípids:** és un monòmer, amb un pes de 18 kDa, que presenta una activitat específica per als fosfolipoperòxids.

#### 4.1.4 Glutatió reductasa i glutatió

La glutatió reductasa (GRd, EC 1.6.4.2) és un flavoenzim depenent de la NADPH que catalitza la reducció del glutatió oxidat (GSSG) a GSH, el qual serà utilitzat com a substrat per la glutatió peroxidasa (**Figura 4**) [145]. L'enzim és un homodímer amb un pes molecular de 104 kDa. En el lloc actiu conté la molècula flavina adenina dinucleòtid i un disulfur [146].

El glutatió és un tripèptid, que a més d'actuar com antioxidant, intervé en la detoxificació de compostos xenobiòtics, el metabolisme de leucotriens i prostaglandines i en la funció immunitària [145].

#### 4.2 Estrès oxidatiu i exercici físic

La resposta de l'organisme davant l'exercici físic respon a una hormesi fisiològica. Segons aquest concepte, l'exposició a un agent a dosis elevades té un efecte nociu, però a dosis baixes induïx una resposta adaptativa i/o un efecte beneficiós per a l'organisme [147]. L'activitat física moderada té un efecte beneficiós mitjançant una reacció d'adaptació a l'estrès oxidatiu, que comporta un augment de l'expressió i de l'activitat d'enzims antioxidants o de la reparació d'estructures danyades per l'activitat oxidant, entre d'altres efectes [139]. Per altra banda, l'exercici físic intens induïx l'acumulació excessiva de ROS, fet que comporta un dany oxidatiu irreparable i incrementa el risc a desenvolupar malalties [148].

L'efecte de l'exercici físic sobre l'estrès oxidatiu és dosi-depenent. Quan l'exercici induïx quantitats nanomolars de peròxid d'hidrogen, aquest actua com a senyalitzador i estimula el creixement i la proliferació cel·lulars, mentre que en quantitats micromolars, el peròxid d'hidrogen promou l'aturada del cicle cel·lular i induïx processos protectors a través de la modulació de l'expressió de diferents gens. A concentracions més elevades (milimolars), el peròxid d'hidrogen induïx estrès oxidatiu a la cèl·lula i provoca la seva mort [149,150].

Durant un exercici físic moderat i regular s'estimula la senyalització del *peroxisome proliferator-activated receptor γ co-activator 1 α* (PGC-1α), que regula l'expressió gènica dels enzims antioxidants, com la SOD, la catalasa i la GPx. A més, promou la biogènesi mitocondrial i és un agent antiinflamatori [151]. Així mateix, la producció de ROS en resposta a l'exercici moderat actua com a senyal activador de la família de les proteïnes quinases activades per mitògens (MAPK), concretament de p38 i ERK1/ERK2, les quals activen el NF-κB que també incrementa l'expressió gènica d'enzims antioxidants com la SOD, de la NOS induïble o de la *gamma-glutamylcysteine synthetase* [152].

Durant l'exercici físic intens es produeixen elevats nivells de ROS que afecten a l'estat redox de les cèl·lules [153]. Aquests canvis poden comportar una sèrie d'alteracions en diferents processos i senyalitzacions cel·lulars. Així, per exemple, es pot potenciar l'apoptosi dels limfòcits T, a causa de la inducció de la proteïna activadora 1, l'activació excessiva de la via del NF-κB, el que incrementa l'expressió gènica de citocines proinflamatòries i quimiocines, i la supressió de l'expressió del gen PGC-1α [151].

### 4.3 Estrès oxidatiu i polifenols

#### 4.3.1 Antioxidants exògens: polifenols

Els polifenols són un conjunt de compostos bioactius derivats del metabolisme secundari de les plantes que es caracteritzen per presentar diversos grups fenol a la seva estructura. Els polifenols es troben principalment en fruites, verdures i algunes begudes derivades de vegetals, en les quals proporcionen color i sabor. A més, també contribueixen a la protecció contra agents externs, com la radiació ultra-violeta o els patògens, entre d'altres [154].

Els polifenols es poden classificar en diferents categories segons la seva estructura química: flavonoides, àcids fenòlics, estilbens, lignans i alcohols fenòlics. Els flavonoides són els polifenols més abundants i es poden

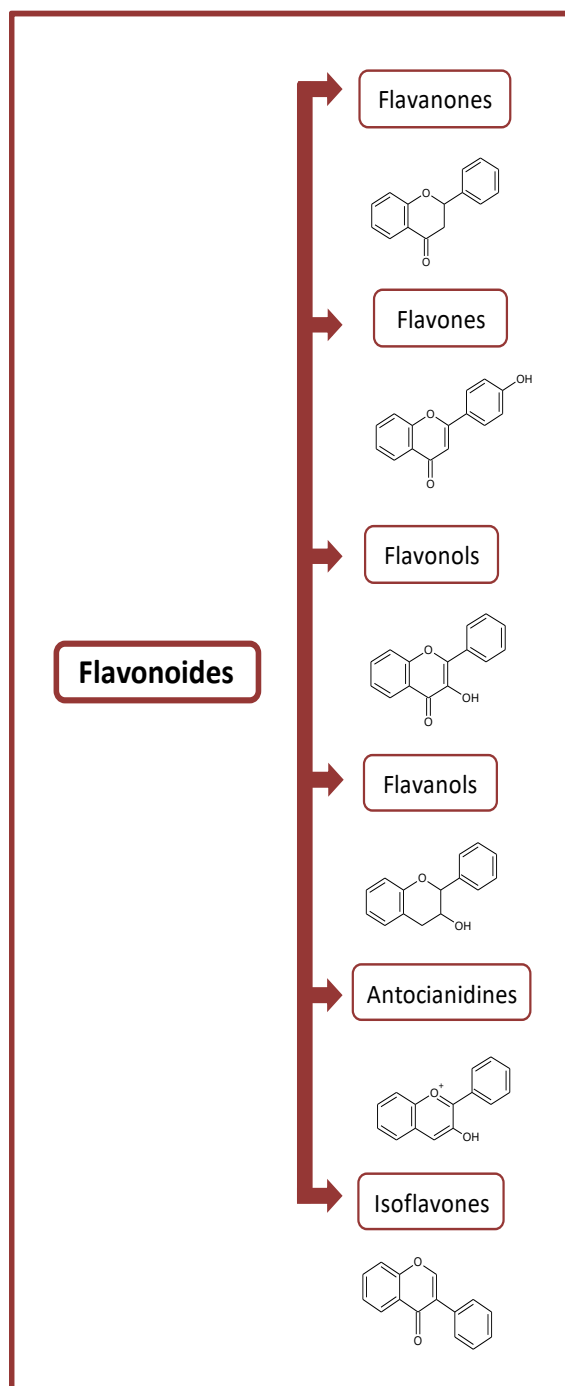
classificar en isoflavones, antocianidines, flavanols, flavonols, flavones i flavanones (**Figura 5**) [154].

En el camp de la nutrició, els polifenols tenen molt interès pels seus efectes beneficiosos sobre l'organisme, principalment degut a l'elevada activitat antioxidant que presenten. Molts estudis epidemiològics, estudis *in vivo* i *in vitro*, així com assajos clínics, han demostrat que la ingesta de polifenols té un gran impacte en la reducció del risc a patir malalties cròniques com càncer, diabetis, malalties cardiovasculars o malalties neuro-degeneratives com el Parkinson o l'Alzheimer [155,156]. A títol d'exemple, s'ha relacionat la ingesta dels flavonoides del tomàquet amb una reducció en la concentració de l'antigen prostàtic específic en pacients amb càncer de pròstata [157], i s'ha descrit el paper preventiu dels flavonoides del te verd sobre el càncer de pròstata i de pit [158,159]. Per altra banda, en persones amb sobrepès i amb resistència a la insulina, els polifenols de maduixes i de nabius milloren la sensibilitat a la insulina [160]. De forma similar, els flavonoides presents en el te negre redueixen la glucosa sanguínia postprandial [161]. A més, un estudi transversal realitzat amb més de 9.000 persones demostra la relació inversa entre el consum de flavonoides i l'obesitat així com la PCR [162]. Així mateix, els flavonoides del cacau destaquen pel seu paper com a potents agents antioxidants i pel seu efecte immunomodulador [163,164].

La ingesta de polifenols també s'ha relacionat amb una millora en el rendiment esportiu a nivell muscular i en la capacitat de recuperació [165,166]. Així mateix, recentment s'ha descrit que la suplementació amb un extracte de la llavor del raïm comporta una disminució en la concentració de la creatina quinasa en sang, fet que podria significar una reducció del dany cel·lular [167] i que la suplementació amb polifenols de garrofer (*Ceratonia siliqua* L.) en atletes de taekwondo millora la capacitat aeròbica de l'organisme [168].



*Figura 5. Estructura i tipus dels flavonoides*



#### 4.3.2 Flavonoides i sistema immunitari

Els flavonoides presenten activitat immunomoduladora, tal com s'ha evidenciat pel seu efecte sobre la regulació de cèl·lules immunitàries, la síntesi de citocines proinflamatòries i l'expressió gènica [169].

Alguns flavonoides poden modular l'expressió de citocines *in vitro* i *in vivo* [169,170]. Així, per exemple, la luteolina i l'apigenina inhibeixen la producció de citocines de tipus Th2, com IL-4, IL-5 o IL-13, en basòfils humans activats [171], i la quercetina i el kaempferol són capaços d'inhibir l'expressió gènica i la secreció de TNF- $\alpha$ , IL-1 $\beta$  o IL-6 en mastòcits humans activats [172].

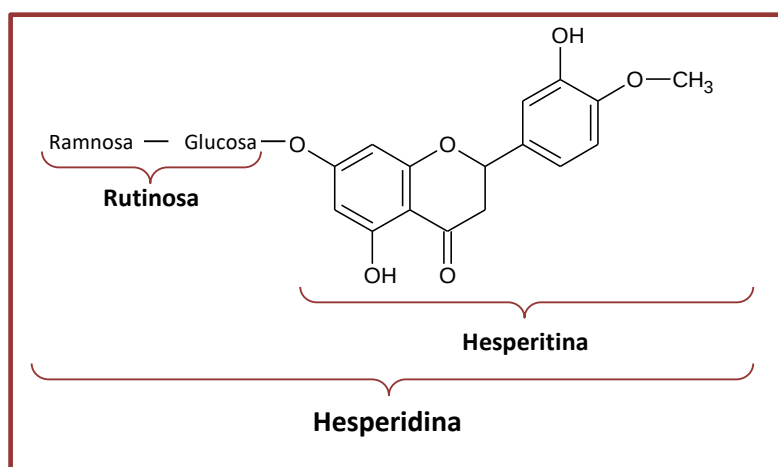
A més, alguns flavonoides poden inhibir la síntesi de citocines de tipus Th2 *in vivo* [173]. Concretament, s'ha demostrat que la quercetina inhibeix l'alliberament d'histamina, redueix la producció de citocines proinflamatòries i d'IL-4 [174]. Aquestes activitats són de gran interès per a la prevenció i/o tractament de l'asma o d'altres al·lèrgies. Alguns flavonoides també són capaços de modular l'expressió de TLR i NF- $\kappa$ B, molècules implicades en l'activació de la resposta inflamatòria [175]. Així, el kaempferol, a través de la inhibició de NF- $\kappa$ B, és capaç de reduir la síntesi de citocines proinflamatòries com la IL-6 i d'incrementar la producció de la citocina antiinflamatòria IL-10 [176].

L'activitat antiinflamatòria del flavonoides pot ser útil en el tractament de malalties neurodegeneratives, com la malaltia de Parkinson, la malaltia d'Alzheimer i l'esclerosi múltiple, la patogènia de les quals inclou un estat inflamatori continuat a nivell cerebral [177,178]. Així mateix, la inflamació crònica pot ser clau en el desenvolupament de l'aterosclerosi i de malalties cardiovasculars. En aquest sentit, els flavanols del cacau són beneficiosos per a persones amb alt risc a patir aquestes malalties [179].

### 4.3.3 Hesperidina: descripció, absorció i efectes

L'hesperidina és el flavonoide més abundant en els fruits cítrics. És una flavanona formada per una aglicona, anomenada hesperitina (3', 5, 7-trihydroxy-4'-methoxy flavanona) i el disacàrid rutinòsid, constituït per una molècula de ramnosa i una de glucosa (**Figura 6**). Per tant, l'hesperidina és l'hesperitina- $\beta$ -7-rutinòsid [180].

*Figura 6. Estructura de l'hesperitina i l'hesperidina*



L'hesperidina va ser aïllada per primera vegada l'any 1827 pel francès Lebreton [181]. Tot i que la font més important de la flavanona és el taronger amarg (*Citrus aurantium*), també es pot trobar en altres espècies de cítrics, com el *C. sinensis* (taronger dolç) o el *C. unshiu* (cítric típic del Japó). A més a més, també està present en altres plantes, com en els gèneres *Fabaceae*, *Betulaceae*, *Lamiaceae* i *Papilionaceae* [182].

La concentració d'hesperidina a les taronges varia en funció del seu estat de maduració: a les taronges més immadures, l'hesperidina pot arribar al 14% del pes fresc de la fruita [182]. Tanmateix, la distribució d'aquesta flavanona també varia segons les diferents parts de la fruita: hi ha concentració superior a les membranes i a l'albedo que al suc de taronja [182].

L'absorció dels flavonoides depèn de la seva estructura, així com de la matriu alimentària i la dosi ingerida [183]. Aquests compostos són parcialment metabolitzats al tracte gastrointestinal i posteriorment s'absorbeixen i distribueixen [184]. L'hesperidina passa per l'intestí prim sense ser absorbida i arriba al còlon, on la microbiota s'encarrega de separar la ramnosa i la glucosa el que afavorirà la seva absorció [183]. En aquest sentit, s'ha vist que *Bifidobacterium longum* R0175 (*B. longum*) i *Lactobacillus rhamnosus* subsp. *Rhamnosus* NCTC 10302 (*L. rhamnosus*) estan involucrats en el catabolisme de les flavanones del suc de taronja [185]. L'absorció de l'hesperidina com a tal en humans sans és bastant baixa (d'un 20%), mentre que l'absorció d'hesperetin-7-O-glucòsid arriba al voltant del 97% [186].

Durant els darrers anys, l'estudi dels efectes beneficiosos de l'hesperidina ha augmentat i s'han descrit efectes anticancerígens, neuroprotectors, antiolesterolèmics i antimicrobians. L'hesperidina, mitjançant l'activació de processos d'apoptosi, pot ser efectiva com a coadjuvant en el tractament de diversos tipus de càncer, com el de pulmó, fetge, pit, estómac i còlon [184,187]. Així mateix, gràcies a les seves propietats antioxidants, el seu paper estimulador de la proliferació epidèrmica i producció lipídica, l'hesperidina protegeix la pell contra els raigs UV i redueix el risc a patir càncer de pell (revisat a [188]). A més, l'hesperidina té un paper protector en malalties neurodegeneratives com la malaltia de Parkinson o la malaltia d'Alzheimer, a través de la millora de la funcionalitat dels factors de creixement neuronals, del sistema antioxidant endogen i per una disminució de la neuroinflamació (revisat a [189]). Per altra banda, s'ha descrit que els flavonoides del suc de taronja són capaços de modular diferents enzims implicats en la via de senyalització de síntesi del colesterol i reduir la seva formació [190]. També s'ha relacionat el consum d'hesperidina amb l'activitat antimicrobiana, tant pel que fa a activitat antibacteriana, antifúngica i antiviral [182,184].

En referència a sistema immunitari, diversos estudis mostren la capacitat immunomoduladora que presenta l'hesperidina. Concretament, s'ha vist que l'hesperidina a una concentració de 3,12  $\mu$ M és capaç d'incrementar

la proliferació limfocítica, així com l'activitat NK en esplenòcits de rata [191]. A més, l'hesperidina modula la funcionalitat dels macròfags peritoneals de rata *in vitro*, tant pel que fa a la seva activitat lisosomal com a l'alliberament d'òxid nítric [191]. A més a més, s'ha observat que en rates amb una dieta que incloïa un 0,5% d'hesperidina durant 1 mes és capaç de modificar la composició dels limfòcits de l'epiteli intestinal i de la làmina pròpia [192]. En rates immunitzades amb ovoalbúmina (OVA), l'administració oral de 100 i 200 mg/kg d'hesperidina canvia la composició dels limfòcits de ganglis limfàtics mesentèrics i incrementa la producció d'IFN- $\gamma$  [192]. Així mateix, en un model d'asma, l'hesperidina és capaç de reduir el desequilibri Th1/Th2 [193]. En ratolins amb esquistosomiasi, l'hesperidina incrementa la producció d'IL-4, IL-10 i IFN- $\gamma$  i disminueix la secreció d'IL-1, IL-12, IL-13, IL-17 i TNF- $\alpha$  per part dels esplenòcits [194]. A nivell clínic, però, la ingesta de 500 mL de suc de taronja que contenen 292 mg d'hesperidina o bé amb la ingesta d'aquesta quantitat d'hesperidina no influeix de forma significativa sobre diversos biomarcadors del sistema immunitari en humans sans [195].

Per altra banda, s'ha relacionat l'hesperidina com a agent protector de l'estrès oxidatiu. En aquest sentit, un estudi *in vitro* ha demostrat que l'hesperidina protegeix del dany oxidatiu induït per peròxid d'hidrogen en la membrana dels eritròcits [196]. En estudis *in vivo*, l'hesperidina és capaç de revertir l'estat oxidatiu i la toxicitat hepàtica i renal induïts per un excés de ferro en rosegadors [197,198]. A més, en un model animal de pleuresia, el pretractament amb hesperidina durant 21 dies va modular les activitats dels enzims antioxidants SOD i catalasa, així com també els nivells de ROS [199]. En rates d'edat avançada, l'hesperidina incrementa les activitats de la catalasa i de la GRd hepàtiques [200]. A més a més, s'ha demostrat que l'administració d'hesperidina a rates (100 mg/kg/dosi durant 4 setmanes) és capaç de protegir enfront de l'estrès oxidatiu generat per un exercici de natació [201]. Finalment, un recent estudi en humans demostra que el suplement amb hesperidina (500 mg/dia) durant quatre setmanes augmenta el rendiment esportiu en una bicicleta estàtica [202].



## OBJECTIVES



The harmful impact of intense and exhausting exercise on the immune system has been established both in elite athletes after intense training periods and in general population after their participation in competitive intensive sporting events, such as marathons. However, the influence of different exercise intensities on the immune system's composition and functionality remains uncertain.

On the other hand, the relationship between diet and health plays a key role in the maintenance or improvement of well-being. Thus, bioactive components, such as polyphenols with antioxidant capacity, can provide many healthy beneficial effects, which can be better evidenced in special situations, such as those inducing oxidative stress.

During the last few years, our research group has focused on the study of the effects of flavonoids on the immune system. In this context, hesperidin, the main flavonoid in citrus fruits, has demonstrated immunomodulatory effects in the intestinal and systemic immune systems in a model of allergy.

Based on this background, the hypothesis that supports the current thesis is that intensive and exhausting exercise can produce an immune system imbalance and hesperidin's supplementation could counteract this disequilibrium.

The main aim of the current thesis was **to study in depth the influence of both intensive and exhausting exercise and of hesperidin on the rat immune system.**



The specific objectives that would together achieve the main goal were as follows:

**1. To assess the influence of intensive and exhausting exercise on the innate and acquired immune system in rats.**

The results obtained from this objective are part of the following publications:

- *Alterations in the innate immune system due to exhausting exercise in intensively trained rats*, submitted for publication (**Article 1**)
- *Changes in the lymphocyte composition and functionality after intensive training and exhaustion exercise in rats*, submitted for publication (**Article 2**).

**2. To establish the immunomodulatory and antioxidant properties of hesperidin in the intestinal immune system and in a model of oxidative stress.**

The results obtained from this objective are in the followings publications:

- *Hesperidin effects on gut microbiota and gut-associated lymphoid tissue in healthy rats*, *Nutrients*, 2019; 11(2):324 (**Article 3**).
- *Protective effect of hesperidin on the oxidative stress induced by an exhausting exercise in intensively trained rats*, *Nutrients*, 2019, 11(4):783 (**Article 4**).



## RESULTATS



**ARTICLE 1**

***“Alterations in the innate immune system due to exhausting exercise in intensively trained rats”***

Sheila Estruel-Amades, Mariona Camps-Bossacoma, Malén Massot-Cladera, Francisco J. Pérez-Cano, Margarida Castell

Sotmès a publicació

Els resultats del present article han estat presentats als congressos següents:

- **13<sup>th</sup> International Society for Exercise and Immunology (ISEI) Symposium**, Coimbra, juliol de 2017. Estruel-Amades, S.; Massot-Cladera, M.; Camps-Bossacoma, M.; Garcia-Cerdà, P.; Franch, À.; Pérez-Cano, F.J.; Castell, M. **“Exhausting exercise in young rats: effect on systemic and intestinal lymphoid tissues”**.
- **10<sup>th</sup> Anniversary International Symposium on Immunonutrition**, Madrid, juliol de 2017. Estruel-Amades, S.; Azagra, I.; Abril-Gil, M.; Franch, À.; Pérez-Cano, F.J.; Castell, M. **“Seeking a model for immunonutrition: exhausting exercise in rats”**. Publicat a *Annals of Nutrition and Metabolism* 2017; 31-79.
- **10<sup>th</sup> Anniversary International Symposium on Immunonutrition**, Madrid, juliol de 2017. Estruel-Amades, S.; Camps-Bossacoma, M.; Pérez, M.; Franch, À.; Pérez-Cano, F.J.; Castell, M. **“NK activity in spleen cells of rats after exhausting exercise”**. Publicat a *Annals of Nutrition and Metabolism* 2017; 31-79.



**Resum ARTICLE 1**

**Objectiu:** Donat que l'activitat física intensa altera la funcionalitat del sistema immunitari, l'objectiu d'aquest article va consistir en caracteritzar les alteracions en el sistema immunitari innat després de dos programes d'exercici físic intens en rates.

**Material i mètodes:** Rates Wistar mascles i femelles de 4 setmanes d'edat van ser sotmeses a dos programes d'entrenament amb una cinta de córrer. Un programa va incloure dues setmanes d'entrenament intens (dues vegades al dia) i, al final, els animals van realitzar una prova d'extenuació (S-TE). El segon programa es va estendre durant cinc setmanes, i incloïa dues proves d'extenuació i tres entrenaments regulars per setmana i, a dia final, els animals es van sotmetre a una prova d'extenuació final. En aquest segon programa, l'anàlisi de la funcionalitat immunitària innata es va determinar abans (T), immediatament després (TE) i a les 24 h (TE24) de la prova d'extenuació final. Com a biomarcadors del sistema immunitari innat es va determinar l'activitat fagocítica de monòcits, citocines derivades de macròfags i l'activitat citotòxica de les cèl·lules NK.

**Resultats:** L'entrenament intens durant dues setmanes no va induir canvis significatius en els biomarcadors analitzats. Per contra, l'entrenament de cinc setmanes va disminuir l'activitat fagocítica i modificar el patró de citocines secretades per part dels macròfags. Pel que fa a les cèl·lules NK, l'entrenament va disminuir la seva proporció en melsa però, en canvi, en va augmentar la seva activitat citotòxica.

**Conclusions:** L'entrenament intens durant cinc setmanes indueix alteracions en la funcionalitat del sistema immunitari innat.



***Alterations in the innate immune system due to exhausting exercise in intensively trained rats***

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## Abstract

It is known that intensive physical activity alters the immune system's functionality. However, the influence of the intensity and duration of the exercise needs to be studied in more depth. Hence, we aimed to characterize the innate immune response in two programmes of intensive-training in rats. A short training programme included 2 weeks of intensive training, which ended with an exhaustion test (short training with exhaustion, S-TE). A second training program comprised 5-week-training containing two exhaustion tests plus three trainings per week. In this case, immune status was assessed before (T), immediately after (TE) and 24 h after (TE24) an additional final exhaustion test. Biomarkers such as phagocytic activity, macrophage cytokines, and natural killer (NK) cell activity were quantified. S-TE group was not enough to induce changes in the assessed innate immunity biomarkers. However, the second training was accompanied by a decrease in the phagocytic activity, changes in the pattern of cytokine secretion by macrophages and reduced NK cell proportion but increased NK cytotoxic activity. In conclusion, a 5-week intense training programme induced alterations in the innate immune system function, some of them mainly observed after an additional exhaustion test.

**Keywords:** cytokine; cytotoxic activity; NK cells; macrophages; phagocytosis; spleen

## Introduction

Moderate exercise induces many beneficial effects on human health. In particular, it reduces the risk of developing many chronic disorders, such as cardiovascular disease <sup>1</sup>, metabolic syndrome <sup>2</sup>, type 2 diabetes <sup>3</sup> and even cancer <sup>4,5</sup>. Furthermore, moderate exercise has an antioxidant effect <sup>6</sup> and a positive influence on the innate immune system <sup>7,8</sup>. On the other hand, intense exercise can induce adverse effects on health, which enhance the risk of upper respiratory tract infections (URTI) <sup>9,10</sup>.

The relationship between training load and risk of illness has led to the *J-curve* hypothesis, which means that both very low or no training, and very high training are associated with a higher risk of illness compared with a moderate training load <sup>10</sup>. Therefore, intensive exercise exposes the organism to an 'open window' to pathogens <sup>11,12</sup>.

The innate immune system is the first line of organism defence against pathogens. Neutrophils, macrophages and natural killer (NK) cells are the major cellular type of innate immunity susceptible to being altered by exercise. Neutrophils are phagocytic cells able to destroy bacteria by means of the release of enzymes and through the secretion of reactive oxygen species (ROS). Monocytes and macrophages are also phagocytic cells able to secrete a wide range of cytokines, mainly with pro-inflammatory properties <sup>13,14</sup>. NK cells represent a small proportion of the lymphocyte population but, due to their cytotoxic activity and ability to fight against virus-infected cells, play an important role in innate immunity.

It has been shown that blood neutrophil counts decreased after moderate exercise <sup>15,16</sup>, thus contributing to the anti-inflammatory effects of this kind of exercise. Conversely, exhaustive exercise produces an acute leukocytosis, with neutrophil and lymphocyte mobilization <sup>17,18</sup>. Similarly, moderate physical activity is capable of increasing phagocytic activity <sup>19,20</sup> but after an exhaustive exercise, the results are quite controversial because both decreases and increases of macrophage phagocytosis have been reported <sup>21,22</sup>. In addition, intensive training has been linked to an inflammatory profile with an increase in pro-inflammatory macrophage cytokines such as interleukin (IL)-1 $\beta$  and tumour necrosis factor (TNF)- $\alpha$  <sup>23</sup>. Likewise, NK cells can be mobilized and their activity can be altered depending on the intensity and duration of the exercise <sup>24</sup>. In blood, in response to physical exercise, NK cells are rapidly mobilized <sup>25</sup>, but after a very prolonged exercise, blood NK cell counts drop below the pre-exercise levels <sup>26</sup>. However, other authors reported that the proportion of circulating NK cells increases in humans after exhaustive exercise, although these cells seem to belong to a subset with lower cytotoxic activity <sup>27</sup>. Controversial results have also been described by Bigley et al. <sup>28</sup> who observed an increase in NK cell cytotoxicity in trained humans, whereas Campbell et al. <sup>29</sup> showed no differences in NK cell cytotoxic activity in post-menopausal women after 12 months of exercise.

Overall, immune system functions improve with moderate exercise but a training that is too intensive can lead to altered immune functions <sup>30</sup>. In this sense, the alterations of innate immunity due to intensive training are not well established <sup>31</sup>. For this reason, we

aimed to characterize the innate immune response in two programmes of intensive-training in rats, and also at different time points after exhaustion exercise.

## Methods

### Animals

The experimental procedures used were approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (UB) and the Catalonia Government (CEEA/UB ref. 464/16 and DAAM 9257, respectively).

Three-week-old Wistar rats ( $n=22$ , males and females in the first training programme; and  $n=30$ , females in the second training programme) were purchased from Envigo (United Kingdom). Animals were housed in the animal facilities of the Faculty of Biology (UB) in polycarbonate cages (2-3 rats per cage) in a controlled environment of temperature and humidity, in a 12/12h light/dark cycle. Water and food were provided *ad libitum*. Throughout the study, before running, body weight (BW) and food intake were recorded.

### Training programmes

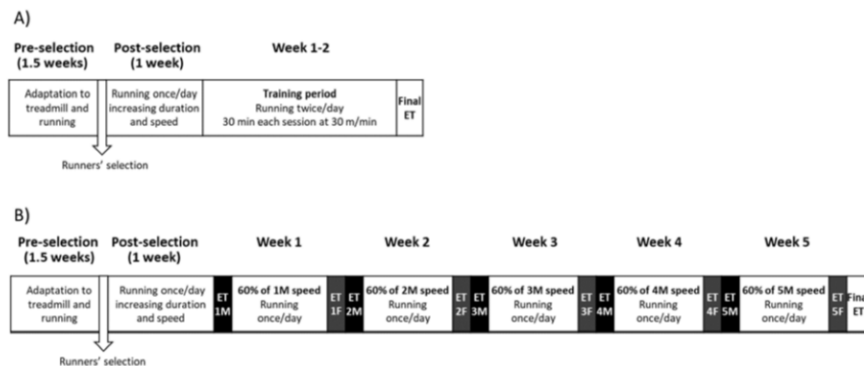
Two types of devices were used: an LE8700 treadmill (Panlab, Harvard, USA) and an Exer3/6 treadmill (Columbus, Ohio, USA). Both devices allow the speed and the exercise length to be controlled. In both approaches, sedentary animals and runners were studied in parallel. After running, as a reward, rats received a 50% solution of condensed milk (100  $\mu\text{L}/100\text{ g BW}$ ); sedentary rats also received this solution.

The exercise training programmes are summarized in Figure 1. Both experimental designs included 3 days of habituation on a turned-off treadmill, followed by 5 days of preselection in which the treadmills were turned on and rats ran on the treadmill once a day with increasing duration and speed. At the end of this period, sedentary animals (SED group) were selected. During the experiment, the SED group was placed in a treadmill receptacle without movement for the same time as the runners' rats.

In the first experimental design, i.e. short intensive training with exhaustion (S-TE), the exercise programme was based on a previously described procedure <sup>32</sup>, with some modifications (Fig. 1A). After selection, the runner group began a period in which the duration and the speed was increased progressively (10 min/session at 5 m/min to a 25 min/session at 25 m/min). In the following 2 weeks (weeks 1 and 2), intensive training started and runner animals ran twice a day (30 min at 30 m/min, 6 h between sessions), 5 days per week. At the end of the 2 weeks, animals were subjected to a final exhaustion test, starting with an initial speed of 5 m/min with a gradual increase of 1.8 m/min every min until exhaustion. The animal was considered exhausted when it could not maintain its normal position or when it touched the shock grid more than three times. Immediately after the final exhaustion test, animals were euthanized for sample collection.

The second experimental exercise programme was based on a previously described procedure<sup>69</sup> (Fig. 1B). After selection, the runner group also began a one-week period in which the duration and the speed on the treadmill were increased progressively. Afterwards, the intensive training began and lasted for 5 weeks (weeks 1 to 5). In each of these weeks, animals carried out an exhaustion test every Monday and Friday, running 15 min at 60% of the speed of the previous Monday's exhaustion test (the initial speed of the first Monday's exhaustion test was 30 m/min), and then the speed was progressively increased by 6 m/min every 2 min until rats' exhaustion. The maximum speed reached on Mondays was used as reference for the three following days, running at 60% of the speed for 20, 25 and 30 min, respectively. Then, the animals were homogeneously distributed into three groups, according to their ability to run: the T group, which was euthanized 24 h after a regular training session, the TE group, which was euthanized immediately after carrying out a final exhaustion test, and the TE24, which was euthanized 24 h after the final exhaustion test to establish the alterations in the recovering period. In the final exhaustion test, animals ran for 15 min at 60% of the speed of the previous Monday's exhaustion test, and then the speed was increased 3 m/min every 2 min until the animal's exhaustion.

**Figure 1. Experimental designs of training programmes.** In the first training programme (A), after 2 weeks of short intensive training (weeks 1 and 2), animals ran a final exhaustion test. In the second training programme (B), animals were intensively trained for 5 weeks, in which they carried out an exhaustion test every Monday and Friday. An additional final exhaustion test was conducted at the end. ET=exhaustion test, M=Monday, F=Friday.



## Sample collection

Rats were anaesthetized using ketamine (Merial Laboratories S.A., Barcelona, Spain) / xylazine (Bayer A.G., Leverkusen, Germany). Blood, peritoneal macrophages, and spleen were collected and immediately processed. Blood was obtained from the heart in heparin- and EDTA-anticoagulated tubes. An ethylenediaminetetraacetic acid (EDTA)-blood sample was used to determine leukocyte differential count, HGB concentration and HCT (automated haematology analyser, Spincell, MonLab Laboratories, Barcelona). Another EDTA-blood sample was used for characterizing blood lymphocyte composition.

Heparinized-blood was devoted to establishing the phagocytic activity. Peritoneal macrophages were used for assessing cytokine secretion. From spleen, lymphocytes were isolated to further assess NK cell proportion and cytotoxic activity.

### **Determination of phagocytic activity**

The phagocytic function of blood monocytes and granulocytes was assessed by flow cytometry analysis using the Phagotest™ kit (Glycotope, Biotechnology GmbH, Heidelberg, Germany) in accordance with the manufacturer's instructions. Briefly, blood was incubated with fluorescein isothiocyanate (FITC)-labelled *Escherichia coli* (10 min, 37 °C). Then, samples were placed on ice and a quenching solution was added to stop phagocytosis. After washing, erythrocytes were lysed. After DNA staining, data were acquired using Gallios™ Cytometer (CCiTUB) and the analysis was performed with FlowJo v.10 software. Monocyte and granulocyte populations were selected according to their forward-scatter and side-scatter characteristics. The percentage of phagocytic monocytes and granulocytes was quantified by means of FITC+ cells. Their corresponding phagocytic activity was measured through mean fluorescence intensity (MFI). Both the proportion of phagocytic cells and their relative phagocytic activity were compared with the SED group, the data of which were considered as 100%.

### **Peritoneal macrophage isolation and stimulation**

Peritoneal macrophages were obtained as previously performed <sup>37</sup>. Briefly, after having injected 40 mL of cold phosphate-buffered saline into the peritoneal cavity and after 2 min of abdominal massage, the peritoneal cells were collected. Following centrifugation (538 g, 10 min, 4 °C), cells were suspended in cold Roswell Park Memorial Institute (RPMI) medium, supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 IU/mL streptomycin-penicillin and 2 mM L-glutamine. Cell counts were assessed by a Sincell haematology analyser.

Peritoneal macrophages (10<sup>6</sup> cells/mL) were plated into 12-well plates and incubated for 2 h. After removing non-attached cells, macrophages were stimulated overnight with 100 ng/mL LPS. Non-stimulated macrophages were included as control. Supernatants were collected for IFN-γ, IL-1β, IL-6, IL-10, IL-12, MCP-1, and TNF-α quantification by using ProcartaPlex® Multiplex Immunoassay (Affymetrix, eBioscience, San Diego, CA, USA), as previously described <sup>70</sup>. Data were acquired by Luminex MAGPIX analyzer (Luminex®) in the Flow Cytometry Unit of the CCiTUB and analyzed with ProcartaPlex® Analyst (Thermo Fisher Scientific, S.L.U, Barcelona, Spain). Results are expressed as percentage considering that the MFI of stimulated macrophages from the SED group was 100%.

## Determination of lymphocyte composition in blood and spleen

After erythrocyte osmotic lysis, blood lymphocyte subsets were determined by mouse anti-rat NKR-P1A, CD45RA, TCR $\alpha\beta$  or TCR $\gamma\delta$  antibodies (BD Biosciences, San Diego, CA, USA) conjugated to FITC, phycoerythrin or brilliant violet 421, as described previously<sup>71</sup>. Briefly, cells were incubated with the mixture of saturating concentrations of the antibodies (4 °C, 20 min) and, after washing, they were fixed (0.5% p-formaldehyde) and stored (4 °C) until flow cytometry analysis. A blank control was included for each cell sample. Data were acquired with a Gallios™ Cytometer (Beckman Coulter, Miami, FL, USA) in the Flow Cytometry Unit of the Scientific and Technological Centres of the UB (CCiTUB) and analysed with FlowJo v.10 software (Tree Star, Inc., Ashland, OR, USA). Blood lymphocytes are represented as subset counts, taking into account the lymphocyte number from the haematology analyser and the subset percentages obtained by flow cytometry.

To isolate spleen lymphocytes, tissues were smashed in a sterile mesh cell strainer (40  $\mu$ m) as previously described<sup>71</sup>. After erythrocyte lysis, cells were suspended in culture medium (RPMI medium supplemented with 10% heat-inactivated FBS, 100 IU/mL streptomycin-penicillin, 2 mM L-glutamine and 0.05 mM 2- $\beta$ -mercaptoethanol, Sigma-Aldrich, Madrid, Spain). NK cell percentage was assessed in spleen suspensions as in blood samples. Changes in the relative proportion of spleen NK lymphocytes are represented considering the SED group value as 100%.

## Assessment of cytotoxic activity by NK cells

The cytotoxic activity of spleen NK cells was quantified by the NKTEST™ kit (Glycotop, Biotechnology GmbH, Heidelberg, Germany) following the manufacturer's protocol. Briefly, 10<sup>6</sup> cells/mL splenocytes, containing effector NK cells, were incubated at 37 °C for 1 h to remove monocytes that could suppress NK cell cytotoxic activity. Afterwards, the spleen cells were mixed with target cells (labelled K562 cells) and incubated for 90 min. Immediately before the flow cytometric analysis, a DNA staining was carried out. Data were acquired by the Gallios™ Cytometer (Flow Cytometry Unit, CCiTUB) and the analysis was performed with FlowJo v.10 software. Spontaneous cell death (without effector cells) was considered as control. Results from the individual cytotoxic activity were calculated according to the total NK activity and the percentage of NK cells of each sample. Final results are expressed as changes in the corrected cytotoxic activity considering that the SED rats had 100% of such activity.

## Statistical analysis

Statistical analysis was performed by the IBM Social Sciences Software Program (SPSS, version 22.0, Chicago, IL, USA). To assess the equality and normality of the results, Levene's and Shapiro-Wilk tests were used, respectively. Once these conditions were confirmed, a one-way ANOVA test was applied. When significant differences were obtained, Bonferroni's post hoc test was carried out between groups. Otherwise, non-parametric tests (Kruskal-Wallis test, followed by Mann Whitney U test) were applied to calculate significance. A repeated measures ANOVA test was carried out to assess time-

dependent parameters (e.g. BW). When comparing two groups (e.g. males and females, and performance within training), unpaired or paired Student's t-test was used. According to the equality and normality of the data, a Pearson or Spearman correlation was used to assess correlations between variables. Significant differences were considered when  $p < 0.05$ .

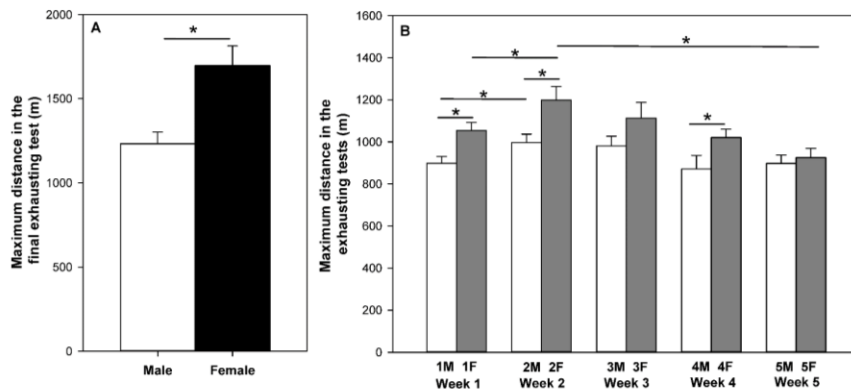
## Results

### Performance of training programmes

The first training programme included male and female rats (S-TE groups), which were intensively trained for 2 weeks (twice a day for 30 + 30 min at a speed of 30 m/min) and afterwards, they carried out a final exhaustion test (Fig. 1). In this final exhaustion test, female rats ran a significantly higher distance (about 1700 m) compared to male runner rats (about 1200 m) (Fig. 2A). Moreover, female rats were also better than the males in terms of adaptability to the treadmill (data not shown). For these reasons, female rats were chosen in the second experimental design.

The second training programme comprised 5-week training containing two exhaustion tests plus three trainings per week (Fig. 1). In this case, immune status was assessed before (T), immediately after (TE) and 24 h after (TE24) a final exhaustion test. In the exhaustion tests during training, animals ran for 21-25 min at increasing speed, achieving maximum values of 58-72 m/min. The time course of the distance achieved in the exhaustion tests carried out every Monday and Friday (Fig. 2B) shows that for the first 2 weeks a longer distance was achieved when comparing those corresponding to Friday vs Monday –in the same week ( $p < 0.001$ ,  $p = 0.003$  for the first and the second week, respectively), Monday vs Monday ( $p = 0.016$ ) and Friday vs Friday ( $p = 0.040$ ). In weeks 4 and 5, maximum distance began to decrease, the distance run on the last Friday being shorter than that on Friday 2 ( $p = 0.005$ ). In the additional final exhaustion test, these animals ran a maximum distance of  $1419.29 \pm 100.03$  m (mean  $\pm$  SEM), which was lower than that run by female runner rats of the S-TE group ( $p < 0.05$ ) in the first training programme.

**Figure 2. Effect of exercise training on maximum distance run by male and female rats at the final exhaustion test of first training programme (A). Effect of exercise training on maximum distance run by female rats throughout the exhaustion tests in the second training programme (B).** Data are expressed as mean  $\pm$  SEM (n=6-23). ET=exhaustion test, M=Monday, F=Friday. Statistical difference: \*  $p < 0.05$  between marked groups (paired –Figure A– and unpaired –Figure B– Student's t-test).



### Effect of exercise training on body weight and food intake

Male runner rats from the S-TE training did not gain as much weight as male sedentary (SED) rats at the end of the study ( $p < 0.05$ ) (Table 1). However, no differences in body weight increase were observed between runner and SED female rats in either of the experimental designs. Likewise, we found that exercise training had an influence on food intake (data not shown).

**Table 1. Effect of the first training programme (short intensive training, lasting 23 days) and the second training programme (intensive training, lasting 55 days) on body weight increase.** Data are expressed as mean  $\pm$  SEM (n=5-8). Statistical difference: \*  $p < 0.05$  versus the corresponding SED group (Student's t-test).

| Training programme       | Group            | Body weight increase (%) |                    |
|--------------------------|------------------|--------------------------|--------------------|
|                          |                  | Days 0 - 23              | Days 0 - 55        |
| Short intensive training | Male sedentary   | 191.71 $\pm$ 5.56        | -                  |
|                          | Male runner      | 149.60 $\pm$ 9.01*       | -                  |
|                          | Female sedentary | 143.65 $\pm$ 3.19        | -                  |
|                          | Female runner    | 139.06 $\pm$ 5.93        | -                  |
| Intensive training       | Female sedentary | 144.04 $\pm$ 10.80       | 231.49 $\pm$ 17.13 |
|                          | Female runner    | 131.78 $\pm$ 5.96        | 226.80 $\pm$ 9.79  |

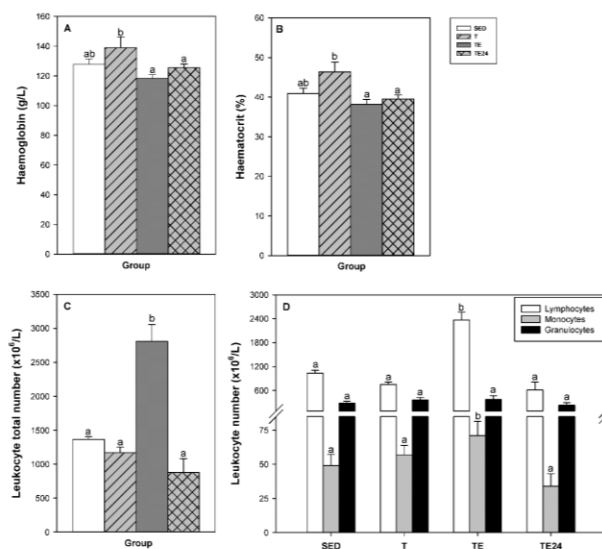


### Effect of exercise training on blood haemoglobin concentration, haematocrit and leukocyte counts

Blood haemoglobin (HGB) concentration and hematocrit (HCT) were assessed only in the second experimental design. The T group tended to increase both HGB concentration and HCT. However, the final exhaustion test (TE group) produced a significant decrease in HGB concentration (Fig. 3A) and HCT (Fig. 3B) compared to the T group, which lasted for at least 24 h (TE24 group).

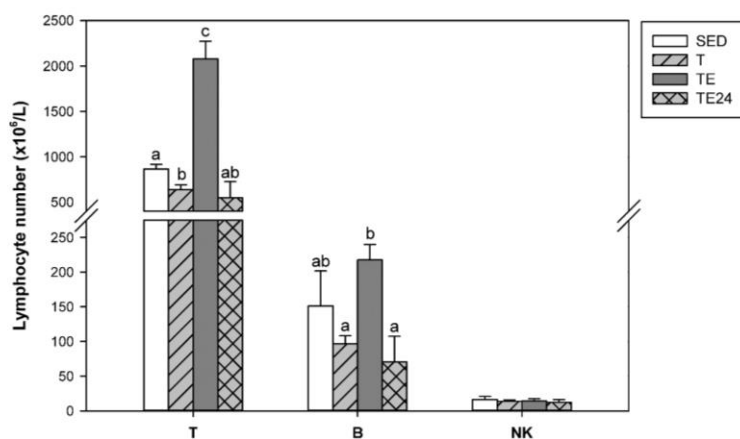
With regard to the blood leukocyte counts, while intensively-trained animals (T group) did not exhibit differences in the total number of leukocytes (Fig. 3C), the final exhaustion test (TE group) induced a twofold increase in the counts of these blood cells ( $p < 0.001$ ), due to higher counts of lymphocytes and monocytes, whereas no changes in the number of blood granulocytes was observed (Fig. 3D). One day after the final exhaustion test (TE24 group), the number of leukocytes returned to the physiological counts (Fig. 3C).

**Figure 3. Effect of exercise training on blood haemoglobin concentration (A), haematocrit (B), number of leucocytes (C) and number of lymphocytes, monocytes and granulocytes (D) in blood.** SED=sedentary rats, T=trained rats, TE=T rats with an additional exhaustion test, and TE24=TE rats 24h after the final exhaustion test. Data are expressed as mean  $\pm$  SEM ( $n=7-8$ ). Values not sharing letters denote significant differences ( $p < 0.05$ ) (Mann–Whitney U test).



To study blood cell changes in depth, the main lymphocyte subsets were determined by flow cytometry. Intensively-trained rats (T group) showed a significant decrease in T-cell counts in comparison with those of the SED animals ( $p=0.032$ , Fig. 4). In contrast, the final exhaustion test (TE group) increased the number of blood T and B lymphocytes ( $p=0.002$  in both cases), which returned to physiological levels 24 h after the final exhaustion test (TE24 group). In the case of NK cells, no changes were detected among the groups (Fig. 4).

**Figure 4. Effect of exercise training on the blood T, B and NK lymphocyte counts.** SED=sedentary rats, T=trained rats, TE=T rats with an additional exhaustion test, and TE24=TE rats 24h after the final exhaustion test. Data are expressed as mean  $\pm$  SEM ( $n=6-12$ ). Values not sharing letters denote significant differences ( $p < 0.05$ ) (Mann-Whitney U test).



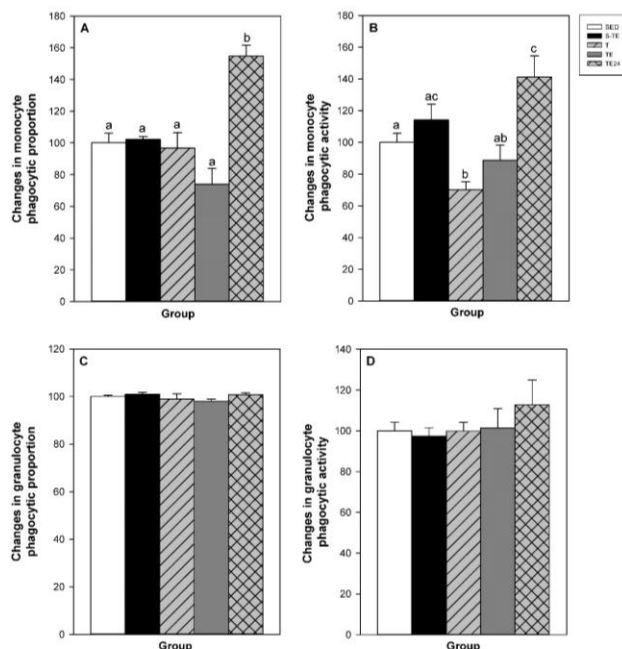
### Effect of exercise training on phagocytic activity

The proportion of phagocytic monocytes and granulocytes and their phagocytic activity were quantified in blood samples (Fig. 5). In the SED group, the percentage of blood phagocytic monocytes was  $50.04 \pm 5.86\%$ , which was considered as 100% to better assess the changes induced by the two training programmes. Phagocytic monocyte proportion increased up to 155% 24 h after the final exhaustion test in trained animals of the second training programme (TE24 group), but no changes were observed beforehand (T and TE groups) (Fig. 5A). With regard to the monocyte phagocytic activity, this was lower in trained animals of the second training programme (T group) than in the SED group (Fig. 5B). However, the TE24 group had a higher phagocytic activity than that in the SED, T and TE groups (Fig. 5B).

With regard to granulocytes, the percentage of phagocytic cells in the SED group was  $95.79 \pm 0.53\%$  (this proportion is considered as 100% to assess changes induced by

training). No changes were observed in any condition either in the proportion of active cells or in their phagocytic activity (Fig. 5C, D).

**Figure 5. Effect of exercise training on blood monocyte phagocytic proportion (A), blood monocyte phagocytic activity (B), blood granulocyte phagocytic proportion (C) and blood granulocyte phagocytic activity (D).** SED=sedentary rats, S-TE= rats with a short intensive training and a final exhaustion test, T=trained rats, TE=T rats with an additional exhaustion test, and TE24=TE rats 24h after the final exhaustion test. Data are expressed as mean  $\pm$  SEM (n=6-12). Values not sharing letters denote significant differences ( $p < 0.05$ ) (one-way ANOVA).



### Effect of exercise training on macrophage cytokine production

To shed some light on macrophage functionality, the cytokine pattern secreted by peritoneal macrophages after lipopolysaccharide (LPS) stimulation was established in the second training programme (Table 2). In intensively trained animals (T group), interferon (IFN)- $\gamma$  and IL-6 concentrations were higher, whereas TNF- $\alpha$ , IL-12 and monocyte chemoattractant protein (MCP)-1 levels were lower than those in SED animals ( $p=0.025$ ,  $p=0.05$ ,  $p=0.027$ ,  $p=0.05$  and  $p=0.038$ , respectively). On the other hand, the final exhaustion test (TE group) produced changes in the cytokine pattern in comparison to the T group by increasing IL-1 $\beta$ , TNF- $\alpha$ , IL-12, MCP-1 and IL-6 concentrations ( $p=0.025$ ,  $p=0.014$ ,  $p=0.014$ ,  $p=0.014$  and  $p=0.05$ , respectively), and decreasing IFN- $\gamma$  and IL-10 levels ( $p=0.05$  and  $p=0.021$ , respectively). One day later (TE24 group), the levels of IFN-

$\gamma$ , IL-1 $\beta$ , TNF- $\alpha$ , MCP-1 and IL-6 were maintained whereas, the secretion of IL-12 and IL-10 increased ( $p=0.027$  and  $p=0.014$ , respectively). There was a positive correlation between IL-10 concentration and monocyte phagocytic activity ( $R=0.777$ ,  $p<0.001$ ).

**Table 2. Effect of exercise training on the cytokine secreted by LPS-stimulated peritoneal macrophages. SED=sedentary rats, T=trained rats, TE=T rats with an additional exhaustion test, and TE24=TE rats 24h after the final exhaustion test. Data are expressed as mean  $\pm$  SEM (n=6-8). Values not sharing letters denote significant differences ( $p < 0.05$ ) (Mann–Whitney U test).**

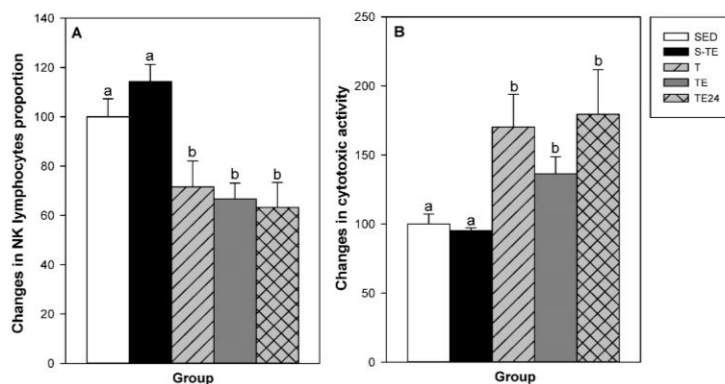
|               | SED group                     | T group                         | TE group                        | TE24 group                       |
|---------------|-------------------------------|---------------------------------|---------------------------------|----------------------------------|
| IFN- $\gamma$ | 100 $\pm$ 34.26 <sup>a</sup>  | 536.49 $\pm$ 4.63 <sup>b</sup>  | 116.69 $\pm$ 17.49 <sup>a</sup> | 115.08 $\pm$ 40.19 <sup>a</sup>  |
| IL-1 $\beta$  | 100 $\pm$ 16.97 <sup>ab</sup> | 59.36 $\pm$ 7.07 <sup>a</sup>   | 118.73 $\pm$ 8.36 <sup>b</sup>  | 102.66 $\pm$ 6.74 <sup>b</sup>   |
| TNF- $\alpha$ | 100 $\pm$ 16.82 <sup>a</sup>  | 45.60 $\pm$ 7.27 <sup>b</sup>   | 106.91 $\pm$ 9.36 <sup>a</sup>  | 122.04 $\pm$ 21.63 <sup>a</sup>  |
| IL-12         | 100 $\pm$ 30.03 <sup>ac</sup> | 32.31 $\pm$ 4.97 <sup>b</sup>   | 81.42 $\pm$ 11.88 <sup>a</sup>  | 171.31 $\pm$ 29.99 <sup>c</sup>  |
| MCP-1         | 100 $\pm$ 9.91 <sup>a</sup>   | 55.91 $\pm$ 2.70 <sup>b</sup>   | 105.98 $\pm$ 1.99 <sup>a</sup>  | 107.13 $\pm$ 3.23 <sup>a</sup>   |
| IL-6          | 100 $\pm$ 19.26 <sup>a</sup>  | 169.81 $\pm$ 16.50 <sup>b</sup> | 242.12 $\pm$ 21.24 <sup>c</sup> | 192.07 $\pm$ 31.88 <sup>bc</sup> |
| IL-10         | 100 $\pm$ 12.93 <sup>ab</sup> | 105.37 $\pm$ 12.37 <sup>a</sup> | 62.70 $\pm$ 2.39 <sup>b</sup>   | 108.17 $\pm$ 10.73 <sup>a</sup>  |

### Effect of exercise training on spleen NK proportion and functionality

The proportion and functionality of spleen NK cells after both training programmes were established (Fig. 6). The NK cell proportion in the spleen of the SED animals was  $9.75 \pm 0.70\%$ , which was considered as 100% to better assess changes induced by exercise. No differences were induced by the short training programme (S-TE group), but all conditions from the second training programme exhibited lower NK cell proportions than the SED group and also than the animals from the S-TE group (Fig. 6A). Interestingly, the spleen NK cell proportion tended to correlate negatively with the total distance run in the final exhaustion test ( $R=-0.569$ ,  $p=0.076$ ), meaning that more distance was accompanied by lower spleen NK cell proportion.

In addition, spleen cytotoxic activity was also modified by exercise. Although the short intensive training programme (S-TE group) did not produce any modification (Fig. 6B), all groups with longer intensive training (T, TE, TE24 groups) markedly increased the spleen NK cytotoxic activity compared to the SED animals ( $p=0.007$ ,  $p=0.021$  and  $p=0.039$ , respectively).

**Figure 6. Effect of exercise training on the spleen NK cell proportion (A) and the spleen NK cytotoxic activity (B).** SED=sedentary rats, S-TE= rats with a short intensive training and a final exhaustion test, T=trained rats, TE=T rats with an additional exhaustion test, and TE24=TE rats 24h after the final exhaustion test. Data are expressed as mean  $\pm$  SEM (n=6-12). Values not sharing letters denote significant differences ( $p < 0.05$ ) (Mann–Whitney U test).



## Discussion

There is no doubt that training when is too intensive can result in an 'open window' period with increasing susceptibility to infectious diseases <sup>10</sup>. In this study we aimed to characterize the innate immune response in two programmes of intensive training in rats. For that purpose, two training programmes using a treadmill were applied: a short intensive training for 2 weeks, including two trainings at day (each lasting 30 min each one) and ending with a final exhaustion test, and the second one with a longer training, lasting 5 weeks including two exhaustion tests plus three trainings per week, in which samples were collected at the end of the training, immediately after an additional exhaustion test and 24 h after this test.

Firstly, in line with other studies <sup>32–34</sup>, Wistar rats were utilized. Male and female rats were tested in the first training program, resulting, as expected, that female rats showed a better performance and adaptation than male rats, as previously reported <sup>35,36</sup>. Accordingly, only female rats were used in the second training programme. The exhaustion tests performed during the second training programme evidenced that the rats improved their exercise performance in the first 2 weeks. However, the distance on the Friday of week 5 was lower than that of week 2, which, together with the lower distance achieved in the additional final exhaustion test compared with those from the short intensive training, evidenced a decline in physical performance possibly due to intensive training. This procedure was previously demonstrated to increase oxidative stress in lymphoid tissues <sup>37</sup>.

With regard to changes in the innate immune functionality, although the first experimental design programme included 2 weeks of intensive training, comprising running twice a day (30 min/session for 5 days per week), it was not able to modify the analysed innate immune functions when assessed immediately after the final exhaustion test. Further studies must clarify whether this short intensive training showed changes before and/or 24 h after the final exhaustion test. In contrast, the second training programme, which consisted of two exhaustion tests per week (running about 21-25 min and achieving speeds of 58-72 m/min) plus 3 days of running once a day for 5 weeks, altered the innate immune functionality. In this second experimental design, we determined innate immune functions in basal conditions (24 h after a standard training, T group), immediately after an additional exhaustion test (TE group) and 24 h later (TE-24 group) to establish what happened in the recovering period. Concerning phagocytic cells and activity, in the T group (basal trained conditions), we found no changes in the proportion of blood phagocytic monocytes and neutrophils, but there was a lower phagocytic activity in monocytes. Moreover, peritoneal macrophage functionality was altered, as observed with a higher production of IL-6 and IFN- $\gamma$ , but lower secretion of IL-12, TNF- $\alpha$  and MCP-1. Higher IL-6 production is in line with increased plasma IL-6 concentrations reported in exercise <sup>38,39</sup>. Even though IL-6 has pleiotropic actions <sup>40</sup>, macrophages are the main cell source of this cytokine, which increased during and after exercise due to muscle damage <sup>41</sup>. Our results suggested that peritoneal macrophages by themselves are able to increase IL-6 secretion in intensively trained rats. On the other hand, macrophages are a heterogeneous cell type able to polarize into M1 and M2 subsets which are associated with inflammatory and anti-inflammatory functions, respectively <sup>14</sup>. M1 macrophages secrete high levels of pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-1, IL-12 and IL-23, and exhibit higher microbicide functions <sup>14,42</sup>. The lower secretion observed in TNF- $\alpha$  and IL-12 could indicate a partial attenuation of M1 macrophage function, which could explain the high susceptibility to infectious diseases reported after high-intensity exercise <sup>43,44</sup>. Previous studies in intensively-trained rats demonstrated a decrease in peritoneal macrophage functions, such as a reduction in phagocytic functions <sup>21,45</sup>, which are partially in agreement with our results. Moreover, we found that peritoneal macrophages from trained rats secreted lower amounts of the chemokine MCP-1 (CCL-2), which is able to regulate migration and infiltration of monocytes/macrophages <sup>46</sup>. These results are in line with the concentrations measured in human plasma after running or performing resistance exercises <sup>47,48</sup>, and would agree with the current decrease of other proinflammatory cytokines. On the other hand, peritoneal macrophages from trained rats secreted higher amounts of IFN- $\gamma$  than those from sedentary animals. The secretion of this proinflammatory cytokine by macrophages is due to IL-12 and IL-18 stimulation <sup>49</sup> and increases M1 activation <sup>50</sup>. The meaning of the higher IFN- $\gamma$  levels in trained rats remains to be elucidated, but it can be suggested as being a counteracting mechanism trying to enhance the decrease in M1 function.

We also assessed what happened in the macrophage functions when these rats performed an additional final exhaustion test, immediately after the test and 24 h later, in the recovery period. The lower blood phagocytic monocyte activity observed in the T group was restored immediately after the final exhaustion test (TE group). However, 24 h later, there was an increase in both the number and the phagocytic activity of blood

monocytes. In fact, in mice running on a treadmill, it has been reported that phagocytic function increases after severe exercise but not after moderate exercise <sup>51</sup>. The enhanced phagocytosis after an exhausting exercise has been related to the influence of glucocorticoids or catecholamines boosting the macrophage function <sup>22</sup>, and could be reflected by an upregulation of the surface receptors <sup>51</sup> as well as by increased cytokine secretion <sup>52,53</sup>. In this sense, immediately after the final exhaustion test, we observed an increase in macrophage IL-1 $\beta$ , TNF- $\alpha$ , IL-12, MCP-1 secretion, which was mainly reduced in the T group whereas IFN- $\gamma$  and IL-10 release decreased. One day after the exhaustion test, we found an increase in IL-10 and IL-12 production by peritoneal macrophages. Both IL-10 and IL-12 are able to enhance the phagocytosis capacity <sup>14,54,55</sup>, which is partially in line with the positive correlation detected between IL-10 and monocyte phagocytic activity in the current results.

Another aspect of the innate immune system considered is the NK cell function, which was determined by both the NK cell proportion and cytotoxic activity in the spleen. NK cells represent an important component of the innate immunity and are widely considered as an important link between exercise and health status <sup>24</sup>. Although different authors have related changes in both the number and activity in blood NK cells <sup>24,56</sup>, there are not so many publications studying the NK population in the spleen. A decreased proportion of blood NK cells has been described in humans <sup>57</sup> and a higher blood NK activity has been reported after chronic exercise (reviewed in <sup>58</sup>). Although we did not detect changes in the number of blood NK cells, we found that T, TE and TE24 groups from the second intensive training had a lower proportion of spleen NK cells. However, these cells had a higher cytotoxic activity, thus suggesting a compensatory immune mechanism in order to maintain the cytotoxic activity in the spleen. In agreement with our results, Blank et al. <sup>59</sup> reported higher NK cell activity in trained mice, although they did not find changes in the splenic NK cell proportion. These changes in rats from the second training programme were not detected in the S-TE group from the first training programme, suggesting that spleen NK percentage could be a biomarker of long intensive training.

Finally, when focusing on blood biomarkers such as haemoglobin concentration, haematocrit and leukocyte counts, we found lower haemoglobin concentrations and haematocrit together with leucocytosis due to higher counts of lymphocytes and monocytes immediately after the exhaustion test. The decrease in haemoglobin concentration and haematocrit could be due to the haemodilution produced for the expansion of blood volume associated with physical activity <sup>60</sup> and might contribute to the decreased performance capacity <sup>61</sup>. Likewise, it is well established that exercise produces leucocytosis <sup>62,63</sup> with an increase in lymphocyte counts <sup>64</sup>. This fact seems to be due to the catecholamines released by exercise <sup>65</sup> that act on lymphocyte adrenergic receptors <sup>66,67</sup>, thus promoting lymphocyte mobilization from both the marginal and the spleen compartments. In our study, the lymphocytosis observed in the TE group was due to increased counts of T and B lymphocytes but not NK cells. These results could be explained by the fact that T cells mobilize faster than other lymphocytes from the spleen compartment <sup>68</sup>.

In summary, our study demonstrated that the intensive training and the exhaustion test carried out in female Wistar rats alter the innate immune system. In particular, intensive training for 5 weeks downregulates M1 macrophage function, but an additional exhaustion test increased monocyte phagocytic activity, which can be related to the altered cytokine pattern from macrophages. Moreover, intensive training for 5 weeks induces a lower proportion of spleen NK cells with a higher cytotoxic activity. These findings could partially explain the decline of the immune system due to intensive exercise. Nevertheless, the current promising results lead us to consider that further studies focused on the acquired immune system are necessary to complete these results.

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**Author Contributions:** M.C. and F.J.P-C. conceived and designed the experiments; S.E-A., M. M-C. and M.C-B. performed the experiments, S.E-A. analysed the data and wrote the paper and M.C. and M.C-B reviewed the manuscript. All authors have read, reviewed and approved the final version of the manuscript.

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ARTICLE 2

***“Changes in lymphocyte composition and functionality after intensive training and exhausting exercise in rats”***

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Sotmès a publicació

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- **XII Congr s de la Societat Catalana d'Immunologia**, Barcelona, novembre de 2018. Ruiz-Iglesias, P.; Estruel-Amades, S.; Massot-Cladera, M.; Garcia-Cerd , P.; Franch, A.; P rez-Cano, F.J.; Castell, M.; Camps-Bossacoma, M. **“Alterations in the spleen immune function due to overtraining and an exhaustive exercise in rats”**.



**Resum ARTICLE 2**

**Objectius:** Donat que l'activitat física intensa altera la funcionalitat del sistema immunitari, l'objectiu d'aquest article va consistir en establir l'efecte de l'exercici físic intens sobre la composició limfocítica i la funcionalitat dels limfòcits de diversos teixits limfoides en rates.

**Material i mètodes:** Rates Wistar mascles i femelles de 4 setmanes d'edat van ser sotmeses a dos programes d'entrenament amb una cinta de córrer. Un programa va incloure dues setmanes d'entrenament intens (dues vegades al dia) i, al final, els animals van realitzar una prova d'extenuació (S-TE). El segon programa es va estendre durant cinc setmanes, i incloïa dues proves d'extenuació i tres entrenaments regulars per setmana i, a dia final, els animals es van sotmetre a una prova d'extenuació final. En aquest segon programa, l'anàlisi dels teixits limfoides es va determinar abans (T), immediatament després (TE) i a les 24 h (TE24) de la prova d'extenuació final. Es va determinar la composició limfocítica en sang, timus i melsa, la funcionalitat dels limfòcits esplènics i la concentració sèrica d'immunoglobulines.

**Resultats:** En sang, només es van detectar canvis limfocítics immediatament després de l'extenuació final en l'entrenament de cinc setmanes. En timus, l'extenuació final després dels dos tipus d'entrenament va disminuir la proporció dels timòcits més madurs. En melsa, l'entrenament durant cinc setmanes va induir un menor percentatge de cèl·lules NK, NKT i TCR $\gamma\delta$ . A més, els animals entrenats (grup T) van mostrar una menor capacitat proliferativa, que va augmentar immediatament després de l'extenuació final. Així mateix, l'entrenament durant cinc setmanes (grup T) va modificar la secreció de citocines per part dels limfòcits esplènics, amb una disminució de la secreció d'IFN- $\gamma$  i IL-10 i un increment de la producció d'IL-6. Per últim, els animals entrenats durant cinc setmanes van mostrar un augment en la concentració sèrica d'IgG.

**Conclusions:** L'entrenament intens durant cinc setmanes canvia la distribució de limfòcits entre els teixits limfoides primaris i secundaris i modifica la seva funcionalitat.





**Changes in lymphocyte composition and functionality after  
intensive training and exhausting exercise in rats**

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### **Abstract**

Exhausting exercise has a deleterious effect on the immune system. Nevertheless, the impact of exercise intensity on lymphocyte composition and functionality remains uncertain. The aim of this study was to establish the influence of intensive training on lymphoid tissues (blood, thymus and spleen) in Wistar rats. Two intensive training programs were performed: a short program, running twice a day for two weeks and ending with a final exhaustion test (S-TE group), and a longer program, including two exhaustion tests plus three runs per week for five weeks. After this last training program, samples were obtained 24 h after a regular training session (T group), immediately after an additional exhaustion test (TE group) and 24 h later (TE24 group). The composition of lymphocytes in the blood, thymus and spleen, the function of spleen cells and serum immunoglobulins were determined. In the blood, only the TE group modified lymphocyte proportions. Mature thymocytes' proportions decreased in tissues obtained just after exhaustion. There was a lower percentage of spleen NK and NKT cells after the longer training program. In these rats, the T group showed a reduced lymphoproliferative activity, but it was enhanced immediately after the final exhaustion. Cytokine secretion was modified after the longer training (T group), which decreased IFN- $\gamma$  and IL-10 secretion but increased that of IL-6. Higher serum IgG concentrations after the longer training program were detected. In conclusion, the intensive training for five weeks changed the lymphocyte distribution among primary and secondary lymphoid tissues and modified their function.

**Keywords:** blood, cytokines, immunoglobulins, lymphocytes, physical activity, spleen, thymus

## **Introduction**

It is well-known that the functionality of the immune system can be modified by physical exercise (Nieman, 2011). In particular, practicing moderate activity enhances immune response but overly intense exercise has a deleterious effect on the immune system (Gleeson, 2007; Leandro et al., 2007). This detrimental effect is mainly observed in the recovery period, which may last from 3 to 72 h, and increases the susceptibility to pathogens, along with the risk of suffering from infectious diseases of the upper respiratory tract (Nieman, 2009).

Changes in the immune system after performing exercise can be found in several lymphoid compartments. In blood, immediately after exhausting exercise, there is leukocytosis, as reported both in humans (Suchánek et al., 2010) and rodents (Krüger et al., 2008). This increase in blood leukocytes comprises higher counts of monocytes, granulocytes and the main subsets of lymphocytes, i.e., B and T (Th and Tc) cells (Dimitrov et al., 2010; Neves et al., 2015). Lymphocytosis is due to the higher release of catecholamines (Graff et al., 2018). Epinephrine and norepinephrine, by binding to adrenergic receptors on lymphocytes, induce the downregulation of adhesion molecules, mainly in mature T lymphocytes, leading to their demargination from reserve pools such as the spleen and other secondary lymphoid organs (Domínguez-Gerpe and Rey-Méndez, 2001; Krüger et al., 2008; Krüger and Mooren, 2007). On the other hand, as long as exercise lasts, there is an increase in the cortisol concentration, which may lead to the release of neutrophils from the bone marrow (Brenner et al., 1998). During the exercise recovery, a secondary lymphopenia appears. This seems to be due to a decrease in Th lymphocyte counts through a redistribution of the cells into the nonlymphoid and lymphoid organs such as the lungs, bone marrow and intestinal Peyer's patches (Guimarães et al., 2017; Krüger et al., 2008). Th cells are a heterogeneous lymphocyte subset, which, after activation, is considered to be mainly differentiated into Th1 and Th2 cells according to the cytokines they release and the consequent activated functions. In terms of physical exercise, a downregulation of Th1 response has been demonstrated 1-24 h after exhausting exercise, but no changes are detected in Th2 lymphocytes (Xiang et al., 2014; Zhao et al., 2012). In addition, apoptosis also seems to occur unevenly between highly differentiated T cells (Krüger et al., 2016a).

With regard to B lymphocytes, although the mobilization pattern is similar to that of T lymphocytes, it happens with less intensity due to the fewer adrenergic receptors they have. This makes the intensity of exercise an important factor in the mobilization of B cells (Walsh et al., 2011). Furthermore, it has been shown that the production of immunoglobulins is also inhibited after exhausting exercise (Gleeson, 2007; Krüger et al., 2016b).

The spleen is an important lymphoid organ where both innate and acquired immune responses can be efficiently mounted. It is crucial for the capture and destruction of pathogens as well as for the induction of the acquired immune response (Mebius and Kraal, 2005). As previously stated, the spleen has a key role as a lymphocyte donor, contributing to the lymphocytosis induced by exercise (Nielsen, 2003). Moreover, excessive exercise has been associated with an abnormal splenic structure (Yuan et al., 2018), along with alterations in its functionality, such as a decrease in T lymphocyte percentage or the mitogenic response of B lymphocytes (Leandro et al., 2007). In addition, exercise influences other lymphoid compartments, such as the thymus. The thymus is responsible for the processes of tolerance, immune reactivity and the production of immunologically competent T cells (Zdrojewicz et al., 2016). The thymic output in elite athletes, evaluated by the circulating levels of T cell receptor excision circles (TRECs), has been reported to be reduced, suggesting a pro-immunosenescence effect of endurance exercise (Prieto-Hinojosa et al., 2014).

Currently, despite the reported harmful effects of exhausting exercise on the immune system, the influence of different exercise intensities and duration on immune system composition and functionality remains uncertain. Previously, we have reported on the alterations in the innate immune system and in oxidative status in intensively trained rats (Estruel-Amades et al., 2019a, 2019b). Nevertheless, the alterations observed cannot totally explain the immunosuppression period observed after an exhausting exercise. Therefore, the aim of this study was to establish the influence of two intensive training programs on the acquired immune system, particularly on the composition and functionality of lymphoid tissues (blood, thymus and spleen) in rats.

## **Material and methods**

### **Animals**

Male and female 3-week-old Wistar rats, provided by Envigo (United Kingdom), were used. The animals were kept in the facilities of the Faculty of Biology (University of Barcelona) in polycarbonate cages (2-3 rats per cage) in a controlled environment in terms of temperature and humidity, in a 12/12 h light/dark cycle. The animals had unrestricted access to food (Teklad Global 14% Protein Rodent Maintenance Diet, Teklad, Madison, WI, USA) and water. After one week of acclimation, the study began. The body weight (BW) of all the rats was monitored throughout the study.

All animal procedures were in accordance with the institutional guidelines for the Care and Use of Laboratory Animals and approved by the Ethical Committee for Animal Experimentation of the University of Barcelona and the Catalonia Government (CEE/UB ref. 464/16 and DAAM 9257, respectively).

### **Experimental designs**

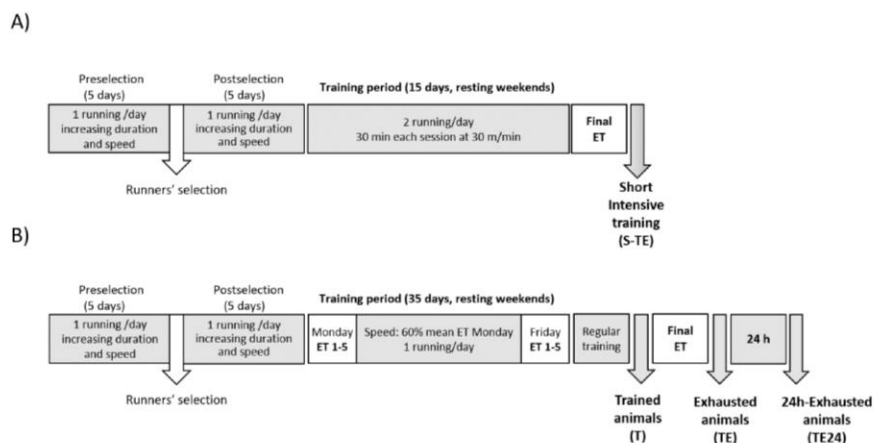
Exercise was enforced by treadmills (LE8700, Panlab, Harvard, USA, and Exer3/6 treadmill Columbus, Ohio, USA). Two training programs, such as those reported previously (Estruel-Amades et al., 2019a), were carried out. In brief, both training programs included three days of treadmill habituation, followed by two weeks (5 days/week) of running once a day with increasing speed (5-25 m/min) and duration (10-25 min). Thereafter, a group of rats (including 6 males and 6 females) followed a short intensive training program that lasted for two weeks (5 days/week) running twice a day (6 h between sessions) for 30 min at 30 m/min (Figure 1A). At the end, the animals were subjected to a final exhaustion test, starting with an initial speed of 5 m/min and with a gradual increase of 1.8 m/min every minute until exhaustion. Rats from this training program constituted the *short intensive training with exhaustion* group (S-TE group).

A second group of rats (only females) were trained for a longer intensive training program that lasted for 5 weeks (5 days/week) (Figure 1B). During these weeks, the animals performed an exhaustion test every Monday and Friday, and on Tuesday, Wednesday and Thursday they ran at 60% of the maximum speed average achieved on the previous Monday for 20, 25 and 30 min, respectively. The exhaustion test on each

Monday and Friday began with running for 15 min at 60% of the speed of the previous Monday's exhaustion test (the speed of the first Monday's exhaustion test was 30 m/min), and then the velocity was increased by 6 m/min every 2 min until animal exhaustion. On the last day, the rats were homogeneously distributed into three groups according to their ability to run: the T group, which were euthanized 24 h after a regular training session; the TE group, which were euthanized immediately after carrying out an additional final exhaustion test; and the TE24 group, which were euthanized 24 h after the additional final exhaustion test (n= 7-8 per group). In the additional final exhaustion test, the animals ran for 15 min at 60% of the speed of the previous Monday's exhaustion test, and then the speed was increased by 3 m/min every 2 min until the animal was exhausted.

Sedentary (SED) groups of rats (5 male and 5 female rats in the short training program, and 8 female rats in the longer training program) were randomly selected at the beginning of the training programs and were exposed to the same conditions of isolation as the rats in the two training programs. As a reward to positively reinforce their running, both runner and SED rats received a 50% solution of condensed milk (100  $\mu$ L/100 g BW).

**Figure 1.** Experimental designs. In the first training program (A), after 2 weeks of intensive training (twice a day, 5 days per week), animals performed a final exhaustion test. In the second training program (B), animals were intensively trained for 5 weeks by carrying out an exhaustion test every Monday and Friday and running the other three days during the week. An additional final exhaustion test was conducted in the 6th week. ET=exhaustion test, S-TE=short intensive training followed by a final exhaustion test, T=trained rats, TE=T rats with a final exhaustion test, TE24=TE rats 24 h after the final exhaustion test.



### Sample collection and processing

The animals were anesthetized using ketamine (Merial Laboratories S.A., Barcelona, Spain) / xylazine (Bayer A.G., Leverkusen, Germany) and exsanguinated. Heart blood was collected and plasma and serum were obtained and kept at -80 °C or -20 °C until cortisol and immunoglobulin quantification, respectively. Hearts, thymuses and spleens were collected and weighed. Spleens and thymuses were immediately processed for lymphocyte isolation.

In the longer training program, blood from the saphenous vein was obtained one week before the final exhausting test in order to study the proportion of T-activated and T-regulatory lymphocytes by flow cytometry.



### **Quantification of cortisol concentration**

Plasma cortisol concentration was measured using DetectX® Cortisol ELISA (Arbor Assays, Michigan, USA) in accordance with the manufacturer's instructions.

### **Lymphocyte isolation from blood, thymus and spleen**

In blood samples, the buffer EL (Qiagen, Hilden, Germany) was added for erythrocyte lysis and lymphocyte purification. Thymuses and spleens were passed by a sterile mesh cell strainer (40  $\mu$ m, Thermo Fisher Scientific, S.L.U, Barcelona, Spain) as previously described (Camps-Bossacoma et al., 2016). In the spleens, osmotic lysis was carried out in order to remove erythrocytes. Lymphocytes from blood, thymuses and spleens, suspended in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL streptomycin-penicillin, 2 mM L-glutamine and 0.05 mM 2-mercaptoethanol (all from Sigma-Aldrich, Madrid, Spain), were counted and their viability was assessed using a Countess™ Automated Cell Counter (Invitrogen™, Thermo Fisher Scientific, S.L.U, Barcelona, Spain).

### **Lymphocyte phenotypic analysis**

The phenotype of blood, thymus and spleen lymphocytes was assessed by using fluorescent monoclonal antibodies, as previously described (Camps-Bossacoma et al., 2016). The antibodies used were specific to rat TCR $\alpha\beta$ , NKR-P1A, CD8 $\alpha$ , CD8 $\beta$ , TCR $\gamma\delta$ , CD45RA, CD4, CD25 (BD Biosciences, Madrid, Spain) and Foxp3 (eBioscience, Frankfurt, Germany), and were conjugated either to fluorescein isothiocyanate, phycoerythrin, peridininchlorophylla protein, allophycocyanin or brilliant violet 421. Briefly, after incubating lymphocytes with saturating concentrations of fluorochrome-conjugated antibodies, cells were fixed with 0.5% p-formaldehyde and stored (4 °C, in darkness) until flow cytometry analysis.

In blood lymphocytes obtained one week before the additional final exhausting test, an intracellular staining was carried out. Cells were incubated with fluorochrome-conjugated anti-CD4 and anti-CD25 antibodies (20 min, 4 °C, in darkness), treated with a fixation-permeabilization solution (eBioscience) (30 min, 4 °C, in darkness) and incubated with fluorochrome-conjugated anti-Foxp3 antibody (eBioscience) (30 min, 4 °C, in darkness) as previously described (Ramos-Romero et al., 2012).

Analyses were performed with a Gallios™ Cytometer (Beckman Coulter, Miami, FL, USA) in the Scientific and Technological Centers of the University of Barcelona (CCiTUB) and data were analyzed by Flowjo v10 software (Tree Star, Inc., Ashland, OR, USA). The percentage of positive cells in the lymphocyte population selected according to their forward-scatter characteristics (FSC) and side-scatter characteristics (SSC) or the proportion of positive cells in a particular lymphocyte population was established. Changes in lymphocyte phenotype are represented considering the SED group values as 100%.

### **Spleen lymphocyte stimulation, proliferation and cytokine release**

Spleen lymphocytes ( $10^5$  cells/well) obtained from the longer training program were stimulated for 48 h, with either concanavalin A (ConA), or pokeweed mitogen (PWM) (5 and 10  $\mu$ g/ml, respectively; Sigma-Aldrich), or incubated with no stimulus. The assay was performed in quadruplicate.

Proliferative cells were quantified using a BrdU Cell Proliferation Assay kit (Merck Millipore, Darmstadt, Germany), as previously described (Grases-Pintó et al., 2018). Results are expressed as the proliferative rate, calculated by dividing the absorbance of stimulated cells by the absorbance of nonstimulated cells.

Supernatants from ConA-, PWM-stimulated and nonstimulated spleen lymphocytes were used to assess the concentration of interferon (IFN)- $\gamma$ , interleukin (IL)-2, tumor necrosis factor (TNF)- $\alpha$ , IL-6, IL-4 and IL-10 by means of ProcartaPlex® Multiplex Immunoassay (Affymetrix, eBioscience, San Diego, USA) as detailed in a previous study (Camps-Bossacoma et al., 2016). Analyses were performed in the CCiTUB using a MAGPIX Cytometer and ProcartaPlex Analyst v1.0 software (Affymetrix). Results are expressed as the ratio of mean fluorescence intensity (MFI) under stimulus with respect to nonstimulated conditions.

### **Immunoglobulin quantification**

Immunoglobulin G and M (IgG and IgM) concentrations in serum and supernatants from nonstimulated spleen cells were determined using a sandwich ELISA (Bethyl Laboratories, Montgomery, TX, USA) following the manufacturer's instructions. Data were analyzed with Ascent v.2.6 software (Thermo Fisher Scientific, S.L.U, Barcelona,

Spain) using the respective standard curves. Changes in immunoglobulin concentrations are represented considering the SED group values as 100%.

### **Statistical analysis**

Statistical analysis was carried out using the IBM Statistical Package for the Social Sciences (SPSS, v22.0, Chicago, IL, USA). The Levene and Shapiro-Wilk tests were used to determine the equality of variances and normal distribution of the data, respectively. After their verification, a one-way ANOVA test was applied. When significant differences were determined, Bonferroni's post hoc test was carried out between groups. The Kruskal-Wallis test was used when data were neither equals nor normally distributed. In the case of significant differences between groups, the Mann-Whitney U test was applied. On the other hand, a repeated-measures ANOVA test was performed to assess time-dependent variables (e.g. BW). Data are represented as mean  $\pm$  standard error. Significant differences were considered when  $P < 0.05$ .

### **Results**

#### **Performance of training programs**

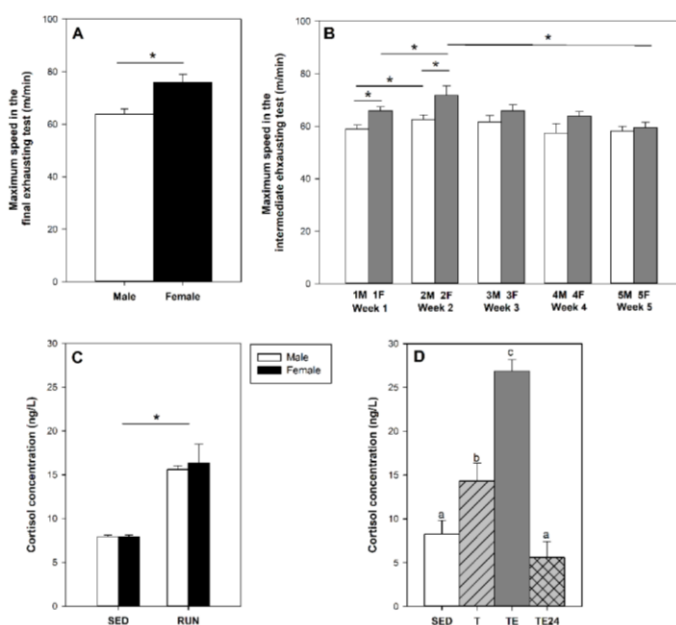
After 2 weeks of intensive exercise, animals from the short training program (S-TE group) completed a final exhausting test with progressively increasing speed. Female rats ran for 36-44 min and achieved a maximum speed ( $\sim 73$  m/min) significantly higher than that of male rats ( $\sim 65$  m/min), which ran for 33-36 min (Fig. 2A).

The longer intensive training program included an exhaustion test every Monday (M) and Friday (F) for five weeks (Fig. 2B). A better performance was progressively observed in the first two weeks of training ( $P=0.005$  between first M and F,  $P=0.048$  between first and second M,  $P=0.006$  between first and second F). However, thereafter, performance decreased gradually and the maximum speed in week 5 was lower than that in week 2 ( $P=0.009$  between second and fifth F). In the final exhausting test performed after the five-week training program, the rats ran for 25-42 min at a maximum speed of  $61.90 \pm 2.22$  m/min (mean  $\pm$  SEM), which was lower than that achieved in the final exhaustion test carried out by female runner rats from the first training program ( $P<0.007$ ).

### Influence of training programs on cortisol concentration

The final exhaustion test in the short training programs (S-TE group) induced a two-fold increase in cortisol levels with respect to the SED group ( $P=0.001$ ) (Fig. 2C). The training in the longer program also doubled the concentration of this hormone ( $P=0.028$ ) and the additional final exhaustion test increased plasma cortisol levels threefold ( $P<0.001$ , Fig. 2D). This effect did not remain after 24 h, when the cortisol levels detected were similar to those from the SED group.

**Figure 2.** Maximum speed achieved by male and female rats at the end of the short intensive training program (A). Maximum speed achieved by female rats throughout the longer intensive training program (B). Cortisol concentration after the short intensive training (C) and the longer intensive training (D). F=Friday, M=Monday, SED=sedentary rats, S-TE=short intensive training followed by a final exhaustion test, T=trained rats, TE=T rats with a final exhaustion test, TE24=TE rats 24 h after the final exhaustion test. Data are expressed as mean  $\pm$  standard error ( $n=5-23$ ). Statistical differences (unpaired –A, C– and paired –B– Student t test, one-way ANOVA –D–):  $P<0.05$  between groups marked with \* and values not sharing common letters.



### **Influence of training programs on body weight and heart, thymus and spleen relative weights**

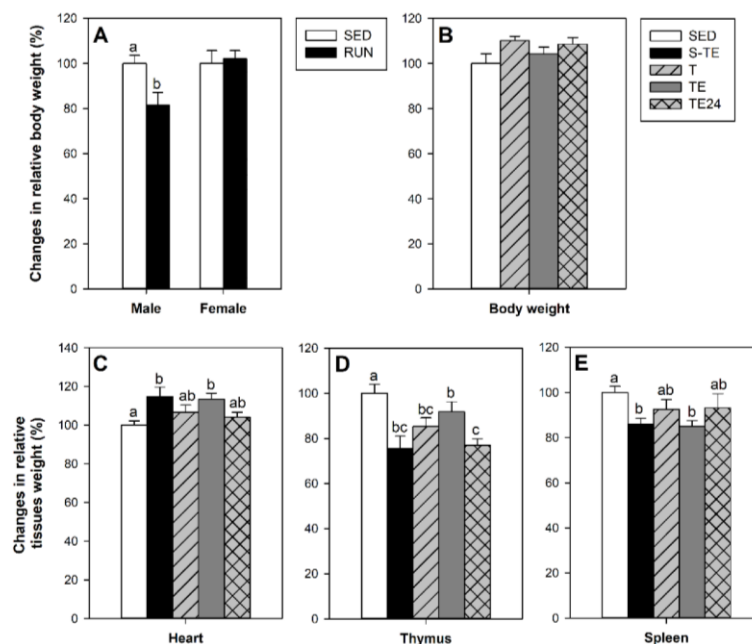
At the end of the study of the short training program, male, but not female, runner rats presented lower BW than their counterparts in the SED group ( $P < 0.05$ , Fig. 3A). In the longer training program, applied only in female rats, no differences were seen between SED and runner rats (Fig. 3B).

The heart relative weight of the SED group was  $0.39 \pm 0.02\%$ , and it was higher in both exhausted groups (S-TE and TE groups,  $P = 0.031$  and  $P = 0.009$  vs SED group, respectively) (Fig. 3C). This difference was not observed in the other runner groups (T and TE24 groups).

The thymus relative weight in the SED group was  $0.31 \pm 0.02\%$ . Both training programs induced a significant reduction in thymus weight with respect to the SED group (S-TE and T groups,  $P = 0.005$  and  $P = 0.017$ , respectively, Fig. 3D). However, immediately after the exhaustion test, the thymus weight in rats from the longer training (TE group) tended to increase and showed a higher thymus weight than that observed 24 h later (TE24 group,  $P = 0.025$ ).

The spleen relative weight in the SED group was  $0.29 \pm 0.02\%$ . The spleen weight was reduced in both exhausted groups (S-TE and TE groups,  $P = 0.008$  and  $P = 0.004$ , respectively, Fig. 3E) in comparison to the SED animals. This difference was not observed in the other runner groups (T and TE24 groups).

**Figure 3.** Changes in body weight at the end of the short intensive training program (A) and the longer intensive training program (B) in comparison to the sedentary group (considered as 100%). Changes in the relative weight of the heart (C), thymus (D) and spleen (E) in comparison to the sedentary group (considered as 100%). SED=sedentary rats, S-TE=short intensive training followed by a final exhaustion test, T=trained rats, TE=T rats with a final exhaustion test, TE24=TE rats 24 h after the final exhaustion test. Data are expressed as mean  $\pm$  standard error (n=5-12). Statistical differences (unpaired Student t test –A–, one-way ANOVA –B– and Mann–Whitney U test –C, D, E–):  $P < 0.05$  between values not sharing common letters.



### Influence of training programs on blood lymphocyte composition

In SED animals, blood lymphocytes comprised  $56.97 \pm 2.54\%$  of Th cells,  $20.04 \pm 1.19\%$  of Tc cells,  $15.34 \pm 3.12\%$  of B lymphocytes,  $2.75 \pm 0.52\%$  of NK cells,  $2.50 \pm 0.22\%$  of NKT cells and  $2.16 \pm 0.19\%$  of TCR $\gamma\delta^+$  lymphocytes. Although neither training program significantly modified the percentage of B cells (Fig. 4A), the proportion of the Th and Tc subsets was altered in the longer training program. In particular, the Th cell percentage increased immediately after the exhaustion test (TE group,  $P=0.005$ ) with respect to the

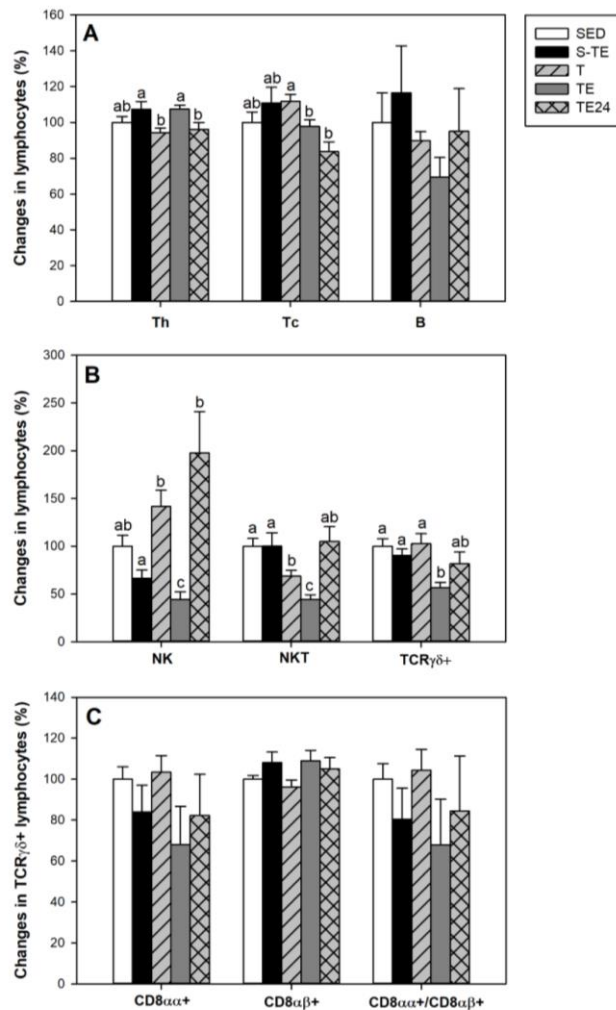
T group, and decreased 24 h later. On the other hand, the Tc cell proportion decreased in the TE group with respect to the T group and remained lower 24 h later.

With regard to blood NK cells, their proportion was markedly reduced after the performance of an exhaustion test in the longer training program and there was a tendency to decrease in the short training program ( $P=0.068$ ) in comparison to the SED group and the other runner groups ( $P=0.003$ , Fig. 4B). In addition, in trained rats and immediately after the exhaustion test (T and TE groups) there was a decrease in the proportion of NKT cells in comparison to the SED animals.

Exhausted animals from the longer training showed a reduction in the percentage of blood  $\text{TCR}\gamma\delta^+$  cells ( $P=0.002$ ) (Fig. 4B), which was due to changes in both  $\text{CD8}\alpha\alpha^+$  and  $\text{CD8}\alpha\beta^+$  subpopulations (Fig. 4C).

The proportion of blood-activated T cells ( $\text{CD25}^+ \text{Foxp3}^-$ ) and regulatory T (Treg) cells ( $\text{CD25}^+ \text{Foxp3}^+$ ) was evaluated in blood  $\text{CD4}^+$  lymphocytes in the longer training program one week before the end of the study. The proportion of activated T cells was  $0.60 \pm 0.09\%$  in the SED group and this did not vary as a result of the intensive training ( $0.78 \pm 0.10\%$  in runner rats). Similarly, no changes were found in the percentage of Treg cells ( $3.22 \pm 0.04\%$  and  $2.94 \pm 0.11\%$  for the SED and runner groups, respectively).

**Figure 4.** Changes in the proportion of blood lymphocytes at the end of the short and the longer intensive training programs compared to the sedentary group (considered as 100%). SED=sedentary rats, S-TE=short intensive training followed by a final exhaustion test, T=trained rats, TE=T rats with a final exhaustion test, TE24=TE rats 24 h after the final exhaustion test. Data are expressed as mean  $\pm$  standard error ( $n=7-12$ ). Statistical differences (Mann–Whitney U test):  $P<0.05$  between values not sharing common letters.



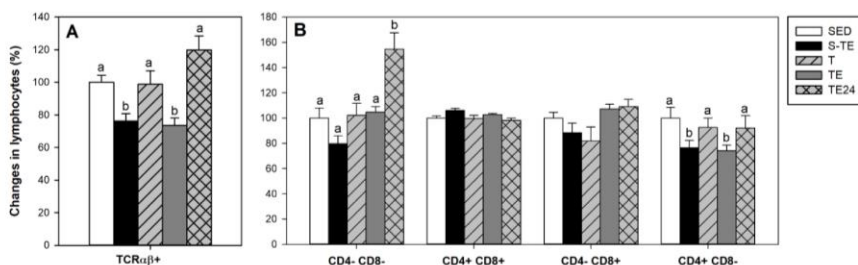


### Influence of training programs on thymus lymphocyte composition

In order to characterize the thymocyte maturation status, the expression of TCR $\alpha\beta$  receptor and CD4 and CD8 coreceptors was analyzed (Figure 5). In the SED group, the proportions of TCR $\alpha\beta$ - and TCR $\alpha\beta$ + thymocytes were  $85.04 \pm 1.13\%$  and  $15.56 \pm 1.05\%$ , respectively. The proportion of TCR $\alpha\beta$ + cells decreased after both exhaustion tests with respect to that of the SED group and animals from the T group ( $P < 0.05$ , Fig. 5A), and this depletion was recovered 24 h later.

On the other hand, the percentages of thymus CD4-CD8-, CD4+CD8+, CD4-CD8+ and CD4+CD8- cells in the SED group were  $3.60 \pm 0.70\%$ ,  $81.80 \pm 2.60\%$ ,  $8.90 \pm 1.47\%$  and  $5.60 \pm 0.50\%$ , respectively. Twenty-four h after the exhaustion test, there was a higher proportion of the most immature subset (CD4-CD8-) in comparison to the SED and runner groups. In addition, both exhausted groups (S-TE and TE groups) showed a lower proportion of the mature CD4+CD8- population than the other groups ( $P < 0.05$ ) (Fig. 5B).

**Figure 5.** Changes in the proportion of thymic lymphocytes at the end of the short and the longer intensive training programs compared to the sedentary group (considered as 100%). SED=sedentary rats, S-TE=short intensive training followed by a final exhaustion test, T=trained rats, TE=T rats with a final exhaustion test, TE24=TE rats 24 h after the final exhaustion test. Data are expressed as mean  $\pm$  standard error ( $n=7-12$ ). Statistical differences (one-way ANOVA):  $P < 0.05$  between values not sharing common letters.



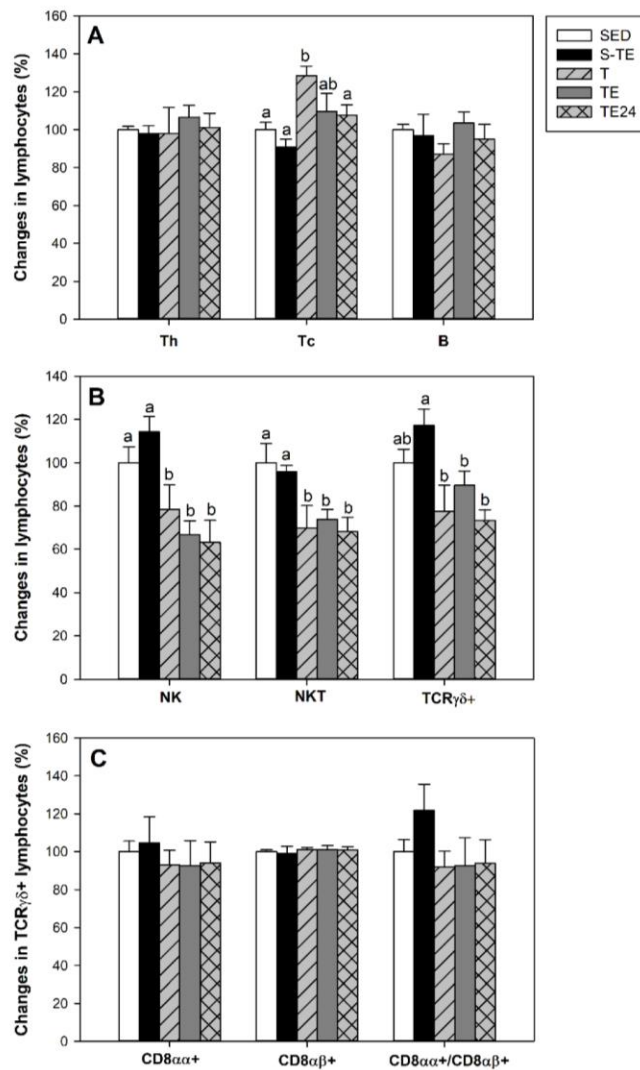
**Influence of training programs on spleen lymphocyte composition and function**

The spleen of SED animals was composed of Th ( $29.51 \pm 0.52\%$ ), Tc ( $13.57 \pm 0.96\%$ ), B ( $28.93 \pm 2.59\%$ ), NK ( $9.30 \pm 0.78\%$ ), NKT ( $4.38 \pm 0.42\%$ ) and TCR $\gamma\delta^+$  ( $4.37 \pm 0.32\%$ ) lymphocytes.

Although the exercise training did not have any effect on the B and Th lymphocyte proportions, the T group had a higher proportion of Tc cells ( $P=0.005$ , Fig. 6A) than SED animals. This higher proportion disappeared after the exhaustion test ( $P=0.028$  between T group and TE24 group). Moreover, the longer training induced a decrease in the proportions of NK and NKT cells ( $P<0.05$ ) which remained reduced with the additional exhaustion test (TE and TE24 groups) in comparison to the SED group ( $P<0.05$ ) (Fig. 6B).

With regard to the TCR $\gamma\delta^+$  cell population, only runner rats in the longer training program showed a tendency for this proportion to decrease (Fig. 6B). The proportion of TCR $\gamma\delta^+$ CD8 $\alpha\alpha^+$  and TCR $\gamma\delta^+$ CD8 $\alpha\beta^+$  subpopulations did not alter either with any tested training (Fig. 6C).

**Figure 6.** Changes in the proportion of spleen lymphocytes at the end of the short and the longer intensive training programs compared to the sedentary group (considered as 100%). SED=sedentary rats, S-TE=short intensive training followed by a final exhaustion test, T=trained rats, TE=T rats with a final exhaustion test, TE24=TE rats 24 h after the final exhaustion test. Data are expressed as mean  $\pm$  standard error (n=7-12). Statistical differences (Mann–Whitney U test):  $P < 0.05$  between values not sharing common letters.

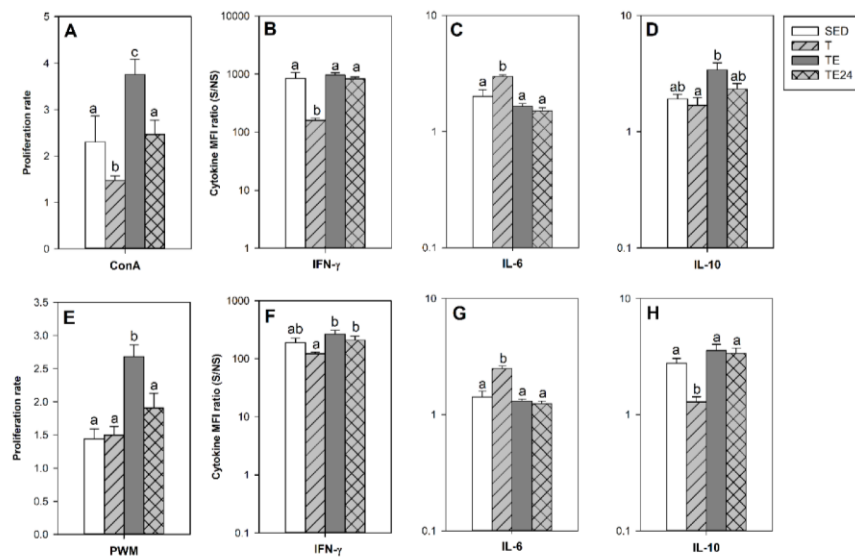


To assess the functionality of the spleen lymphocytes, the proliferative response after ConA and PWM stimulation was established in the longer training program (Fig. 7). After ConA stimulation, the T group showed a lower proliferative rate than SED animals ( $P=0.05$ ). Nevertheless, the cells obtained immediately after the exhaustion test (TE group) had an enhanced proliferation rate which decreased 24 h later ( $P<0.05$  with respect to SED, T and TE24 groups).

The cytokine pattern after ConA stimulation differed when considering the release of IFN- $\gamma$ , IL-6 and IL-10 (Fig. 7B-D). In particular, cells from the T group secreted lower amounts of IFN- $\gamma$  and higher levels of IL-6 ( $P<0.05$  with respect to the SED group). These changes were not observed after the exhaustion test. On the other hand, cells obtained immediately after the exhaustion test (TE group) released higher IL-10 amounts than the cells from the T group ( $P=0.030$ ). There were no significant changes concerning the secretion of IL-2, TNF- $\alpha$  and IL-4 (data not shown).

The proliferative activity after PWM stimulation increased in cells obtained immediately after the exhaustion test (TE group) ( $P<0.05$ ; Fig. 7E). PWM-stimulated cells from the T group increased IL-6 secretion and decreased IL-10 production ( $P=0.004$  and  $P=0.034$ , respectively), as well as having a tendency to decrease IFN- $\gamma$  levels ( $P=0.068$ ; Fig. 7F-H). Similarly, to what occurred in ConA-stimulated cells, these changes disappeared in the TE and TE24 groups. No significant changes in the secretion of IL-2, TNF- $\alpha$  and IL-4 were observed (data not shown).

**Figure 7.** Proliferative response and relative cytokine concentration released by spleen lymphocyte stimulated by concanavalin A (ConA) (A-D) or pokeweed mitogen (PWM) (E-H) at the end of the longer intensive training program. MFI=mean fluorescence intensity, S/NS= stimulated versus nonstimulated, SED=sedentary rats, T=trained rats, TE=T rats with a final exhaustion test, TE24=TE rats 24 h after the final exhaustion test. Data are expressed as mean  $\pm$  standard error (n=7-8). Statistical differences (Mann-Whitney U test):  $P<0.05$  between values not sharing common letters.

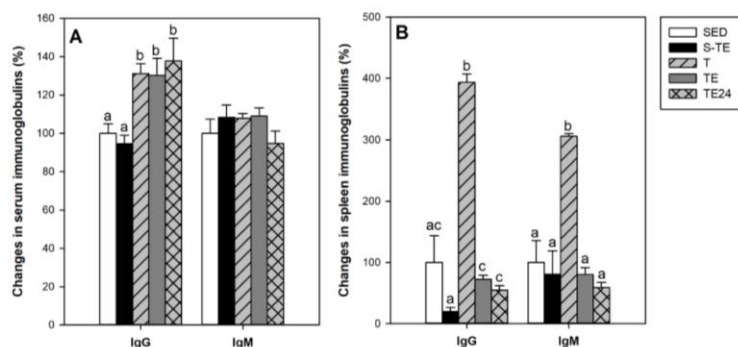


### Influence of training programs on serum and spleen immunoglobulins

At the end of the study, short intensive training did not induce any significant change in the serum IgG and IgM concentrations (Fig. 8A). However, all the groups that followed the longer intensive program had higher serum IgG concentrations than the SED animals ( $P<0.05$ ), with no modifications in serum IgM levels (Fig. 8A).

With regard to the immunoglobulins released by the spleen lymphocytes *in vitro*, in the groups that followed the intensive training program, a marked increase in IgG and IgM production was observed in the T group with respect to the SED animals. However, cells obtained after the exhaustion test (TE and TE24 groups), showed lower immunoglobulin secretion than those obtained before the exhaustion test (T group) ( $P<0.05$ ; Fig. 8B).

**Figure 8.** Changes in immunoglobulin concentrations in serum (A) and in spleen lymphocyte supernatants (B). S-TE=short intensive training followed by a final exhaustion test, T=trained rats, TE=T rats with a final exhaustion test, TE24=TE rats 24 h after the final exhaustion test, SED=sedentary rats. Data are expressed as mean  $\pm$  standard error (n=7-12). Statistical differences (Mann–Whitney U test):  $P < 0.05$  between values not sharing common letters.



## Discussion

A large and growing body of literature has investigated the benefits of moderate exercise for health (Deresz et al., 2007; Janssen and LeBlanc, 2010; Leung et al., 2016; Lujan and DiCarlo, 2013; Warburton et al., 2006). Nevertheless, there is little research focused on the detrimental effects of intensive exercise on the immune system. Thus, the present study was designed to assess the influence of intensive training and exhausting exercise on the acquired immune system in rats. In order to accomplish this objective, two training programs were performed on a treadmill: a short intensive training program, in which male and female rats were intensively trained for two weeks (twice a day, 5 days/week) and ending with a final exhaustion test, and a longer intensive training program, in which female rats were trained intensively by means of two exhaustion tests plus three trainings per week for five weeks before performing an additional final exhaustion test. In this longer training, samples were obtained at the end of the exercise program, immediately after the final exhaustion test and 24 h later,

to assess the immune system before, immediately after the final exhaustion test and after recovery one day later, respectively.

Exercise performance after the two training programs was assessed by the maximum speed achieved by animals in the performed exhaustion tests. In the short training program, female rats achieved better scores than male rats, as previously reported (Estruel-Amades et al., 2019a; Lanza et al., 2015). In spite of this difference, the plasma concentrations of cortisol, a hormone released by the adrenal gland in response to stress situations (Taverniers et al., 2010), which increases proportionally to the exercise intensity (Hill et al., 2008), did not differ between sexes in these intensively trained rats. In the longer intensive training program, performed only in female rats due to the results obtained in the short training, the maximum speed in the exhaustion tests led to the detection of a peak in performance at week 2 followed by a progressive decrease in the following weeks. At the end of the training program, the plasma cortisol concentration was higher in running animals in line with chronic increased cortisol concentrations in the plasma of athletes (Greenham et al., 2018). Moreover, immediately after the additional final exhausting test, the concentration of this adrenal hormone was even higher. All these higher cortisol levels, together with other stress hormones not determined here but known to be released in these conditions (Brenner et al., 1998), must influence the immune system function involving immunosuppression (Vitlic et al., 2014). To assess specifically the acquired immunity after intensive training and exhaustion, lymphocyte composition was determined in three compartments, blood, thymus and spleen, and the function of splenocytes was also established.

We found that the short intensive training program performed, ending with an exhaustion test, and the longer intensive training did not modify blood NK, T and B cell proportions, including the percentage of activated T cells and regulatory T cells. Our results concerning regulatory T cells are in line with those reported in athletes (Minuzzi et al., 2017), although there were other reported changes after the running of a marathon (Clifford et al., 2017). In the exhaustion exercise after the longer training, the blood lymphocyte subset proportions were altered, suggesting that the length and type of exercise are important in the blood lymphocyte response. It is well established that, immediately after intensive exercise, there is a fast lymphocytosis (Suchánek et al.,

2010). As we show here, this lymphocytosis was accompanied by a higher proportion of Th cells, whereas that of Tc, NK, NKT and TCR $\gamma\delta$ + lymphocytes decreased. These results are in line with those reported both in young people after intensive cycling exercise (Kakanis et al., 2010; Park et al., 2016) and in elderly people after walking 30-50 km per day for four consecutive days (van der Geest et al., 2017). Nevertheless, our results did not agree with the higher proportion of blood Tc lymphocytes reported in humans immediately after an endurance exercise (Jin et al., 2015) and, in fact, we found a lower blood Tc proportion that was maintained even 24 h after the exhaustion test. Lower counts of CD8+ T cells have been reported in the recovery period following intense exercise due to the preferential movement of lymphocyte subtypes with potent effector functions out of the blood (Peake et al., 2017), which could explain the current results. As the rats trained for 5 weeks without the final extenuation exercise did not show significant changes in the considered cell subset proportion, the exhaustion test seems a necessary challenge to evidence such blood changes. This challenge can be the result of stress hormones release that influence lymphocyte distribution between lymphoid organs, and that would depend on the cell subset. In fact, lymphocytosis associated with intensive training responds to a higher release of catecholamines (Graff et al., 2018), which induce the downregulation of adhesion molecules producing lymphocyte demargination and release from reserve pools such as the spleen among other secondary lymphoid organs (Domínguez-Gerpe and Rey-Méndez, 2001; Krüger et al., 2008; Krüger and Mooren, 2007). Increased catecholamines are also responsible for modifications in the diameter of blood vessels, producing a higher blood flow in skeletal muscle and the heart and vasoconstriction in splanchnic organs (Nielsen, 2003). Thus, vasodilation must be the cause of higher relative heart weight, whereas vasoconstriction must be responsible for the lower relative spleen weight, both detected immediately after an exhaustion exercise after the short and long intensive training.

Exercise was able to affect the thymus, the primary lymphoid tissue where T cell maturation is completed. We observed that in all the studied groups of both training programs, rats showed lower thymic weight, which is in line with results reported in rats undertaking repeated daily swimming for 21 days (Živković et al., 2005) and under stress induced by 1 h restraint followed by 15 min of forced swimming exercise for two, four



or eight weeks (Sarjan and Yajurvedi, 2019). This decrease has been attributed to thymocyte apoptosis due to chronic stress (Sarjan and Yajurvedi, 2019; Savino and Dardenne, 2000). In the current study the lower thymus weight was accompanied by a lower proportion of TCR $\alpha\beta$ + cells in the S-TE and TE groups, due to a lower proportion of the CD4+CD8- subset, which would also suggest the mobilization of this mature thymic population to the blood, similarly to what occurs in secondary lymphoid organs (24). In addition, our findings suggest that as a consequence of this decrease, 24 h after the exhaustion test there was an increase in the most immature thymocyte population, the CD4-CD8- subset, which could indicate a fast thymus response to Th lymphocyte migration to blood.

The composition and function of splenic lymphocytes were modified by the longer intensive exercise program but not by the short training. In particular, we found that intensive training for 5 weeks was able to increase the proportion of spleen Tc cells whereas there was a decrease in that of NK and NKT cells. Although with the current relative results we are not able to conclude that such a reduction was due to a reduced number of these cellular types, it is important to point out that the proportion of the last cellular types was reduced in all the studied time points of the longer intensive training protocol, suggesting their importance as biomarkers of exercise-derived immunosuppression. Ru *et al.* (Ru and Peijie, 2009) also suggested that the reduced spleen NKT cell proportion after chronic excessive exercise in rats even seven days after the exercise, could play an important role in post-training immunosuppression.

Apart from the changes in the distribution of spleen lymphocytes, intensive training was able to modify their *in vitro* function as assessed by quantifying lymphocyte proliferative activity, cytokine release and antibody production. We found that spleen cells from intensively trained rats responded differently to those of sedentary animals after the T cell stimulus ConA, particularly concerning their proliferation rate, and IFN- $\gamma$  and IL-6 production. Cells from trained rats (T group) showed a lower proliferation rate than those from sedentary rats, and they produced lower IFN- $\gamma$  (Th1 cytokine) but higher IL-6 (Th2 cytokine). These characteristics disappeared after the additional final exhaustion test, when the proliferation rate of spleen cells increased even more than in sedentary animals, producing similar IFN- $\gamma$  and IL-6 amounts to the nontrained rats (TE and TE24

groups), but higher IL-10 secretion (TE group). Similarly, an increase in plasma IL-10 levels was reported in runners immediately after running a marathon (Nielsen et al., 2016). Spleen cells from intensively trained rats showed a similar proliferative ability under the B cell stimulus PWM but secreted higher amounts of IL-6 and lower amounts of IL-10 than sedentary animals. Similarly, as after ConA stimulation, the exhaustion changed these characteristics leading to a higher PWM-stimulated proliferative activity and normalized the pattern of cytokine release. These results suggest that T lymphocytes, but not B cells, from the trained T group have a lower proliferative activity and lower IFN- $\gamma$  as a consequence of the long intensive training, which is in line with results obtained from blood lymphocytes in humans after aerobic physical training for several months (Patiño et al., 2018) and has been associated with strenuous exercise (Shaw et al., 2018). This lower T cell activity with lower ability to secrete cytokines such as IFN- $\gamma$  would have a deleterious effect on the immune system response against pathogens such as viruses. This defect could be enhanced by increased levels of IL-10, which has an immunosuppressive action (Shaw et al., 2018). Stress hormones such as catecholamines and/or cortisol released by exhaustion exercise could increase lymphocyte proliferation and IL-10 secretion, and normalize the production of other cytokines. Moreover, spleen lymphocytes from animals that carried out the longer intensive training were able to synthesize higher amounts of antibodies, both IgG and IgM. It has been reported in this regard that spleen lymphocytes are sensitive to repetitive stress, as was induced here by intensive exercise, which induces upregulation of receptors for corticosterone and noradrenaline in this lymphoid tissue (Li et al., 2018). In addition, exercise can induce a higher production of Th2 cytokines (Crotty and Ahmed, 2004; Smith, 2003), as we found here with increases in IL-6 and IL-10 synthesis, as well as higher cortisol levels that could explain the higher production of antibodies by spleen cells.

Finally, in order to assess the overall functionality of systemic B cells, the serum levels of IgG and IgM were quantified. We found a higher serum concentration of IgG due to the intensive training, with no modifications in IgM concentrations, results that were maintained after the final exhaustion test. These results partially match those of Mckune et al. (Mckune et al., 2005), which reported an increase of 12% in serum IgG but a

decrease of 23% in serum IgM immediately after an ultramarathon. A higher serum IgG concentration may be partially due to an increase in IgG-producing cells and a prolonged serum IgG half-life due to exercise as has been described (Suzuki and Tagami, 2005).

In conclusion, intensive training for five weeks in female Wistar rats, followed or not by an exhaustion test, has an impact on the acquired immune system. In particular, intensive training increases the serum IgG concentration and influences the lymphocyte distribution among compartments, including both primary and secondary lymphoid tissues. These alterations are more evident immediately after an exhaustion test. Moreover, although intensive training decreases T cell proliferative ability, modifies the released cytokine pattern, and increases IgG and IgM *in vitro* production, these changes are counteracted by exhaustion. In spite of these results, a clear immunosuppression associated with intensive training was not found, and further research using either more intensive or longer exercise training must be performed in order to obtain higher exercise-derived immunosuppression.

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**ARTICLE 3**

***“Hesperidin effects on gut microbiota and gut-associated lymphoid tissue in healthy rats”***

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**Resum ARTICLE 3**

**Objectius:** Donat que l'hesperidina presenta diferents propietats beneficioses a l'organisme, l'objectiu d'aquest article va ser aprofundir en els efectes de l'administració oral d'hesperidina sobre el sistema immunitari i, concretament, sobre el teixit limfoide associat a intestí i la composició de la microbiota cecal en rates sanes.

**Material i mètodes:** Per assolir aquest objectiu es van utilitzar rates Lewis mascles de 3 setmanes d'edat, que van rebre hesperidina per via oral tres vegades la setmana durant quatre setmanes a dosis de 100 o 200 mg/kg. Es va determinar la composició de la microbiota i la proporció de bacteris units a IgA en el contingut cecal. També es van establir les proporcions de les principals poblacions de limfòcits en ganglis limfàtics mesentèrics (GLM), així com la seva capacitat proliferativa i secretora de citocines. A l'intestí prim, es van analitzar les citocines i immunoglobulines en rentat intestinal, i l'expressió gènica de diverses molècules implicades en la secreció d'IgA.

**Resultats:** L'administració oral de 200 mg/kg d'hesperidina va augmentar el nombre de bacteris i la proporció de *Lactobacillus* a nivell cecal. A més, l'hesperidina va incrementar el nombre de bacteris units a IgA a nivell cecal i el contingut d'IgA a nivell intestinal. Aquests canvis es van acompanyar d'una disminució de la concentració d'IFN- $\gamma$  i MCP-1 a l'intestí prim. Tanmateix, l'hesperidina va augmentar la proporció de limfòcits TCR $\alpha\beta$ + en GLM, encara que no es van veure canvis ni en la seva capacitat proliferativa ni en la secreció de citocines.

**Conclusions:** L'hesperidina, administrada per via oral a una dosi de 200 mg/kg a rates sanes, presenta efectes immunomoduladors sobre el teixit limfoide associat a intestí, i actua com a prebiòtic modulant la composició de la microbiota cecal.





## Article

# Hesperidin Effects on Gut Microbiota and Gut-Associated Lymphoid Tissue in Healthy Rats

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**Abstract:** Hesperidin, found in citrus fruits, has shown a wide range of biological properties. Nonetheless, a more in-depth investigation is required on the effects on the immune system, and in particular, on the gut-associated lymphoid tissue, together with its relationship with the gut microbiota. Therefore, we aimed to establish the influence of oral hesperidin administration on the intestinal lymphoid tissue and on the gut microbiota composition in healthy animals. Lewis rats were orally administered 100 or 200 mg/kg hesperidin three times per week for four weeks. Microbiota composition and IgA-coated bacteria were determined in caecal content. Mesenteric lymph node lymphocyte (MLNL) composition and functionality were assessed. IgA, cytokines, and gene expression in the small intestine were quantified. Hesperidin administration resulted in a higher number of bacteria and IgA-coated bacteria, with changes in microbiota composition such as higher *Lactobacillus* proportion. Hesperidin was also able to increase the small intestine IgA content. These changes in the small intestine were accompanied by a decrease in interferon- $\gamma$  and monocyte chemotactic protein-1 concentration. In addition, hesperidin increased the relative proportion of TCR $\alpha\beta$ + lymphocytes in MLNL. These results show the immunomodulatory actions of hesperidin on the gut-associated lymphoid tissue and reinforce its role as a prebiotic.

**Keywords:** flavanone; flavonoids; immunoglobulin A; intestinal immunity; prebiotic; polyphenol

## 1. Introduction

Polyphenols, extensively found in plants as a product of their secondary metabolism [1] can be classified into different groups regarding their chemical structure: Phenolic acids, flavonoids, anthocyanidins, stilbenes, and lignans [2]. Within the flavonoid family, the most distinguished subgroups are flavonols (e.g., quercetin, kaempferol, and myricetin), flavanones (e.g., eriodictyol, hesperetin, and naringenin), isoflavones (e.g., daidzein, genistein, and glycitein), flavones (e.g., apigenin, and luteolin), flavan-3-ols (e.g., catechin), and anthocyanins (e.g., cyanidin, delphinidin, and malvidin) [3]. The biological activity of flavonoids in human or animal health and their protective role in several diseases have been widely described [4,5]. There is a growing interest in flavonoids for their anti-inflammatory [6] and anti-diabetic [7] properties, as well as their microbial modulatory actions [8].

Hesperidin is the major flavanone present in citrus fruits, such as orange [9,10]. It is composed by hesperetin being conjugated to rutinose. After hesperidin intake, in the small intestine this flavanone is poorly absorbed via the paracellular pathway and it is highly dependent on the conversion to hesperetin [5,11]. Hesperidin reaches the large intestine where gut microbiota cleaves the attached rutinose moiety, forming hesperetin for further colonic absorption [12]. Hesperidin is gaining attention



due to its different biological activities [3]. In this context, it has been considered a potential protective factor in neurodegenerative diseases [13], by reducing neuro-inflammation in experimental stroke [14]. Moreover, its anti-oxidant [15,16], anti-depressive [17], anti-cancer [18], and immunomodulatory properties have also been described [19–23]. In particular, hesperidin has been shown to increase the production of anti-inflammatory cytokines in vitro [19], to exert an anti-asthmatic effect [20,21], to alter the CD4/CD8+ T cell ratio in the intestine wall of mice infected with *Aeromonas hydrophila* [22], and to influence the lymphocyte composition and functionality of the gut-associated lymphoid tissue in immunized rats [23]. Overall, the immunomodulatory properties of hesperidin were observed in vitro or in infection/immunization models in which the immune system was triggered. Nevertheless, no studies have shown the immune effects of this flavanone in health status.

On the other hand, as far as we know, Unno et al. [24] in the only existing study on the influence of citrus flavanones on the gut microbiota, included them in rat food and showed the prebiotic-like effects of a hesperetin-enriched diet, but not a diet containing hesperidin. In this context, the relationship between the gut microbiota and the function of the gut-associated lymphoid tissue must be emphasized, as its close interaction are well established [25]. Indeed, the intestinal mucosa may be considered as an immunological niche as it hosts a complex immune-functional organ comprised of immunocompetent cells, their products, such as secretory IgA, and the microbiota [25].

While some studies have focused on the influence of hesperidin on the immune response, an in-depth investigation is needed into the effects of hesperidin on the gut-associated lymphoid tissue, which hesperidin reaches first, and moreover, where it can interact with gut microbiota contributing to the crosstalk between gut bacteria and intestinal immune tissue. Therefore, the aim of the present study was to establish the influence of oral hesperidin administration on the function of the gut-associated lymphoid tissue, including the mesenteric lymph node lymphocyte phenotype characterization, and on the microbiota composition in healthy rats. In fact, even in good health, intestinal immune tissue is continuously active, distinguishing innocuous antigens (from food and gut microbiota) from pathogenic microorganisms [26]. The dosage used in the current intervention (100 and 200 mg/kg body weight by oral gavage, three times per week for four weeks) had already been used in a previous study, producing higher immunomodulatory effects than the incorporation of the hesperidin in the rat food [22].

## 2. Materials and Methods

### 2.1. Animals and Diets

The experimental procedure of this study was approved by the Ethical Committee for Animal Experimentation of the University of Barcelona and the Catalonia Government (CEEAA/UB Ref. 464/16 and DAAM 9257, respectively).

Three-week-old male Lewis rats ( $n = 18$ ) were purchased from Janvier Labs (Saint-Berthevin Cedex, France) and housed in polycarbonate cages (3 animals per cage) with large fibrous-particle bedding and tissue papers as enrichment, and monitored daily in a controlled environment of temperature and humidity, in a 12/12 h light/dark cycle in the Faculty of Pharmacy and Food Science animal facility. Water and food (Teklad Global 14% Protein Rodent Maintenance Diet, Teklad, Madison, WI, USA, Supplementary Table S1) were provided ad libitum throughout the study. Body weight (BW) was monitored during the study, as well as the food and water consumption in each cage. Animals were randomly assigned into three groups (six animals/group): Reference (REF), H100, and H200 groups. The H100 group received 100 mg/kg BW hesperidin, the H200 group received 200 mg/kg BW hesperidin, and the REF group received the same volume of 0.5% carboxymethylcellulose that was used as a vehicle (1 mL/100 g BW). Hesperidin was given by oral gavage three times a week for four weeks. The hesperidin, kindly provided by Ferrer HealthTech (Murcia, Spain), had a purity of 95.5%, containing 2% isonaringine, 1.5% didimine, and other impurities, as determined by high-performance liquid chromatography.

## 2.2. Sample Collection and Processing

Blood and feces were collected weekly throughout the study. Serum was kept at  $-20^{\circ}\text{C}$  until immunoglobulin quantification. Fecal samples were dried overnight at  $37^{\circ}\text{C}$  and weighed in order to obtain the fecal homogenates (20 mg/mL), as previously described [23].

After four weeks, animals were anaesthetized intramuscularly with ketamine (Merial Laboratories S.A. Barcelona, Spain) and xylazine (Bayer A.G., Leverkusen, Germany) (90 mg/kg and 10 mg/kg, respectively). In addition to blood and feces, urine (directly from urine bladder) and caecal samples were collected. Moreover, small intestine and mesenteric lymph node (MLN) samples were obtained.

Caecal content was weighed and homogenized to establish microbiota composition as well as to determine IgA content. For microbiota analysis, a part of the caecal homogenates was fixed overnight using 4% paraformaldehyde (Merck, Madrid, Spain). After a low centrifugation, pellets were resuspended with 1:1 phosphate buffered saline (PBS):ethanol, stored at  $-20^{\circ}\text{C}$  for at least 1 h, and maintained at  $-80^{\circ}\text{C}$  until analysis. For caecal IgA, the rest of the caecal homogenate was centrifuged (538 g, 5 min,  $4^{\circ}\text{C}$ ) and supernatants were kept at  $-80^{\circ}\text{C}$  until analysis.

To obtain small intestine washes, the distal small intestine was obtained and processed as previously described [27]. Moreover, a middle part of the small intestine was kept in RNAlater® (Ambion, Life Technologies, Austin, TX, USA) until the determination of gene expression of some molecules by real-time polymerase chain reaction (RT-PCR).

## 2.3. Lymphocyte Isolation from Mesenteric Lymph Nodes

Lymphocytes from MLNs (MLNL) were isolated, as previously detailed [28]. Briefly, MLNs were obtained in aseptic conditions and immediately immersed in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL streptomycin-penicillin, 2 mM L glutamine and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Madrid, Spain). MLNs were smashed in a sterile mesh cell strainer (40  $\mu\text{m}$ , Thermo Fisher Scientific, Barcelona, Spain). After centrifugation, the recovered pellet was resuspended with Dulbecco's Modified Eagle Medium (DMEM-GlutaMAX, Gibco™, Thermo Fisher Scientific). Cell counting and viability were assessed by a Countess™ automated cell counter (Invitrogen™, Thermo Fisher Scientific, Barcelona, Spain).

## 2.4. Quantification of Urine Total Phenolic Content

Total phenolic content in urine was determined according to Folin–Ciocalteu's method using a gallic acid standard curve (0–32  $\mu\text{g/mL}$ ), as previously described [28].

## 2.5. Fluorescence In Situ Hybridization of Gut Microbiota

The bacterial groups present in caecal content were characterized by means of the fluorescent in situ hybridization (FISH) technique coupled to flow cytometry (FCM) analysis using group- or genus-specific fluorochrome-conjugated probes (Table 1), which target the bacterial 16S rRNA (Sigma-Aldrich, Madrid, Spain), as previously established [29–31]. FCM analysis (FacsAria SORP sorter, BD, San José, CA, USA) was carried out in the flow cytometry unit of the Scientific and Technological Centres of the University of Barcelona (CCiT-UB). Analysis was performed using FlowJo v10 software (Tree Star, Inc., Ashland, OR, USA). Microbiota data were expressed as total bacteria counts/g caecal content, as well as the relative percentage of each bacterial group (the sum of all percentages of each studied probe was considered as 100%).

**Table 1.** Oligonucleotide probes and hybridization conditions used in the analysis of intestinal bacteria by FISH-FCM. Y = (C/T), R = (A/G).

| Phylum         | Bacterial Group Targeted                                      | Sequence (5'-3')             | Hybridization Temperature (°C) | Reference |
|----------------|---------------------------------------------------------------|------------------------------|--------------------------------|-----------|
| Firmicutes     | <i>Clostridium coccoideus</i> /<br><i>Eubacterium rectale</i> | GCTTCTTAGTCARGRACCG          | 50                             | [32]      |
|                | <i>Clostridium histolyticum</i> /<br><i>C. perfringens</i>    | TTATGCGGTATTAATCT(C/T)CCCTTT | 50                             | [33]      |
|                | <i>Lactobacillus/Enterococcus</i>                             | GGTATTAGCAYCTGTTCCA          | 50 (lysozyme)                  | [34]      |
|                | <i>Staphylococcus</i> spp.                                    | TCCTCCATATCTCTGCGC           | 46                             | [35]      |
|                | <i>Streptococcus</i> spp.                                     | CACTCTCCCTTCTGCAC            | 46                             | [35]      |
| Bacteroidetes  | <i>Bacteroides/Prevotella</i>                                 | CCAATGTGGGGACCTT             | 46                             | [36]      |
| Actinobacteria | <i>Bifidobacterium</i> spp.                                   | CATCCGGCATTACCACCC           | 50                             | [37]      |
| Proteobacteria | <i>Escherichia coli</i>                                       | CACCGTAGTGCCTCGTCATCA        | 37                             | [38]      |

## 2.6. Quantification of Bacteria Coated to IgA

IgA-coated bacteria in caecal content were determined, as previously described [31]. After centrifuging the diluted caecal homogenates in 1% (v/v) FBS/PBS, the resulting pellet was resuspended in a dilution of fluorescein isothiocyanate (FITC)-anti-rat Ig antibodies (Abcam, Cambridge, UK) in 1% FBS/PBS and incubated for 30 min at 4 °C in the dark. Labelled samples were mixed with propidium iodide (1 mg/mL, Sigma-Aldrich) 15 min prior to FCM analysis in order to label total bacteria. The counts of IgA-coated bacteria were established from the IgA-coated bacteria proportion and the counts of bacteria of each sample using Commercial Flow Check™ Fluorospheres (Beckman Coulter, Inc., Hialeah, FL, USA) combined with propidium iodide. IgA-coated bacteria results were expressed both as caecal IgA+ bacteria counts/g caecal content and as the relative percentage of IgA+ bacteria.

## 2.7. MLN Lymphocyte Composition

MLNLs (500,000 cells/tube) were stained using mouse anti-rat monoclonal antibodies conjugated to FITC, phycoerythrin (PE), peridinin-chlorophyll-protein (PerCP), allophycocyanin (APC) or APC-cyanine (Cy)7. The antibodies used herein were anti-TCRαβ, anti-CD8α, anti-CD4, anti-TCRγδ and anti-CD45RA (BD Biosciences, San Diego, CA, USA). Cells were mixed with PBS containing 2% FBS and 1% NaN<sub>3</sub> and stained, as previously described [28]. The data were acquired with a Gallios™ Cytometer (Beckman Coulter, Miami, FL, USA) in the CCiT-UB and assessed by the Flowjo v10 software (Tree Star, Inc., Ashland, OR, USA). Results are expressed as percentages of positive cells in the lymphocyte population, selected according to their forward-scatter characteristics (FSC) and side-scatter characteristics (SSC).

## 2.8. MLN Lymphocyte Proliferative Capacity

T-lymphocyte activation was carried out in 96-well plates previously coated with anti-CD3/CD28, as previously described [39]. MLNLs (10<sup>5</sup> cells/well) were incubated in quadruplicate with or without stimulus. After 48 h (37 °C, 5% CO<sub>2</sub>), lymphocytes were incubated with the pyrimidine analogue bromodeoxyuridine (BrdU) in order to determine the proliferative capacity using the BrdU Cell Proliferation Assay kit (Merck Millipore, Darmstadt, Germany) following the manufacturer's instructions.

## 2.9. Cytokine Quantification

Concentrations of interleukin (IL)-4, IL-10, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and monocyte chemotactic protein (MCP)-1 were quantified in MLNL supernatants after 48 h of anti-CD3/CD28 monoclonal antibody stimulation, and in small intestine washes by ProcartaPlex®

Multiplex Immunoassay (Affymetrix, eBioscience, San Diego, CA, USA), as detailed in previous studies [23].

#### 2.10. Immunoglobulin Quantification by ELISA

IgA concentration in small intestine washes, serum, faecal and caecal homogenates was quantified using a sandwich enzyme-linked immunosorbent assay (ELISA) technique with a rat IgA ELISA Quantification Set (A110-102) from Bethyl Laboratories (Montgomery, TX, USA). Intestinal and serum IgM and IgG concentrations were quantified using rat IgM and IgG ELISA Quantification sets (Bethyl Laboratories, A110-100 and A110-1364, respectively). The kits were applied following the manufacturer's instructions. Absorbance was measured on a microplate photometer (Labsystems Multiskan, Helsinki, Finland) at 492 nm. Data were interpolated by means of Ascent v.2.6 software (Thermo Fisher Scientific, S.L.U, Barcelona, Spain), according to the respective standard curves.

#### 2.11. Gene Expression by Real-Time Polymerase Chain Reaction (RT-PCR)

The RNA isolation from small intestine samples was done by the RNeasy<sup>®</sup> mini kit (Qiagen, Madrid, Spain) after homogenization in a FastPrep<sup>®</sup>-24 (MP Biomedicals) for 30 s as previously detailed [40]. After RNA concentration and purity determination by a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), the RNA was reverse-transcribed using random hexamers and TaqMan<sup>®</sup> Reverse Transcription Reagents (Applied Biosystems, AB, Weiterstadt, Germany). The RT-PCR was performed using the ABI Prism 7900 HT quantitative RT-PCR system with the following specific PCR TaqMan<sup>®</sup> primers from AB: IgA (331943, made to order); TGF  $\beta$ 1 (Rn00572010\_m1; Inventoried (I)); CCR9 (Rn00597283\_m1, I); CD40 (Rn01423584\_g1, I); CCL25 (Rn01403352\_m1, I); and CCL28 (Rn00586715\_m1, I). Quantification of the target genes was normalized with the housekeeping gene HPRT (Rn01527840\_m1, I). The SDS s2.4 software (AB) was used to analyze the expression data. Relative gene expression levels are represented as a percentage compared with the REF group (considered 100% of gene expression) using the  $2^{-\Delta\Delta Ct}$  method, as previously described [37]. Results are expressed as the mean  $\pm$  standard error of the percentage of these values.

#### 2.12. Statistical Analysis

Scientific data analysis and graphing software SigmaPlot (version 12.0, San Jose, CA, USA), was used to create graphics. Statistical analysis of the data was performed using IBM Social Sciences Software Program (SPSS, version 22.0, Chicago, IL, USA). Variance equality and homogeneity of the data were assessed by Levene's and Shapiro-Wilk tests, respectively. When the equality and normality of the results were established, a one-way ANOVA test was carried out. When significant differences were obtained, Bonferroni's post hoc test was carried out between groups. Kruskal-Wallis (KW) test was used when results were neither equally nor normally distributed. When significant differences were obtained by the KW test, a Mann-Whitney *U* test was performed between groups. To assess differences in parameters throughout the study (e.g., body weight or serum immunoglobulins), a repeated measure ANOVA test was performed. When significant differences were obtained, a Mann-Whitney *U* test was carried out between groups. To assess the correlation between variables, a Pearson correlation was used. Significant differences were considered when  $p < 0.05$ .

### 3. Results

#### 3.1. Influence of Hesperidin on Body Weight, Food Intake and Urine Flavonoid Content

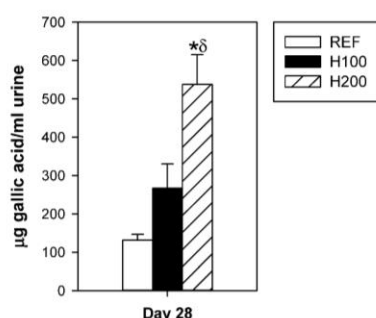
The administration of 100 mg/kg or 200 mg/kg of hesperidin three times per week for four weeks did not modify the body weight compared to the REF group (Table 2). Similarly, no changes were observed in either food or water intake due to the hesperidin during the intervention (Table 2).



**Table 2.** Effect of hesperidin administration on body weight, food and water intake throughout the study. Body weight is expressed as mean  $\pm$  standard error ( $n = 6$ ). Food and water intake data are expressed as mean  $\pm$  standard error obtained from two cages (3 rats/cage).

| Day | Body Weight (g) |                 |                 | Food Intake (g/100 g rat/day) |                |                | Water Intake (g/100 g rat/day) |                |                |
|-----|-----------------|-----------------|-----------------|-------------------------------|----------------|----------------|--------------------------------|----------------|----------------|
|     | REF             | H100            | H200            | REF                           | H100           | H200           | REF                            | H100           | H200           |
| 0   | 48.8 $\pm$ 1.6  | 48.5 $\pm$ 0.8  | 48.4 $\pm$ 0.9  | -                             | -              | -              | -                              | -              | -              |
| 7   | 77.2 $\pm$ 2.6  | 75.3 $\pm$ 1.4  | 74.8 $\pm$ 1.4  | 14.3 $\pm$ 0.5                | 14.7 $\pm$ 0.1 | 14.7 $\pm$ 0.2 | 10.8 $\pm$ 0.1                 | 11.1 $\pm$ 0.2 | 13.8 $\pm$ 2.5 |
| 14  | 117.0 $\pm$ 3.8 | 117.6 $\pm$ 1.9 | 119.2 $\pm$ 2.2 | 11.80 $\pm$ 2.4               | 9.2 $\pm$ 0.0  | 10.0 $\pm$ 0.8 | 10.9 $\pm$ 0.0                 | 12.0 $\pm$ 0.2 | 11.6 $\pm$ 0.3 |
| 21  | 158.0 $\pm$ 3.8 | 162.6 $\pm$ 1.8 | 161.1 $\pm$ 3.0 | 12.6 $\pm$ 0.2                | 12.7 $\pm$ 0.4 | 12.3 $\pm$ 0.2 | 10.1 $\pm$ 0.3                 | 11.6 $\pm$ 0.3 | 10.7 $\pm$ 0.1 |
| 28  | 190.5 $\pm$ 4.7 | 189.9 $\pm$ 1.9 | 189.9 $\pm$ 3.3 | 9.8 $\pm$ 0.3                 | 9.8 $\pm$ 0.0  | 9.4 $\pm$ 0.2  | 11.5 $\pm$ 0.0                 | 11.7 $\pm$ 0.2 | 11.0 $\pm$ 0.8 |

At the end of the study, although both hesperidin administrations resulted in a dose-dependent total polyphenol increase in urine, only the highest dose reached significance (Figure 1). These results evidence its intestinal absorption.



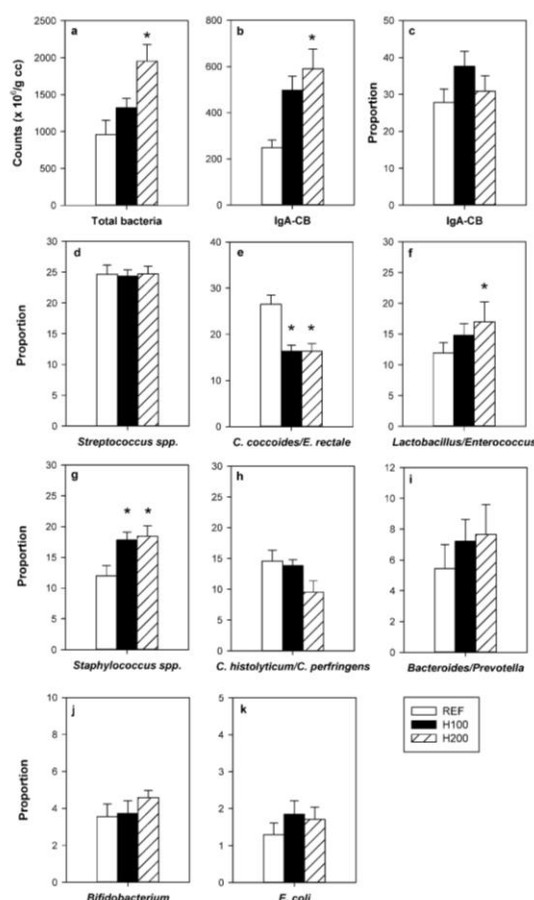
**Figure 1.** Effect of hesperidin administration on total polyphenol content in urine. Data are expressed as mean  $\pm$  standard error ( $n = 5-6$ ). Statistical difference: \*  $p < 0.05$  versus REF group,  $\delta$   $p < 0.05$  versus H100 group (one-way ANOVA).

### 3.2. Influence of Hesperidin on Caecal Microbiota

The administration of the highest dose of hesperidin increased, by twofold, the amount of total bacteria in the caecum compared to that found in the REF group (Figure 2a). Such effect was not observed in the H100 group. No significant correlation between caecal bacteria and urine polyphenol content was found ( $r = 0.505$ ,  $p = 0.113$ ).

In addition to increasing the total amount of caecal bacteria, hesperidin administration promoted the growth of particular bacterial groups as observed by the increased proportions of *Lactobacillus/Enterococcus* (Figure 2f) and *Staphylococcus* (Figure 2g), whereas decreased that of *Clostridium coccoides/Eubacterium rectale* (Figure 2e). Moreover, when considering the bacterial counts, the highest dose of hesperidin induced increases in *Streptococcus*, *Lactobacillus/Enterococcus*, *Staphylococcus*, *Bacteroides/Prevotella*, *Bifidobacterium*, and *Escherichia coli* groups (Supplementary Figure S1).

The number of caecal bacteria coated with IgA significantly increased after the administration of hesperidin ( $p = 0.008$  H200 vs. REF group and  $p = 0.065$  H100 vs. REF group). Nevertheless, the proportion of bacteria coated with IgA was similar between groups (Figure 2b,c).

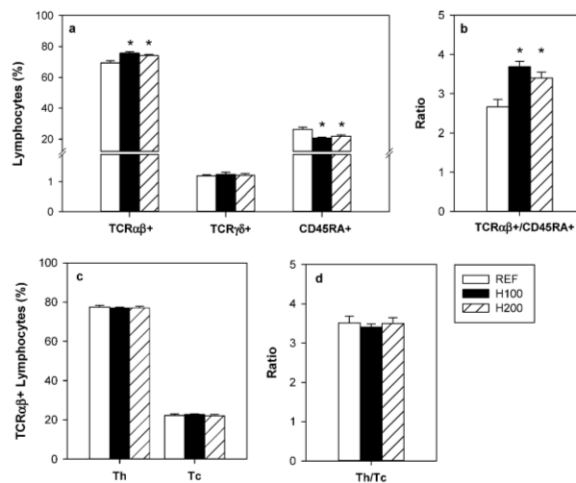


**Figure 2.** Effect of hesperidin administration on bacterial groups determined by FISH-FCM in caecal homogenates. (a) Counts of total bacteria; (b) counts of IgA-coated bacteria; (c) proportion of IgA-coated bacteria; proportions of (d) *Streptococcus* spp.; (e) *Clostridium coccoide/Eubacterium rectale*; (f) *Lactobacillus/Enterococcus*; (g) *Staphylococcus* spp.; (h) *Clostridium histolyticum/Clostridium perfringens*; (i) *Bacteroides/Prevotella*; (j) *Bifidobacterium*; and (k) *Escherichia coli*. Data are expressed as mean  $\pm$  standard error ( $n = 5-6$ ). Statistical difference: \*  $p < 0.05$  versus REF group (one-way ANOVA).

### 3.3. Influence of Hesperidin on MLNL Composition and Functionality

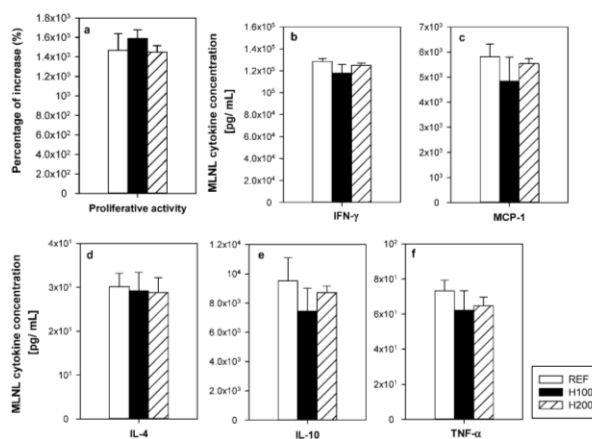
Hesperidin, at both doses, increased significantly the proportion of TCR $\alpha\beta$ + lymphocytes in the MLN without modifying that of TCR $\gamma\delta$ + cells (Figure 3a). Reciprocally, the proportion of B lymphocytes (CD45RA+) was decreased by the hesperidin administration, and consequently the TCR $\alpha\beta$ + /CD45RA+ ratio increased around 33% due to 100 and 200 mg/kg hesperidin (Figure 3a,b).

Regarding the TCR $\alpha\beta$ + cell subsets, no significant differences were observed in either Th (TCR $\alpha\beta$ +CD4+) or Tc (TCR $\alpha\beta$ +CD8+) cells, indicating that the increase in TCR $\alpha\beta$ + cells affected both subsets similarly (Figure 3c). Accordingly, no significant differences were observed between groups in the Th/Tc ratio (Figure 3d).



**Figure 3.** Effect of hesperidin administration on the proportion of mesenteric lymph node lymphocytes. (a) TCR $\alpha\beta$ +, TCR $\gamma\delta$ + and CD45RA+ lymphocytes; (b) TCR $\alpha\beta$ + /CD45RA+ ratio; (c) Th (TCR $\alpha\beta$ +CD4+) and Tc (TCR $\alpha\beta$ +CD8+) lymphocytes; and (d) Th/Tc ratio. Data are expressed as mean  $\pm$  standard error ( $n = 5-6$ ). Statistical difference: \*  $p < 0.05$  versus REF group (one-way ANOVA).

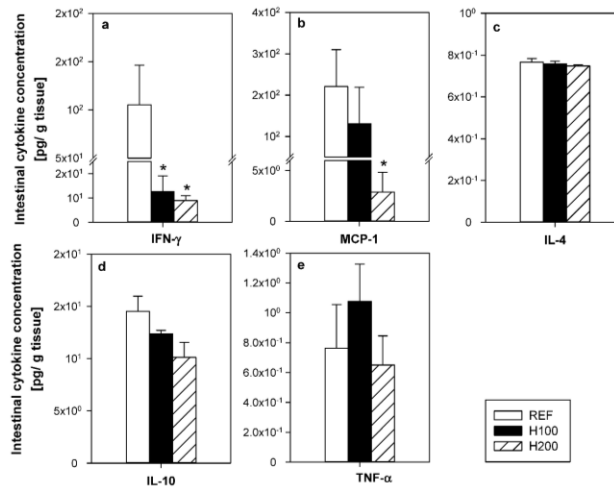
To assess the functionality of the MLNL, their proliferative capacity and the cytokine pattern released after in vitro stimulation were determined (Figure 4a–f). Oral hesperidin administration at any of the doses used did not modify the MLNL ability to proliferate as a result of anti-CD3/CD28 stimulus (Figure 4a). In addition, the hesperidin administration did not significantly alter the secretion pattern of cytokines (IFN- $\gamma$ , MCP-1, IL-4, IL-10, and TNF- $\alpha$ ) released by activated MLNL (Figure 4b–f).



**Figure 4.** Effect of hesperidin administration on (a) the mesenteric lymph node lymphocyte (MLNL) proliferative activity; and (b–f) cytokine release by MLNL after anti-CD3/CD28 stimulation. Data are expressed as mean  $\pm$  standard error ( $n = 5-6$ ).

### 3.4. Effect of Hesperidin on Small Intestine Cytokines and Gene Expression

Hesperidin administration changed the cytokine profile in the small intestine wash (Figure 5a–e). Both doses of hesperidin significantly reduced the concentration of IFN- $\gamma$  in this compartment (Figure 5a), whereas the highest dose significantly decreased that of MCP-1 (Figure 5b). The content of IL-4, IL-10, and TNF- $\alpha$  in the intestinal compartment was not affected by the administration of the flavanone (Figure 5c–e).



**Figure 5.** Effect of hesperidin administration on the cytokine concentration in the small intestine washes (a–e). Data are expressed as mean  $\pm$  standard error ( $n = 5$ –6). Statistical difference: \*  $p < 0.05$  versus REF group (Mann–Whitney  $U$  test).

Regarding the gene expression study, hesperidin intervention did not alter the expression of the genes encoding for IgA, TGF- $\beta$ , CCR9, CD40, CCL25, and CD28 in the small intestine (Table 3).

**Table 3.** Effect of hesperidin administration on gene expression of some molecules in small intestine. The relative mRNA gene expression was calculated assigning the value of 100% to the mean of the rats from the REF group. Data are expressed as mean  $\pm$  standard error ( $n = 5$ –6).

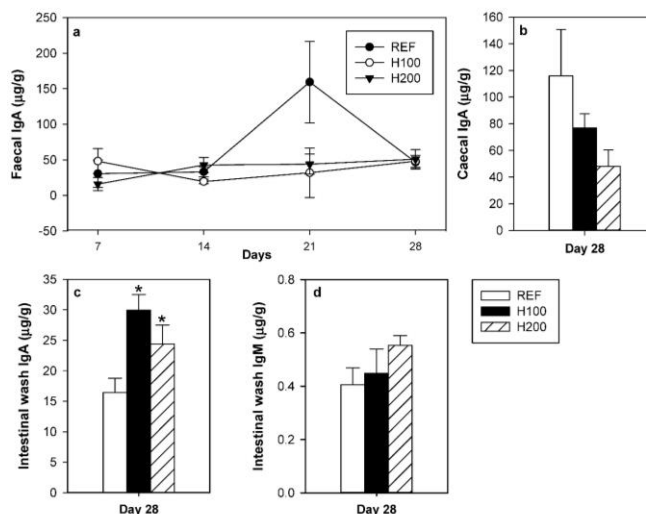
| Gene         | Relative Gene Expression (%) |                    |                    |
|--------------|------------------------------|--------------------|--------------------|
|              | REF                          | H100               | H200               |
| IgA          | 100.00 $\pm$ 13.24           | 148.46 $\pm$ 82.70 | 151.91 $\pm$ 37.14 |
| TGF- $\beta$ | 100.00 $\pm$ 11.88           | 63.00 $\pm$ 14.39  | 85.96 $\pm$ 23.61  |
| CCR9         | 100.00 $\pm$ 12.94           | 69.05 $\pm$ 14.85  | 113.66 $\pm$ 31.96 |
| CD40         | 100.00 $\pm$ 36.80           | 82.97 $\pm$ 30.91  | 62.36 $\pm$ 12.78  |
| CCL25        | 100.00 $\pm$ 13.80           | 66.57 $\pm$ 22.26  | 102.75 $\pm$ 17.79 |
| CCL28        | 100.00 $\pm$ 15.62           | 104.00 $\pm$ 14.08 | 100.00 $\pm$ 14.14 |

### 3.5. Effect of Hesperidin on Intestinal Immunoglobulins

The effect of hesperidin administration on immunoglobulin content at several intestinal compartments was established (Figure 6a–d). Flavanone administration did not modify either the time-course of faecal IgA during the four weeks (Figure 6a) or the caecal IgA content at the end of the study (Figure 6b). Interestingly, both hesperidin doses resulted in a nearly twofold increase of the small intestine content of IgA (Figure 6c). There was no correlation between small intestine IgA content



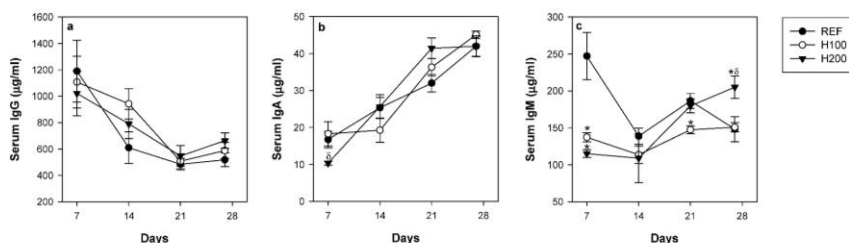
and the urine polyphenol concentration ( $r = 0.247$ ,  $p = 0.491$ ). However, a positive correlation was observed between small intestine IgA levels and T/B ratio in MLNL ( $r = 0.644$ ,  $p = 0.013$ ). No effect on small intestinal IgM was detected (Figure 6d).



**Figure 6.** Effect of hesperidin administration on (a) IgA concentration in faeces collected throughout the study; (b) caecum content; (c) small intestine wash; and on (d) IgM concentration in the small intestine wash. Data are expressed as mean  $\pm$  standard error ( $n = 5$ – $6$ ). Statistical difference: \*  $p < 0.05$  versus REF group (one-way ANOVA).

### 3.6. Influence of Hesperidin on Serum IgG, IgM and IgA Concentrations

The influence of hesperidin on systemic immunity was assessed by determining the concentrations of serum IgG, IgA, and IgM throughout the study (Figure 7a–c). None of the hesperidin administrations altered the time-course of serum IgG (Figure 7a) and IgA concentrations (Figure 7b) in comparison to the REF group. On the contrary, one week of both doses of hesperidin was able to significantly reduce the amount of serum IgM (Figure 7c), but such effect was not observed over the entire study. In fact, and interestingly, serum IgM concentration was increased after four weeks of the highest dose of hesperidin (Figure 7c).



**Figure 7.** Effect of hesperidin administration throughout the study on serum (a) IgG; (b) IgA; and (c) IgM. Data are expressed as mean  $\pm$  standard error ( $n = 5$ – $6$ ). Statistical difference: \*  $p < 0.05$  versus REF group,  $\delta$   $p < 0.05$  versus H100 group (Mann–Whitney  $U$  test).

#### 4. Discussion

Previous studies showed the effect of hesperidin administration on the intestinal immune system in infected mice [22] and in immunized rats [23]. Moreover, the influence of hesperetin, the hesperidin aglycone, on the gut microbiota has been reported [24]. However, as far as we know, the influence of hesperidin in the gut-associated lymphoid tissue on health status as well as its association with gut microbiota has not been previously described. Here we aimed to investigate in-depth the effects of hesperidin on the immune system, focusing on the gut-associated lymphoid tissue and the gut microbiota in healthy rats.

The hesperidin doses used in this study (100 or 200 mg/kg) were established with the aim of observing the effects on both the intestinal immune system and on the microbiota, and taking into account that in a previous study a diet including hesperidin did not alter the gut microbiota composition in rats [24]. The dosage used (oral gavage, three times per week for four weeks) had already been used in a previous study, producing higher immunomodulatory effects than the incorporation of hesperidin in the rat food [23], and as observed here, it did not affect the food and water intake of the animals, nor did their body weight increase. The equivalence between rat and human dosage can be established by taking into account the body surface area [41]. Therefore, 100 mg/kg hesperidin in rat would be equal to 16.22 mg/kg hesperidin in humans, which would be supplied by more than a litre of orange juice for an adult (given that 1 L of orange juice provides about 600 mg hesperidin [42]).

Interestingly, we found that hesperidin administration influenced the gut microbiota composition. The H200 group increased the total bacteria number in caecal content nearly twofold when compared to the reference group. In particular, the administration of 200 mg/kg hesperidin promoted the growth (total counts) of *Streptococcus* spp., *Lactobacillus/Enterococcus*, *Staphylococcus* spp., *Bacteroides/Prevotella*, *Bifidobacterium*, and *E. coli*. Furthermore, this high hesperidin dose did not affect the growth of *C. coccoides/E. rectale* and *C. histolyticum/C. perfringens* groups. In addition, considering their proportions, there was a higher percentage of *Lactobacillus* and *Staphylococcus* spp. Given that *Lactobacillus* genus is associated with many beneficial effects for the host, promoting anti-inflammatory [43,44], anti-viral [45], and anti-diabetic actions [46], our results suggest the potential prebiotic-like effects of hesperidin. In partial agreement with our results, a recent in vitro study demonstrated a rise in bacterial growth, particularly on *Lactobacillus rhamnosus* and also *Bifidobacterium longum* cultures, by the presence of 25 µg/mL of hesperidin and also with its aglycone form [47]. However, another in vitro study showed no impact of hesperidin on *Lactobacillus* spp. cultures, and even though its aglycone form, hesperetin, inhibited their growth [48]. Considering in vivo studies, our findings are in line with a study in which a hesperidin derivate (neohesperidin dihydrochalcone) together with saccharin increased the content of *Lactobacillus* in caecal samples from piglets [49]. Likewise, we found that hesperidin increased the proportion of *Staphylococcus* spp., which does not agree with in vitro studies using berry phenols [50] and in vivo studies carried out with cocoa flavonoids [30,51]. While *Staphylococcus* genus could include opportunistic species such as *S. aureus*, it is worthy to note that an increase in *Staphylococcus* spp. counts has been described in rats after prebiotic fibre-enriched diets [52]. In addition, hesperidin induced a lower proportion of *C. coccoides* group, which was also found in a mouse model treated with a probiotic strain of *Lactobacillus* [53]. On the other hand, Unno et al. [24] showed that the ingestion of a hesperetin-enriched diet for three weeks, but not one containing hesperidin, modified the microbiota composition in rats. The difference in the results between hesperetin and hesperidin was attributed to the higher inhibitory effect of hesperetin on starch digestion, increasing the short-chain fatty acid production in the large intestine. These authors postulated that the attachment of the rutinose moiety to the flavanone structure diminished its effectiveness [24]. On the other hand, the discrepancies with the reported and current results could be attributed to the dosage of the flavanone, which could influence its bioavailability, and which could also be affected by the food matrix. In addition, the different techniques used to assess microbiota composition, as well as the bacterial genera and species investigated cannot be discarded as factors influencing the outcomes.

In relation to IgA, although no differences were found in faecal and serum IgA as a result of the nutritional intervention, it is worth noting that both doses of hesperidin were able to increase the IgA content in the small intestine. Interestingly, such an effect had already been observed with the administration of 100 mg/kg hesperidin, thus behaving differently than on the microbiota. As IgA is the main immunoglobulin in the mucosal surfaces providing a first line of non-inflammatory immune defense in the organism [54], our results suggest that hesperidin may play a principal role in the maintenance of the gut homeostasis by increasing intestinal IgA in healthy animals. In line with this, the ingestion of a 0.5% hesperidin-enriched diet increased small intestine IgA levels in orally sensitized rats [23], as did other dietary polyphenols such as curcumin and those extracted from Japanese fruits [55,56]. On the other hand, no differences were observed, quantifying caecal and faecal IgA, suggesting that the rise in the small intestine IgA content was coated by the higher number of caecal bacteria, as shown by the increase in the number of caecal IgA-coated bacteria. In any case, a higher amount of small intestine IgA and bacteria coated to IgA, as well as beneficial changes in the caecal microbiota, will enhance the intestinal barrier to protect against bacterial invasion, and to compete with pathogenic microorganisms for enterocyte adherence.

Regarding the hesperidin mechanism involved in increasing small intestine IgA content, the study of the gene expression of molecules implicated in the proliferation, differentiation, and migration of IgA+ B cells was inconsistent. Other mechanisms, such as IgA transcytosis and also T cell-independent mechanisms associated with TLR expression, deserve further attention. While the intestinal cytokines were not useful in elucidating the implicated mechanism, we found a decrease in the pro-inflammatory cytokines IFN- $\gamma$  and MCP-1, which agrees with the anti-inflammatory properties of hesperidin reported in vitro [57] and in a model of neuroinflammation [14]. These downregulatory effects on pro-inflammatory cytokines could be useful even in health status, as in the current study, avoiding lower-grade inflammation due to innocuous antigens and promoting tolerance. The effects on IFN- $\gamma$  and MCP-1 do not agree with a previous study carried out in rats immunized with ovalbumin and adjuvants challenging Th2-response [23], indicating the different responses of the immune system when it was specifically triggered to be polarized for a particular immune response. Nonetheless, modifications in the gut associated lymphoid tissue (GALT) were detected through changes in MLN. In this sense, a difference in the T and B lymphocyte proportions were observed, with a higher T cell percentage, which positively correlates with the increase in small intestine IgA levels, showing a major sensibility in the lowest dose of hesperidin. These changes in the T and B cell proportions did not impact on their functionality assessed by the proliferation test and the cytokine secretion. Therefore, further studies should be focused on the hesperidin mechanism of action on the GALT, which finally enhances small intestine IgA content. The effects of hesperidin on the intestinal IgA are not reflected at systemic level. Serum IgG and IgA concentrations were not modified by the flavanone, and only punctual effects on serum IgM levels were observed. In fact, effects on serum immunoglobulin or specific antibody concentrations by hesperidin have not been reported before [23], with the exception of an attenuating action on ovalbumin-specific serum levels of IgE in an asthma mouse model [58].

## 5. Conclusions

In conclusion, our study demonstrates that the administration of hesperidin (200 mg/kg, three times/week for four weeks) is able to influence gut microbiota by increasing the total bacteria number due to, among others, the increased proportions of *Lactobacillus* and *Bifidobacterium* genera, which suggests its potential prebiotic effect. Even though hesperidin showed no effect on serum IgG, IgA, and IgA concentrations, it could play a key role in the gut homeostasis maintenance by increasing intestinal IgA at a lower dose than that required to induce the prebiotic effect. These effects were observed in healthy animals and suggest the potential of hesperidin in preventing and treating some immune-mediated diseases.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6643/11/2/324/s1>, Figure S1: Effect of hesperidin administration on counts of each bacterial group determined by FISH-FCM in



caecal homogenates, Table S1: Composition of the diet (Teklad Global 14% Protein Rodent Maintenance Diet, Madison, USA).

**Author Contributions:** M.C., M.C.-B., A.F. and F.J.P.-C. conceived and designed the experiments, M.C.-B., M.M.-C. and S.E.-A. performed the experiments, S.E.-A., M.M.-C. and M.C.-B. analysed the data and wrote the paper and M.C., F.J.P.-C. and M.C.-B. reviewed the manuscript. All authors have read, reviewed and approved the final version of the manuscript.

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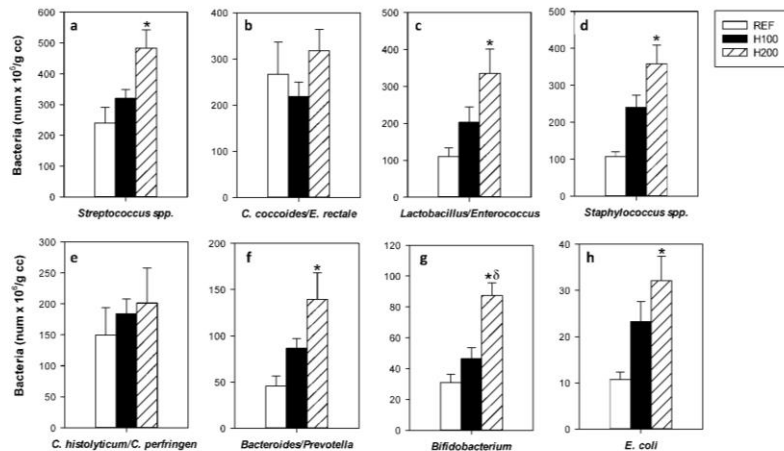
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**Table S1.** Composition of the diet (Teklad Global 14% Protein Rodent Maintenance Diet, Madison, USA).

| Components               | %    |
|--------------------------|------|
| Crude Protein            | 14.3 |
| Fat (ether extract)      | 4.0  |
| Carbohydrate (available) | 48.0 |
| Crude Fibre              | 4.1  |
| Neutral Detergent Fibre  | 18.0 |
| Minerals                 | 2.8  |
| Humidity                 | 8.8  |



**Figure S1.** Effect of hesperidin administration on counts of each bacterial group determined by FISH-FCM in caecal homogenates. (a) *Streptococcus* spp.; (b) *Clostridium coccoides*/*Eubacterium rectale*; (c) *Lactobacillus*/*Enterococcus*; (d) *Staphylococcus* spp.; (e) *Clostridium histolyticum*/*Clostridium perfringens*; (f) *Bacteroides*/*Prevotella*; (g) *Bifidobacterium*; and (h) *Escherichia coli*. Data are expressed as mean  $\pm$  standard error (n = 5–6). Statistical difference: \* p < 0.05 versus REF group, δ p < 0.05 versus H100 group (one-way ANOVA)





**ARTICLE 4**

***“Protective effect of hesperidin on the oxidative stress induced by an exhausting exercise in intensively trained rats”***

Sheila Estruel-Amades, Malén Massot-Cladera, Pau Garcia-Cerdà,  
Francisco J. Pérez-Cano, Àngels Franch, Margarida Castell, Mariona  
Camps-Bossacoma

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Els resultats del present article han estat presentats al congrés següent:

- **XI International Conference on Immunonutrition 2018: Immunonutrition in Health and Disease**, Londres, setembre de 2018. Camps-Bossacoma, M.; Estruel-Amades, S.; Garcia-Cerdà, P.; Massot-Cladera, M.; Periz, M.; Castell, M. **“Overtraining and exhausting exercise alter redox status, composition and functionality of lymphoid tissues in rats”**. Publicat a Ann Nutr Metab 2018, 197-198.



**Resum ARTICLE 4**

**Objectiu:** Donat que l'activitat física intensa comporta estrès oxidatiu, l'objectiu d'aquest article va ser avaluar l'impacte de l'hesperidina sobre l'estat redox dels teixits limfoides després d'un exercici físic intens i extenuant en rates.

**Material i mètodes:** Rates Wistar femelles de 4 setmanes d'edat van ser entrenades intensament durant cinc setmanes. Aquest entrenament consistia en la realització de dues proves d'extenuació i tres entrenaments regulars per setmana i, a dia final, els animals es van sotmetre a una prova d'extenuació final. Durant aquest període, els animals van rebre hesperidina a una dosi de 200 mg/kg o vehicle tres cops per setmana. L'avaluació de l'estat redox es va realitzar abans (T), immediatament després (TE) i a les 24 h (TE24) de la prova d'extenuació final. L'estat oxidatiu es va determinar per la producció d'espècies reactives d'oxigen (ROS) per part de macròfags peritoneals, les activitats superòxid dismutasa (SOD) i catalasa en melsa, timus i fetge, i l'activitat glutatíon peroxidasa hepàtica.

**Resultats:** L'administració d'hesperidina va evitar l'augment de la producció de ROS observada després de la prova d'extenuació final. A més, també va evitar la disminució de l'activitat de catalasa a melsa. Per altra banda, el suplement d'hesperidina es va associar amb un rendiment esportiu superior.

**Conclusions:** L'hesperidina, administrada oralment tres cops a la setmana durant un entrenament intens, evita l'estrès oxidatiu derivat de l'exercici intens i millora el rendiment esportiu.





## Article

# Protective Effect of Hesperidin on the Oxidative Stress Induced by an Exhausting Exercise in Intensively Trained Rats

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**Abstract:** Intensive exercise can lead to oxidative stress, which can be particularly deleterious for lymphoid tissues. Hesperidin has demonstrated its antioxidant activity, but few studies focus on its influence on intensive training. The aim of this study was to assess the impact of hesperidin on the oxidant/antioxidant status of lymphoid tissues after an intensive training program. Wistar rats were trained for five weeks (five days per week), including two exhaustion tests plus three trainings per week. During this period, animals were orally administrated with 200 mg/kg of hesperidin or vehicle (three days per week). The oxidative status was determined before, immediately after and 24 h after an additional exhaustion test. The production of reactive oxygen species (ROS) by peritoneal macrophages, superoxide dismutase (SOD) and catalase activities in spleen, thymus and liver, and hepatic glutathione peroxidase activity (GPx) were assessed. Hesperidin prevented an increase in ROS production induced by the additional exhaustion test. Likewise, hesperidin avoided a decrease in SOD and catalase activities in the thymus and spleen that was found after the additional exhaustion test. The antioxidant effects of hesperidin were associated with a higher performance in the assessed training model. These results suggest that hesperidin, acting as an antioxidant, can prevent oxidative stress induced by exercise and improve exercise performance.

**Keywords:** antioxidant; catalase; exercise; glutathione peroxidase; hesperidin; oxidative stress; ROS; superoxide dismutase; training

## 1. Introduction

Reactive oxygen species (ROS) include a number of reactive molecules and free radicals derived from molecular oxygen as a result of normal cellular metabolism [1]. It is well known that low to moderate levels of ROS are necessary for many physiological processes in an organism, including gene transcription, signaling transduction, redox signal pathways and immune response [2,3]. However, the overproduction of ROS induces adverse effects on lipids, proteins and DNA that eventually lead to cell death. Under physiological conditions, ROS production is counterbalanced by both endogenous or externally supplemented antioxidants [1,3]. The main endogenous antioxidant enzymes are superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) [4]. SOD converts superoxide radicals into H<sub>2</sub>O<sub>2</sub>, which is catalyzed to H<sub>2</sub>O through the action of catalase and/or GPx. The latter enzyme

can also be the donor of electrons to reduce peroxidase. While SOD and GPx are ubiquitously expressed [5], catalase is highly present in liver, kidney and red blood cells [6]. The fact that the membranes of immune cells are made of a high percentage of polyunsaturated fatty acids makes them highly sensitive to oxidative stress [7]. For that reason, since membrane-related signaling and gene expression are critical to maintaining immune cell functionality, they present high concentrations of antioxidant enzymes.

Oxidative stress occurs as a consequence of an imbalance between the production of ROS and antioxidant defense mechanisms [4]. As a result of this imbalance, a wide range of physiological processes can be altered, including immune function [8]. It is widely accepted that exercise, at any intensity, results in ROS synthesis, since it occurs naturally as a condition of oxidative metabolic processes [9,10]. Moderate exercise has been reported to increase ROS levels along with the body's antioxidant defenses, contributing to the maintenance of a healthy oxidant status [11] and activating the immune system [12]. Such beneficial effects of moderate exercise seem to be due to the body's adaptation to oxidative stress, increasing the expression and activity of endogenous antioxidant enzymes [4]. It has been reported that moderate exercise in mice induces a higher expression of catalase and GPx in intestinal lymphocytes [13], and induces higher catalase, SOD and GPx activities in the hippocampus of swimmer rats [14]. However, intensive exercise induces greater ROS production, overtaking the antioxidant system's capacity and leading to oxidative stress [15]. A lot of studies have shown excessive ROS production due to strenuous exercise training in athletes [2,16,17]. The higher ROS production in intensive exercise, among other effects, can compromise muscular force and result in muscle damage, thus decreasing muscle performance [2]. In addition, intensive exercise can enhance glucocorticoid production, which will cause immune suppression [18,19]. The current knowledge about the effect of intensive training on antioxidant enzyme activities in different compartments is quite controversial. Some authors have reported that exhausting exercise decreases thymic and splenic catalase and SOD concentrations [20], as well as lowering blood SOD activity [21]. However, other authors have associated exhaustive exercise with higher gene expression and activity of antioxidant enzymes [22–25]. In any case, an adequate redox balance is essential for the functionality of the immune system, even in conditions of intensive and exhausting exercise.

Polyphenols are products of plant secondary metabolism which are associated with antioxidant activities as well as other beneficial health effects [26–28]. In this sense, polyphenol supplements can improve antioxidant defenses and decrease ROS production. An *in vitro* study demonstrated that total polyphenolic fraction of olive oil increased glutathione levels in HeLa and HepG2 cell cultures [29]. Similarly, an extract of apple peel or cocoa polyphenols produced an increase of GPx, SOD and catalase activities in liver and thymus in rodents [30,31]. In addition, the flavanol catechin increases glutathione reductase (GR) activity in mice immediately after downhill running, a typical eccentric exercise [32].

Hesperidin and its aglycone form, hesperetin, are a type of polyphenols found mainly in citrus fruits [33]. Besides having an antioxidant ability [34], they also exert beneficial biological activities in obesity [35], cancer [36] and in systemic and intestinal immunity [37,38]. The antioxidant activity of hesperidin has been observed in a rat model of pleurisy by reducing liver ROS production and increasing both catalase and SOD liver activities [39]. Furthermore, in older rats, hesperetin has been demonstrated to improve antioxidant status, mainly by increasing both catalase and GR activities in the liver [40]. Hesperidin also protects against oxidative damage induced by exercise through a decrease in lipid peroxidation in swimmer rats [41]. However, although in previous studies hesperidin has shown its effects in the immune system [37,38], there are no studies focused on the oxidative status in lymphoid tissues under intensive exercise-induced oxidative stress. For this reason, the aim of the present study is to assess the impact of hesperidin supplementation on the oxidant and antioxidant status of lymphoid tissues in rats after an intensive training program and after an additional exhaustion test.

## 2. Materials and Methods

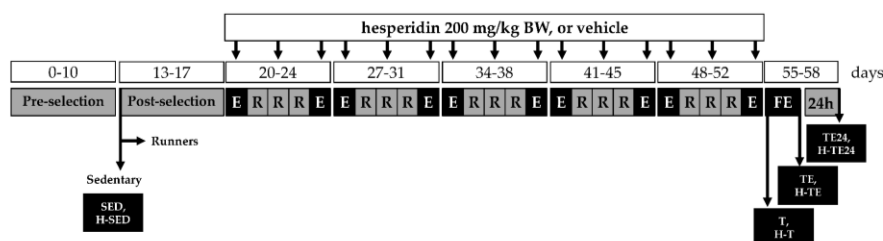
### 2.1. Animals

All animal procedures performed in this study were approved by the Ethical Committee for Animal Experimentation of the University of Barcelona and the Catalonia Government (CEEA/UB ref. 464/16 and DAAM 9257, respectively).

A total of 64 three-week-old female Wistar rats were purchased from Envigo (Huntingdon, United Kingdom) and maintained in the animal facilities of the Faculty of Biology from the University of Barcelona. Experiments began after a 7-day adaptation period. Animals were housed in polycarbonate cages (3 rats per cage) in a controlled environment of temperature and humidity, in a 12/12 h light/dark cycle. Water and food were provided *ad libitum*.

### 2.2. Training Program

Training was performed in two specialized treadmills for rodents: a LE8700 treadmill (Panlab, Harvard, USA) and an Exer3/6 treadmill (Columbus, OH, USA). Both devices allow the speed and exercise length to be controlled. The training program, based on that described by Batatinha et al. [42] and including 5 days per week of training, is summarized in Figure 1. The training program consisted of a preselection period (days 0–10), including 3 days of habituation on a turned-off treadmill, 2 days of resting (weekend) and 5 days in which the treadmill was turned on at a low but increasing speed (0–6 m/min) for an increasing length of time (15–20 min). At the end of this period, sedentary animals (two SED groups,  $n = 8$  each one) were selected including those animals with low ability to run (4/16). Runner animals then began a 5-day period (post-selection period, days 13–17) in which animals ran once a day with increasing conditions of speed and duration (from 10 min/session at a speed of 5 m/min to 25 min/session at 25 m/min). In this period, 3/48 rats were excluded from the study because they were poor runners. Afterwards, the real training program began and lasted 5 weeks, from day 20 to 55 (Figure 1). In each of these 5 weeks, animals carried out an exhaustion test every Monday and Friday and ran for a limited and increasing time on Tuesday, Wednesday and Thursday. Each exhaustion test consisted of running 15 min at 60% of the previous Monday's exhaustion test (the initial speed of the first Monday was 30 m/min), and the speed was progressively increased by 6 m/min every 2 min until exhaustion. The rats were considered exhausted when they could no longer maintain running. The maximum speed reached on Mondays was used as a reference for the following 3 days, running at 60% of such speed for 20, 25 and 30 min, on Tuesday, Wednesday and Thursday, respectively. After each run, the rats received a 50% solution of condensed milk (100  $\mu$ L/100 g body weight (BW)) as a reward to positively reinforce the running. Sedentary animals also received this solution.



**Figure 1.** Experimental design. E = exhaustion test; FE = final exhaustion test; R = running training; T = vehicle-trained group; H-T = hesperidin-trained group; TE = T group with an additional exhaustion test; H-TE = H-T group with an additional exhaustion test; TE24 = TE group 24 h after the exhaustion test; H-TE24 = H-TE group 24 h after the exhaustion test.



At day 55, once the 5-week training program was finished, runner animals were distributed into 3 groups, each one with a similar average in running ability (Figure 1). Two of them carried out an additional final exhaustion test. For this, animals ran for 15 min at 60% of the speed of the previous Monday's exhaustion test, then the speed was increased 3 m/min every 2 min until the exhaustion of the animal.

Sedentary rats were exposed to the same conditions of maintaining, feeding and handling stress without training. BW and food intake were monitored throughout the study.

Oxygen consumption was recorded in representative animals using an OxyletPro system analyzer (Panlab, Harvard, MA, USA) integrated into the LE8700 treadmill. It was recorded in the pre-selection period (considered as basal values) and in every exhaustion test. Metabolism software (Panlab, Harvard, MA, USA) was used to calculate  $O_2$  at 20.8%,  $CO_2$  consumption (according to age, gender and weight of the animal) and the maximal respiratory exchange ratio (RER) (ratio between maximal  $CO_2$  consumption and maximal  $O_2$  consumption).

### 2.3. Hesperidin Supplement

From day 20 to 52 of the training program, a group of sedentary rats (H-SED group,  $n = 8$ ) and a group of runner rats ( $n = 23$ , randomly selected at day 20 among runner rats) received a supplement of the flavanone hesperidin (5,7,3-trihydroxy-4-methoxyflavanone-7-rhamnoglucoside). The hesperidin was provided by Ferrer HealthTech (Murcia, Spain) and had a purity of 88.7% (92% S isomer) containing 5.1% isonaringin, 2.9% didymin, 0.2% hesperetin and 0.4% neohesperidin. The flavanone was administered by oral gavage three times per week at a dose of 200 mg/kg of rat BW. This dosage was chosen following a previous study demonstrating its beneficial effects on the immune system [37,38].

Animals that were not supplemented with hesperidin received the same volume of the vehicle (0.5% carboxymethylcellulose, 1 mL/100 g BW).

### 2.4. Animal Groups and Sample Collection

At day 56, in order to assess the oxidative stress at different time points, both hesperidin-supplemented (H-) and non-supplemented runner rats were distributed into three cohorts, each one with a similar average in the ability to run. The rats from the first cohort (T and H-T groups,  $n = 7-8$  in each group) were euthanized 24 h after a regular training session (day 56). The animals from the second cohort (TE and H-TE groups,  $n = 7-8$  in each group) were euthanized immediately after carrying out an additional final exhaustion test (day 57). The animals from the third cohort (TE24 and H-TE24 groups,  $n = 8$  in each group) were euthanized 24 h after the additional exhaustion test to assess the long-term effects or the recovery (day 58). Likewise, hesperidin-supplemented and non-supplemented SED rats were euthanized randomly over the 3 days.

Animals were anaesthetized using ketamine (Merial Laboratories S.A., Barcelona, Spain)/xylazine (Bayer A.G., Leverkusen, Germany) and exsanguinated. Peritoneal macrophages were collected to assess ROS production. Spleen, thymus and gastrocnemius and soleus muscles were obtained and weighed. Afterwards, a part of the spleen, thymus and liver were frozen at  $-80^\circ\text{C}$  until processing to determine antioxidant activity.

### 2.5. Peritoneal Macrophage Isolation and ROS Production

Peritoneal macrophages were obtained, from five to six representative rats per group, as previously described [43]. Briefly, after the injection of 40 mL of sterile, cold phosphate-buffered saline (PBS) in the peritoneal cavity, a 2-min massage was performed and cells were collected. Once centrifuged ( $538 \times g$ , 10 min,  $4^\circ\text{C}$ ), cells were resuspended with cold Roswell Park Memorial Institute (RPMI) medium without phenol red, and supplemented with 1% heat-inactivated fetal bovine serum, 100 IU/mL streptomycin-penicillin, 2 mM L-glutamine and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Madrid,

Spain). Finally, macrophage counts were assessed by a Spincell hematology analyzer (MonLab Laboratories, Barcelona, Spain) properly calibrated for this type of cell.

ROS production was quantified as previously established [43]. In brief, macrophages were plated ( $10^5$  cells/well) and incubated overnight. Afterwards, the macrophages were washed using warm RPMI medium and incubated for 30 min with  $20\ \mu\text{M}$  of reduced 2',7'-dichlorofluorescein diacetate probe ( $\text{H}_2\text{DCF-DA}$ , Invitrogen, Paisley, UK). Macrophage-derived ROS oxidized  $\text{H}_2\text{DCF-DA}$  to a fluorescent compound (2',7'-dichlorofluorescein), which was quantified by the fluorimeter Modulus<sup>®</sup> Microplate Multimode Reader (excitation 538 nm, emission 485 nm, Turner BioSystems, CA, USA). ROS data were expressed as the area under the curve (AUC) between 0 and 150 min.

#### 2.6. Catalase Activity in Spleen, Thymus and Liver

Spleen, thymus and liver samples were homogenized in 50 mM of potassium phosphate buffer containing 1 mM of EDTA- $\text{Na}_2$  (pH 7.0), using a Polytron<sup>®</sup> PT 10–35 (Kinematica AG, Lucerne, Switzerland), then centrifuged ( $10,000\times g$ , 10 min,  $4\ ^\circ\text{C}$ ). The spleen, thymus and liver supernatants were mixed with an appropriate volume of 0.036%  $\text{H}_2\text{O}_2$  solution, and immediately the absorbance was read at 240 nm (every 10 s for 4 min) using a spectrophotometer (UV-160A, Shimadzu Corporation).

Catalase activity was calculated based on the rate of decomposition of  $\text{H}_2\text{O}_2$ , which was proportional to the reduction in the absorbance. Catalase activity was expressed as units of catalase per g of tissue (U/g).

#### 2.7. SOD Activity in Spleen, Thymus and Liver

SOD activity was measured in the same spleen, thymus and liver homogenates used for catalase determination via the xanthine–xanthine oxidase method. Homogenates were mixed with xanthine oxidase, xanthine and cytochrome C. The xanthine and xanthine oxidase system generated a superoxide, which reduced cytochrome C. The reduction of cytochrome C was monitored spectrophotometrically at 550 nm (every 1 min for 5 min). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. SOD activity was expressed as U/g of tissue.

#### 2.8. Glutathione Peroxidase Activity in Liver

GPx activity was quantified by the Colorimetric Assay Kit (Biovision, Milpitas, CA, USA) following the manufacturer's protocol. Briefly, liver samples were homogenized with the assay buffer, using the Polytron<sup>®</sup> PT 10–35. After centrifugation ( $10,000\times g$ , 15 min,  $4\ ^\circ\text{C}$ ), supernatants were incubated with the reaction mix containing the NADPH, GR and reduced glutathione solutions for 15 min to deplete oxidized glutathione of the sample. Afterwards, cumene hydroperoxide was added to start the reaction, and the absorbance was read at 340 nm immediately and 5 min later. Results are expressed as AU/g of tissue.

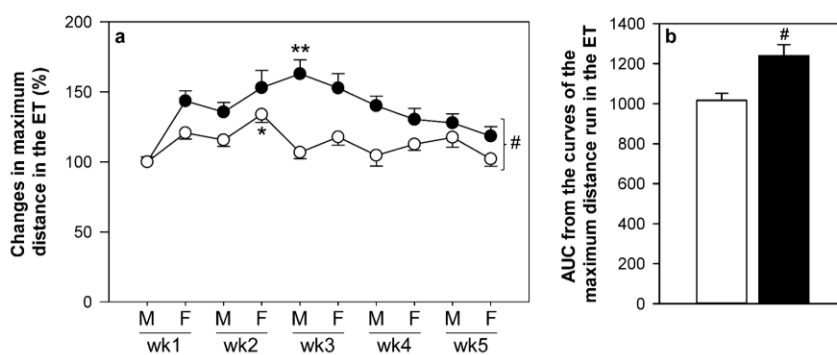
#### 2.9. Statistical Analysis

Analysis of the data was carried out using the IBM Social Sciences software program (SPSS, version 22.0, Chicago, IL, USA). After confirming the equality and normality of the data by Levene's and Shapiro–Wilk's tests, respectively, a two-way ANOVA test was applied. When significant differences were detected, Tukey's post hoc test was performed. A Kruskal–Wallis test was used when results were neither equally nor normally distributed, followed by a Mann–Whitney U test in the case of significant differences among groups. To compare variables during the study (e.g., maximum distance in the exhaustion tests), a repeated-measures ANOVA was applied followed by Student's *t*-test. Significant differences were considered when  $p \leq 0.05$ . With regard to multiple comparisons (Student's *t*-test), *p* value was corrected, dividing it by the number of applied tests (Bonferroni correction).

### 3. Results

#### 3.1. Training Program

The training program lasted five weeks, in which animals ran an exhaustion test every Monday and Friday. The distance in the first exhaustion test was  $898.17 \pm 32.57$  m (mean  $\pm$  SEM) and  $814.43 \pm 34.20$  for non-supplemented and hesperidin-supplemented animals, respectively. The distance run for each rat in the first exhaustion test was considered as 100% and, therefore, the time-course of the exercise performance in the five weeks of the training program was expressed accordingly (Figure 2a). The exercise performance of non-supplemented animals increased gradually up to the Friday of week two ( $p < 0.001$ ), when rats ran about 134% in comparison with the first exhaustion test. Thereafter, performance decreased and ranged between 102% and 127% compared with the first exhaustion test (Monday, week one).



**Figure 2.** Changes in maximum distance run in the exhaustion tests throughout the study in percentage in comparison with the first day (a), and AUC from these curves showing changes in maximum distance run in the exhaustion tests (b). AUC = area under the curve, ET = exhaustion test, M = Monday, F = Friday, wk = week. The non-supplemented group is represented by white symbols and bars, and the hesperidin group by black symbols and bars. Data are expressed as mean  $\pm$  SEM ( $n = 22$ – $23$ ). Statistical difference: \*  $p < 0.001$  in the non-supplemented group vs. the first and the last day (peak of performance); \*\*  $p < 0.001$  in the hesperidin group vs. the first and the last day (peak of performance); #  $p < 0.005$  between non-supplemented and the hesperidin-supplemented groups (Student's  $t$ -test).

Hesperidin-supplemented animals showed a better performance than non-supplemented animals ( $p < 0.005$ ). In fact, the highest performance of hesperidin-supplemented rats was achieved on the Monday of week three, when rats ran about 158% in comparison with the first exhaustion test ( $p < 0.001$ ). Later, performance decreased and ranged between 121% and 152% compared to the first exhaustion test. The higher performance in the hesperidin-supplemented rats was also observed when considering the AUC throughout the study (Figure 2b). In addition, the absolute distance ran for hesperidin-supplemented and non-supplemented animals is summarized in Supplementary Figure S1, which also shows a higher performance in those hesperidin-supplemented animals in the middle of the study.

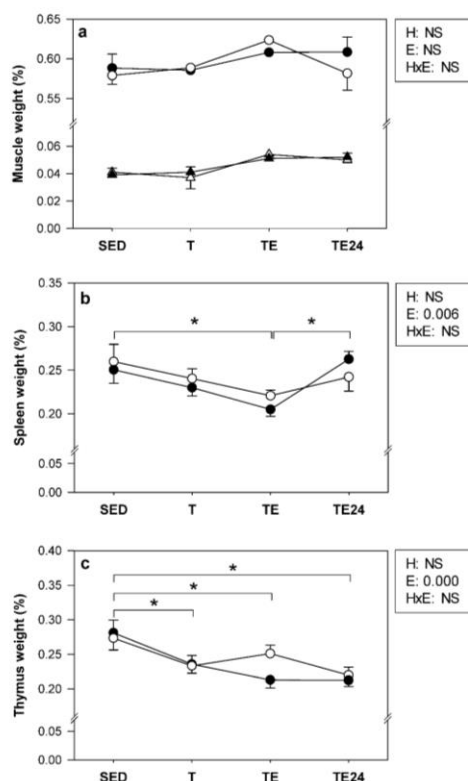
RER values throughout the exhaustion tests showed values around 1, meaning that the intensive training applied was mainly aerobic and neither exercise nor hesperidin-supplementation influenced  $O_2$  uptake.

In spite of these previous results, the final additional exhaustion test performed by the TE, H-TE, TE24 and H-TE24 groups showed no differences between interventions: non-supplemented animals ran a maximum distance of  $1419.3 \pm 100.0$  m, while hesperidin-supplemented rats ran  $1474.9 \pm 138.7$  m.

### 3.2. Body Weight, Food Intake and Organ Weight

No differences were observed in BW resulting from the hesperidin administration at the end of the intensive training program (day 52). However, both hesperidin- and non-supplemented runner animals showed higher BW compared with the corresponding SED group (Table 1). Regarding food intake, no differences were observed due to exercise training or hesperidin supplementation (Table 1).

At the end of the study, the relative weight of the gastrocnemius and soleus muscles, spleen and thymus were considered (Figure 3). No changes were detected in either muscle's relative weight from the exercise training or hesperidin administration. In the case of the spleen, hesperidin did not affect its weight. However, the TE condition caused a decrease in the spleen relative weight with respect to SED and TE24 animals. Regarding the weight of the thymus, T, TE and TE24 had a lower thymic weight compared to the SED animals, both in hesperidin- and non-supplemented animals, without any effect as a result of hesperidin administration being observed.



**Figure 3.** Relative weight of gastrocnemius muscle (a, circles), soleus muscle (a, triangles), spleen (b) and thymus (c) at the end of the study. The non-supplemented groups are represented by white symbols (○) and the hesperidin groups by black symbols (●). SED = sedentary groups, T = trained groups, TE = T groups with an additional exhaustion test, TE24 = TE groups 24 h after the exhaustion test, NS = no statistically significant differences detected. Data are expressed as mean ± SEM ( $n = 7-8$ ). Statistical difference: the inset table shows two-way ANOVA results when applied (H, hesperidin; E, exercise, HxE, interaction between hesperidin and exercise). \*  $p < 0.05$  between exercise conditions for both the non-supplemented and the hesperidin-supplemented groups.



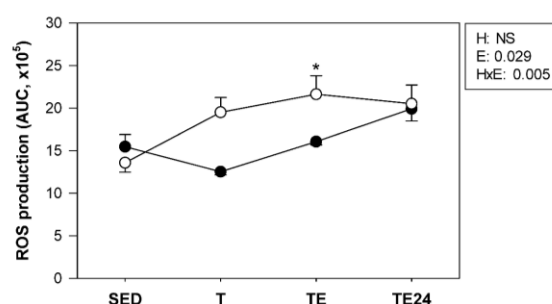
**Table 1.** Body weight of the animals at the beginning of the study, and before and after the five-week training program and hesperidin supplementation (day 20 and 52, respectively), and food intake before and after the five-week training program and hesperidin supplementation.

| Group | Body Weight (g) |               |                 | Food Intake (g/day/rat) |              |
|-------|-----------------|---------------|-----------------|-------------------------|--------------|
|       | Day 0           | Day 20        | Day 52          | Day 20                  | Day 52       |
| SED   | 67.67 ± 3.41    | 144.80 ± 3.62 | 206.57 ± 8.22   | 15.76 ± 0.29            | 15.68 ± 0.10 |
| H-SED |                 |               | 205.00 ± 4.46   |                         | 15.77 ± 0.19 |
| Run   | 71.85 ± 1.91    | 154.33 ± 1.59 | 222.17 ± 3.09 * | 15.27 ± 0.21            | 14.86 ± 0.49 |
| H-Run |                 |               | 221.65 ± 3.61 * |                         | 15.59 ± 0.45 |

SED = non-supplemented sedentary group; H-SED = hesperidin sedentary group; Run = non-supplemented runner condition; H-Run = hesperidin runner condition. Data are expressed as mean ± SEM (*n* = 8–23). Statistical difference: \* *p* < 0.05 vs. the corresponding SED group (Student's *t*-test).

### 3.3. ROS Production by Peritoneal Macrophages

ROS production tended to be higher in the non-supplemented trained animals (Figure 4), the significant difference being reached immediately after the additional final exhaustion test in the non-supplemented animals. This increase was prevented by the hesperidin administration.



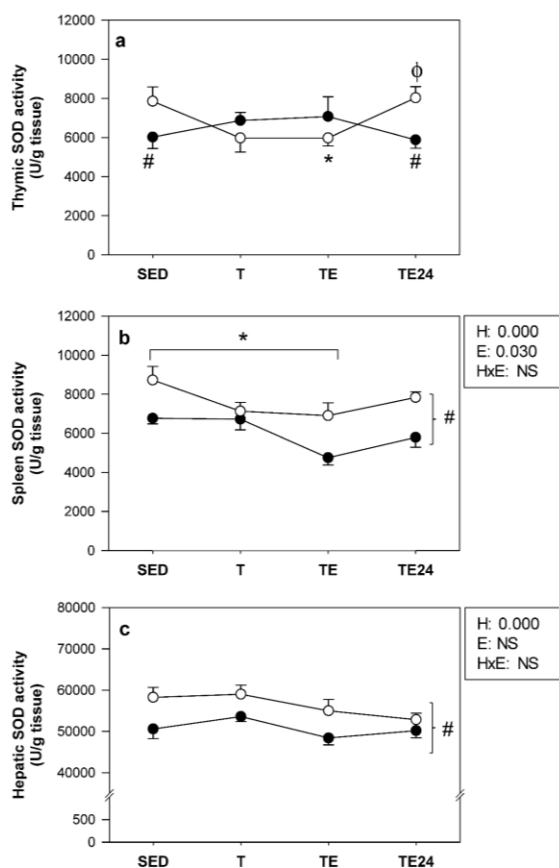
**Figure 4.** Production of ROS by peritoneal macrophages at the end of the study (AUC from 0 to 150 min). The non-supplemented groups are represented by white symbols (○) and the hesperidin groups by black symbols (●). SED = sedentary group, T = trained group, TE = T group with an additional exhaustion test, TE24 = TE group 24 h after the exhaustion test, NS = no statistically significant differences detected. Data are expressed as mean ± SEM (*n* = 5–6). Statistical difference: the inset table shows two-way ANOVA results when applied (H, hesperidin; E, exercise; HxE, interaction between hesperidin and exercise). \* *p* < 0.05 vs. the SED group just in the non-supplemented animals.

### 3.4. SOD Activity

SOD activity detected in the thymus, spleen and liver is summarized in Figure 5. In non-supplemented animals, a decrease in thymic SOD activity was detected in the TE group compared with the SED group, which returned to basal levels after 24 h. The hesperidin supplementation resulted in lower SOD activity in the H-SED and H-TE24 groups in comparison with their respective non-supplemented groups (Figure 5a).

Exercise training caused a reduction in spleen SOD activity just after the additional final exhaustion test in both hesperidin-supplemented and non-supplemented animals compared to the SED groups. Moreover, hesperidin administration decreased such activity with respect to the non-supplemented groups (Figure 5b).

Regarding the liver, exercise training did not modify SOD concentration. However, hesperidin administration resulted in lower SOD activity in comparison with the non-supplemented groups (Figure 5c).



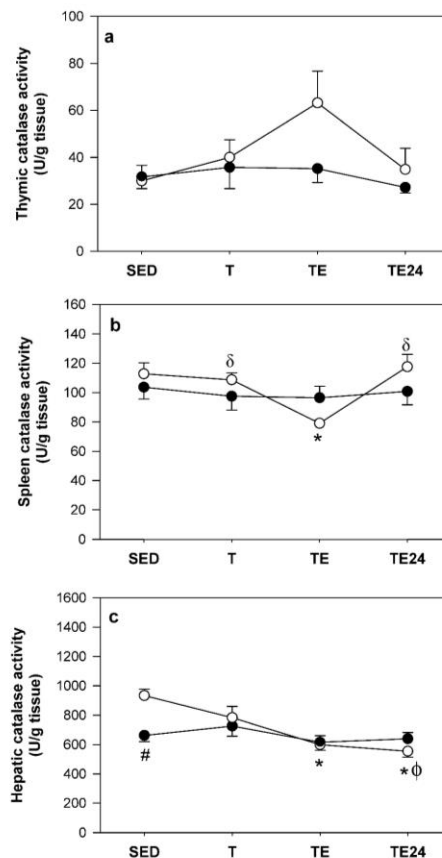
**Figure 5.** Superoxide dismutase (SOD) activity in the thymus (a), spleen (b) and liver (c) at the end of the study. The non-supplemented groups are represented by white symbols (○) and the hesperidin groups by black symbols (●). SED = sedentary group, T = trained group, TE = T group with an additional exhaustion test, TE24 = TE group 24 h after the exhaustion test, NS = no statistically significant differences detected. Data are expressed as mean  $\pm$  SEM ( $n = 7-8$ ). Statistical difference in (a) (Mann–Whitney U test): #  $p < 0.05$  between non-supplemented and the hesperidin-supplemented groups; \*  $p < 0.05$  vs. SED group just in the non-supplemented animals;  $\phi$   $p < 0.05$  vs. T group just in non-supplemented animals. Statistical difference in (b,c): the inset table shows two-way ANOVA results when applied (H, hesperidin; E, exercise; HxE, interaction between hesperidin and exercise); #  $p < 0.05$  between non-supplemented and the hesperidin-supplemented groups; \*  $p < 0.05$  vs. exercise conditions.

### 3.5. Catalase Activity

No significant changes were detected in thymic catalase activity due to either training, the final exhaustion test or hesperidin administration (Figure 6a).

In non-supplemented animals there was a marked drop in splenic catalase activity immediately after the additional final exhaustion test (Figure 6b). This activity was reestablished 24 h later. Hesperidin administration avoided these changes in splenic catalase activity.

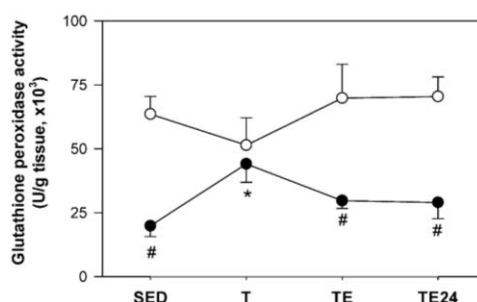
The non-supplemented animals that followed the additional final exhaustion test (TE group) presented a decreased hepatic catalase activity with respect to the SED animals. This drop was maintained for at least 24 h (Figure 6c). Hesperidin administration prevented changes due to exercise, but the hepatic catalase activity in SED rats was lower than that in the non-supplemented SED group.



**Figure 6.** Catalase activity in the thymus (a), spleen (b) and liver (c) at the end of the study. The non-supplemented groups are represented by white symbols (○) and the hesperidin groups by black symbols (●). SED = sedentary group, T = trained group, TE = T group with an additional exhaustion test, TE24 = TE group 24 h after the exhaustion test. Data are expressed as mean ± SEM ( $n = 7-8$ ). Statistical differences (Mann-Whitney U test): #  $p < 0.05$  between non-supplemented and the hesperidin-supplemented groups; \*  $p < 0.05$  vs. SED group just in the non-supplemented animals; δ  $p < 0.05$  vs. TE group just in the non-supplemented animals; φ  $p < 0.05$  vs. T group just in the non-supplemented animals.

### 3.6. Hepatic Glutathione Peroxidase Activity

There were no significant changes in hepatic GPx activity in non-supplemented animals resulting from training or the additional final exhaustion test. Nevertheless, exercise training in hesperidin-supplemented animals (H-T group) showed significantly higher GPx activity than the H-SED group (Figure 7). On the other hand, hesperidin administration significantly reduced hepatic GPx activity compared to its respective non-supplemented group in all the considered experimental conditions, with the exception of the T group (Figure 7).



**Figure 7.** Hepatic glutathione peroxidase (GPx) activity at the end of the study. The non-supplemented groups are represented by white symbols (○) and the hesperidin groups by black symbols (●). SED = sedentary group, T = trained group, TE = T group with an additional exhaustion test, TE24 = TE group 24 h after the exhaustion test. Data are expressed as mean ± SEM ( $n = 6-8$ ). Statistical difference (Mann–Whitney U test): #  $p < 0.05$  between non-supplemented and the hesperidin-supplemented groups; \*  $p < 0.05$  vs. SED group just in the hesperidin animals.

## 4. Discussion

The current study demonstrates the effects of hesperidin supplementation on exercise performance in intensively trained rats, the endogenous antioxidant defenses in their lymphoid tissues and liver and the oxidative stress induced by an additional exhaustion test.

In particular, the administration of 200 mg/kg of hesperidin three times per week for five weeks prompted a better performance in the exhaustion tests carried out during the study in Wistar rats. This effect was more evident in the first four weeks and even hesperidin-supplemented animals achieved the maximum performance one week later, which was higher than that in non-supplemented animals. The amount of hesperidin used was based on previous studies [37,38] evidencing its immunomodulatory effects. This amount in rats could be transformed to a human dosage by taking into account the body surface area [44]; accordingly, 200 mg/rat kg would be equal to 32.43 mg/human kg, and given that 1 L of orange juice provides about 600 mg hesperidin [45], the hesperidin amount per week would be supplied by a total of 13 L of orange juice per week. In this sense, we proposed here to use hesperidin supplements as has been previously described [46]. Previous studies have reported that polyphenols, particularly flavonoids such as quercetin, positively influence exercise performance [47–50]. Additionally, a recent study in humans has demonstrated that supplementation with a hesperetin extract (hesperidin aglycone) for four weeks in trained men improves exercise performance as assessed by a time-trial test on a cycle ergometer [51]. In spite of the better performance obtained, we did not find differences in the gastrocnemius or soleus muscle weight as a result of either the intensive training or the hesperidin supplementation.

Intensive training, together with an additional exhaustion test, increased ROS production of peritoneal macrophages, which may saturate an organism's antioxidant mechanisms leading to oxidative stress [1]. This increase was prevented when rats were supplemented with hesperidin during the training program, a fact that could be due to the scavenging activity of polyphenols [52]



that are able to neutralize ROS, such as superoxide anions, hydroxyl radicals, peroxynitrite and nitric oxide radicals [33,53,54]. It is well established that a decrease in exercise performance may be related to higher ROS production. In particular, higher ROS synthesis in muscles can produce fiber damage, which could contribute to muscle fatigue [55]. In this sense, we can suggest that hesperidin, like other flavonoids [56], can improve exercise performance by preventing muscle ROS accumulation, as we observed in peritoneal macrophages. Further studies are needed to check muscle ROS production in order to determine the influence of hesperidin in muscular tissue.

Under physiological conditions, ROS excess is counterbalanced by endogenous or externally supplemented antioxidants. Overproduction of ROS can produce oxidative stress and, consequently, immune functions—among other physiological processes—will be impaired. This study aimed to assess the influence of hesperidin supplementation in the endogenous antioxidant systems of two lymphoid tissues, the thymus (primary lymphoid tissue) and the spleen (secondary lymphoid tissue). Regarding the thymus, where T-lymphocyte maturation and selection occurs [57], a decrease in the thymic weight due to the intensive-training was observed. This result is in line with those reported in rats following a swimming training program for 21 days [58], and also the result in mice after a high-intensity swimming exercise for four weeks [59]. These results could be associated with thymocyte apoptosis due to the chronic stress induced by exercise [60], and would have consequences on thymic immune function. Although thymocyte apoptosis has been associated with oxidative stress [20], we found that lower thymic weight was also present after hesperidin administration. Moreover, we have detected that thymic SOD activity decreased immediately after the additional final exhaustion test, which agrees with previously reported data [20]. These results suggested that this antioxidant enzyme would be depleted when the additional exhaustion test was carried out, although it quickly recovered 24 h later. Interestingly, hesperidin administration prevented such reduction induced by the exhaustion test, although SOD activity was lower in sedentary animals receiving this flavonoid and remained constant whenever exercise was performed. This result could reflect the reduced need for this endogenous enzyme when an exogenous antioxidant is provided, even in conditions of oxidative stress, as in the intensive training and the additional final exhaustion test applied here.

The spleen is a secondary lymphoid organ acting as a reservoir of lymphocytes, where the immune response to antigens initiated [61]. Spleen weight was reduced in trained rats just after the additional final exhaustion test, being recovered 24 h later. These results agree with those of Yuan et al. [59], who found that excessive exercise in mice caused spleen atrophy. Both results are likely related to the splenic vasoconstriction described when exercising [62], which increases muscle blood flow and is associated with splenic lymphocyte mobilization [63] due sympathetic nervous system activation [64]. Changes in splenic weight were also observed in hesperidin-supplemented rats, meaning that blood redistribution also occurred even with the antioxidant supplement. Apart from changes in splenic weight, the additional final exhaustion test decreased spleen SOD and catalase activities. These SOD results agree with those reported in rats after eight weeks of swimming (1 h/day, 5 days/week) [65] and both SOD and catalase activity decreases in the spleen after acute exercise in mice [20]. Taken all together, this would indicate the depletion of antioxidant defenses in the spleen after certain exercises, which could affect the immune function of this lymphoid tissue. Nevertheless, these results do not agree with those reported in the lymphocytes of active males performing high-intensity interval training who increased their SOD, catalase and GPx activities [66]. When supplemented with hesperidin, both sedentary and intensively trained animals showed, overall, lower levels of spleen SOD activity than were observed in non-supplemented animals, with no changes in catalase activity. As occurred in the thymus, this effect of hesperidin could reflect the lower necessity of SOD when an antioxidant is externally provided.

Antioxidant enzyme activities were also assessed in the liver, with higher SOD, catalase and GPx activities than the thymus or spleen. The training program and additional final exhaustion test applied did not significantly modify the liver SOD and GPx activities. However, the exhaustion test decreased catalase, which did not recover after 24 h. Similar results regarding liver SOD and/or GPx

activities have been reported in both aged and young rats after eight weeks of swimming exercise [67], and in endurance-trained rats in a treadmill either for seven [68] or eight weeks [69]. These results could demonstrate the high antioxidant potential of the liver, which is not affected even in long exercise programs. Results concerning liver catalase activity are controversial. Although we found that the additional final exhaustion test decreased such enzymatic activity, other authors reported no changes in rats trained by swimming exercise for four weeks [70]. Regarding the influence of flavanone intake on liver antioxidant potential, hesperidin supplementation for five weeks decreased SOD and GPx activities in almost all groups, and lowered catalase activity just in sedentary rats. On the contrary, it has been reported that hesperidin was able to increase liver antioxidant activities in rats when administered at doses of 20–80 mg/kg BW for 10 days [71], as well as when it was administered to streptozotocin-induced diabetic rats at doses of 25–50 mg/kg BW for 35 days [72]. The discrepancies between our hesperidin results and those reported may derive from the flavanone dose applied, which was higher in our case and able to counteract oxygen radicals by itself, and not by enhancing endogenous antioxidant defenses. It is postulated that flavonoids, and in particular hesperidin and hesperetin, exert their antioxidant effect through two main mechanisms: by a direct radical scavenging of ROS accumulation, thus preventing oxidative damage, and by enhancing defense of endogenous antioxidants, for example, by increasing antioxidant enzymatic activities [34]. In our case, the protective effect of hesperidin at 200 mg/kg BW (three times per week for five weeks) against oxidative damage, induced by an intensive exercise, was more likely the result of its ability to scavenge ROS overproduction, rather than its influence on enzymatic antioxidant activities.

Overall, we have demonstrated the effect of hesperidin supplementation on intensively trained rats. Although the amount of hesperidin used here was supplied by extracts rather than natural orange juice, further studies must define the minimum dose and the posology able to induce antioxidant effects. Moreover, the current results give cause to consider further research focused on the effects of hesperidin on the immunosuppression derived from intensive exercise.

## 5. Conclusions

Our results indicate that hesperidin supplementation in intensively trained rats improves exercise performance, maintains endogenous antioxidant defenses in lymphoid organs and the liver and prevents the oxidative stress induced by an additional exhaustion test.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6643/11/4/783/s1>, Figure S1: Maximum distance run in the exhaustion tests throughout the study.

**Author Contributions:** M.C., M.C.-B., F.J.P.-C. and A.F. conceived and designed the experiments, S.E.-A., M.M.-C., and P.G.-C. performed the experiments, S.E.-A. analyzed the data and wrote the paper and M.C., M.C.-B. and F.J.P.-C. reviewed the manuscript. All authors have read, reviewed and approved the final version of the manuscript.

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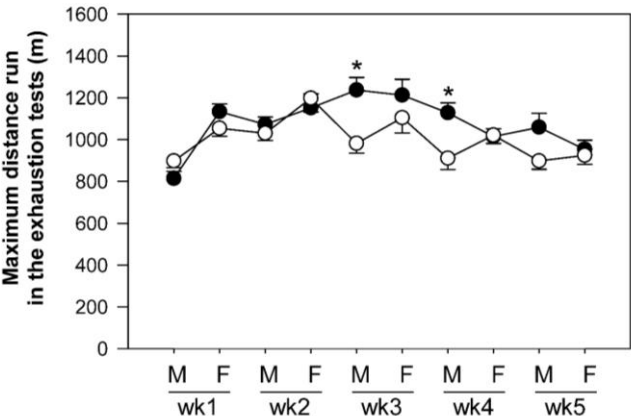
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Supplementary material

**Figure S1.** Maximum distance run in the exhaustion tests throughout the study. M=Monday, F=Friday, wk=week. The non-supplemented group is represented by white symbols, and the hesperidin group by black symbols. Data are expressed as mean  $\pm$  SEM (n=22–23). Statistical difference: \*p < 0.05 between non-supplemented and the hesperidin-supplemented groups (Student's t-test).





DISCUSSIÓ





La pràctica d'exercici físic intens i extenuant indueix sovint un estat d'immunosupressió, amb una durada d'hores o dies després de la realització de l'exercici, que pot comprometre la salut de l'esportista. Fins fa poc, aquesta immunosupressió es produïa bàsicament en esportistes d'elit després de la realització d'exercici intens, el que generava una disminució del seu rendiment esportiu. En els darrers anys, però, ha crescut la participació de la població general en competicions d'alt rendiment i per això ha augmentat la freqüència dels efectes secundaris de la immunosupressió transitòria. Avui en dia està ben establerta l'associació de la realització d'exercici intens amb l'augment d'incidència d'infeccions del tracte respiratori [203]. Tot i que se sap que hi ha afectació del sistema immunitari, és necessari estudiar amb més detall els efectes perjudicials que se'n deriven, i avaluar els efectes d'intervencions nutricionals que puguin contrarestar-los. Per tant, és important caracteritzar les diferents alteracions immunitàries derivades de l'exercici intens i extenuant i avaluar la influència d'intervencions nutricionals. Prèviament, el grup d'Autoimmunitat i Tolerància va establir els efectes potenciadors de l'hesperidina sobre el sistema immunitari sistèmic i intestinal [192]. Amb aquestes premisses, l'objectiu principal d'aquesta tesi se centra en aprofundir en la influència de l'exercici físic intens i extenuant i de l'hesperidina sobre el sistema immunitari en rates.

Per tal d'assolir aquest propòsit, s'han desenvolupat dos objectius secundaris. En el primer objectiu, centrat en avaluar la influència de l'exercici físic intens i extenuant en el sistema immunitari innat i adaptatiu en rates, es van utilitzar dos protocols d'exercici físic, desenvolupats mitjançant una cinta de córrer (*treadmill*) especial per a rosegadors, que diferien en la durada i la pauta de l'entrenament. En ambdós casos, es van emprar rates Wistar, que és una de les soques més utilitzades en l'experimentació sobre exercici físic, amb una edat de 4 setmanes a l'inici de l'entrenament. Aquesta edat, utilitzada també per altres investigadors [204,205] ha estat condicionada per la intervenció nutricional que s'ha realitzat posteriorment, i és la més utilitzada pel grup de recerca en els estudis d'immunonutrició realitzats [192,206].

El primer protocol utilitzat, anomenat S-TE (*short training with exhaustion*), es basa en els procediments descrits per Xu i col·laboradors

[207] i Andrade i col·laboradors [208], els quals inclouen l'entrenament dels animals dues vegades al dia. Concretament, el protocol S-TE va consistir en dues setmanes d'entrenament on, cinc dies a la setmana, els animals realitzaven dues sessions d'exercici (30 min a 30 m/min) separades per un interval de 6 h. Després de les dues setmanes d'entrenament, els animals van ser sotmesos a una prova d'extenuació final, en la qual els animals van començar a córrer a una velocitat de 5 m/min que es va anar incrementant progressivament (1,8 m/min cada min) fins a l'extenuació de l'animal.

Davant dels resultats obtinguts en el protocol S-TE, que no mostraven una clara modificació de la funcionalitat del sistema immunitari, es va procedir a aplicar un segon model d'exercici físic en el qual els animals van ser entrenats a diferent velocitat en funció de la velocitat màxima aconseguida al llarg de l'estudi, de forma similar al protocol descrit per Batatinha i col·laboradors [209]. Aquest segon protocol d'entrenament va tenir una durada de cinc setmanes, en les quals cada dilluns i divendres es realitzava una prova d'extenuació, mentre que dimarts, dimecres i dijous, els animals corrien a un 60% de la velocitat màxima obtinguda a l'extenuació anterior (el que va suposar una velocitat entre 34 i 43 m/min) durant un període de temps creixent (15, 20 i 25 min, progressivament). Després de les cinc setmanes d'aquest entrenament, el següent dilluns es va realitzar un entrenament similar al del dijous anterior i, dimarts, un grup d'animals va fer una prova d'extenuació addicional, en la qual els animals van córrer 15 min a la mateixa velocitat que la setmana anterior i a continuació es va incrementar la velocitat a raó de 3 m/min cada 2 min fins a l'extenuació. En aquest segon protocol, per tal de tenir una visió completa de l'afectació del sistema immunitari dels animals entrenats, es va procedir a obtenir mostres a tres temps diferents: al dia següent després de l'entrenament regular (grup T) per tal d'avaluar l'efecte de l'entrenament intens, immediatament després de la prova d'extenuació final addicional (grup TE) per avaluar l'impacte de l'extenuació, i 24 h més tard (grup TE24) per determinar els canvis en les variables mesurades a llarg termini o en el període de recuperació.

Les proves d'extenuació realitzades en els dos entrenaments van permetre quantificar el rendiment esportiu dels animals, ja que proporcionen dades de temps, velocitat i recorregut màxims de cada animal. En el protocol S-TE, desenvolupat en mascles i femelles, es va poder constatar que el rendiment esportiu de les rates Wistar femelles era superior al dels mascles (**Articles 1 i 2**), fet que coincideix amb el que han descrit altres investigadors [210,211], encara que hi ha estudis en aquesta mateixa soca que només utilitzen rates mascle [124,212]. Malgrat la superior capacitat corredora en femelles, els valors de cortisol en plasma, elevats en animals corredors, van ser similars entre ambdós sexes (**Article 2**). A més, també es va poder apreciar de forma subjectiva, mitjançant l'avaluació de la forma/estil de córrer segons el criteri AEY [213] i de la constància de l'animal durant l'entrenament, que les rates femella s'adaptaven millor a la cinta de córrer. Per aquests motius, en el segon protocol es va disposar només d'animals femella.

Les proves d'extenuació realitzades al llarg del segon protocol van permetre fer un seguiment del rendiment esportiu dels animals durant les cinc setmanes de l'entrenament. Al llarg de les dues primeres setmanes, els animals van mostrar una millora del seu rendiment (**Articles 1 i 2**) però, posteriorment, el rendiment ja no va millorar i fins i tot, a dia final, va arribar a ser inferior a l'aconseguit durant la segona setmana. Aquests resultats ens van fer pensar que, en aquestes condicions, el sistema immunitari podria estar realment compromès.

L'estudi del sistema immunitari en els animals corredors s'ha realitzat principalment a partir de les cèl·lules obtingudes de diferents compartiments: timus (òrgan representant de teixit limfoide primari), melsa (òrgan representant del teixit limfoide secundari) i sang (teixit que mostra la mobilització de les cèl·lules immunocompetents durant l'exercici físic i que és el que s'utilitza en estudis en humans).

En resposta a l'exercici físic, s'estimula el sistema nerviós simpàtic i s'allibera adrenalina i noradrenalina que, a més dels seus efectes fisiològics sobre el cor i els vasos sanguinis, entre d'altres teixits, actuen

sobre els òrgans limfoides i directament sobre els limfòcits. Els limfòcits T i B, presenten receptors adrenèrgics, sobretot del tipus  $\beta_2$  i responen a l'augment de catecolamines en sang i a l'increment de l'estimulació simpàtica sobre el teixit limfoide [28]. Conseqüentment, de forma immediata a l'extenuació final es produeix leucocitosi, tal com s'ha descrit en humans i models animals [214,215] i s'ha observat en la present tesi (**Article 1**). Concretament, l'extenuació final posterior a l'entrenament de cinc setmanes va produir un increment del nombre de leucòcits en sang, causat sobretot per un increment de limfòcits i monòcits (**Article 1**). L'augment en el nombre de limfòcits es pot atribuir principalment a un increment de limfòcits T (**Article 1**) i en concret per un augment de la proporció de limfòcits Th (**Article 2**). Pel contrari, la mateixa extenuació final va disminuir la proporció de cèl·lules Tc, NK, NKT i TCR $\gamma\delta$ + (**Article 2**). Cal remarcar que aquests canvis només es van detectar en els animals extenuats que van seguir el protocol d'entrenament durant cinc setmanes en comparació amb els animals que van seguir el mateix entrenament sense realitzar l'extenuació final. Tot i així, 24 h després de l'extenuació, la població de leucòcits sanguinis, es va recuperar i el nombre de limfòcits, monòcits i granulòcits en sang va ser similar al dels animals sedentaris (**Article 1**). També es va observar que l'augment en la proporció de limfòcits Th i la disminució de la proporció de les cèl·lules NK, NKT i TCR $\gamma\delta$ + detectades immediatament després de l'extenuació desapareixien (**Article 2**). No obstant això, la menor proporció de limfòcits Tc va perdurar a les 24 h de l'extenuació, el que podria ser degut a la sortida preferencial d'aquesta subpoblació cap als teixits [105]. En aquest sentit, s'ha descrit la diferent mobilització dels limfòcits T CD8+ efectors o verges en funció de l'entrenament previ a l'exercici intens [216] i també la seva diferent resposta a un augment d'adrenalina en sang [217]. Per tant, caldria definir la funcionalitat de les subpoblacions de limfòcits T CD8+ per tal de conèixer la seva cinètica durant l'exercici extenuant després d'un entrenament intens.

L'estudi de la composició limfocítica de timus i melsa ha permès comprendre els resultats sobre la distribució de limfòcits sanguinis, i dona una idea de la mobilització dels limfòcits. En el timus s'ha observat que l'entrenament i la prova d'extenuació final van provocar una disminució del seu pes relatiu (**Article 2**) i tal com s'ha suggerit a l'**Article 2**, això pot

ser degut a un augment de l'apoptosi tímica causat per l'estrès [218,219] i/o a un augment de la sortida de timòcits. En aquest sentit, es va observar que la prova d'extenuació final, després dels dos tipus d'entrenament, reduïa la proporció de limfòcits TCR $\alpha\beta$ +, i en concret, d'una de les poblacions més madures, els timòcits CD4+CD8-. Aquesta disminució podria ser causada per la sortida d'aquesta subpoblació del timus, fet que podria contribuir a l'augment del percentatge de limfòcits Th en sang (**Article 2**), si bé d'altres autors no han observat variacions en el nombre relatiu de limfòcits T en timus [104,220]. Per altra banda, al cap de 24 h de l'extenuació final, es va observar un increment de la proporció dels timòcits més immadurs (CD4-CD8-), el que podria ser degut a una resposta fisiològica per tal de recuperar la possible sortida de limfòcits Th.

Pel que fa a la melsa, la prova d'extenuació final en els dos protocols estudiats també va disminuir el pes esplènic relatiu (**Article 2**), el que podria indicar la mobilització limfocítica des d'aquest òrgan, juntament amb la vasoconstricció d'aquest teixit. Malgrat això, l'estudi de la composició de la melsa no va revelar canvis significatius en la proporció de limfòcits Th després de la prova d'exercici físic extenuant (**Article 2**). Fet que suggereix que, a part de la melsa, altres teixits limfoides podrien estar implicats en la mobilització dels limfòcits, com per exemple el timus. Per altra banda, cal remarcar que l'entrenament durant cinc setmanes va comportar un augment en la proporció de limfòcits Tc de melsa, que es va perdre en fer la prova d'extenuació final, i l'entrenament també va comportar una reducció en la proporció de cèl·lules NK i NKT, abans, immediatament després i a les 24 h de l'extenuació final (**Article 2**). La disminució d'aquests tipus cel·lulars podria ser deguda a una mobilització a altres teixits (pulmons, intestí, entre d'altres) i/o a la seva apoptosi. Aquesta apoptosi podria comportar un estat d'immunodeficiència per un exercici excessiu, encara que, tal com es comenta més endavant i es recull a l'**Article 1**, l'activitat citotòxica de les cèl·lules NK esplèniques va incrementar.

Per tal d'aprofundir en la funcionalitat dels limfòcits, es va caracteritzar l'activitat proliferativa i secretora de citocines i immunoglobulines dels esplènòcits en el protocol d'entrenament de cinc setmanes, i l'activitat

citotòxica de les cèl·lules NK d'aquest teixit en ambdós protocols (**Articles 1 i 2**).

Les cèl·lules obtingudes després de l'entrenament durant cinc setmanes van mostrar una capacitat de proliferació reduïda davant de concanavalina A (ConA), una davallada de la secreció d'IFN- $\gamma$  i un augment de la IL-6, mentre davant de l'estimulació amb el mitogen *pokeweed* (PWM) es va observar una reducció de la secreció d'IL-10 i un augment de la d'IL-6 (**Article 2**). Aquests resultats demostren que l'entrenament intens de cinc setmanes va reduir la funcionalitat dels limfòcits T, tant per la seva capacitat per proliferar [221] com per secretar citocines de tipus Th1 com IFN- $\gamma$  [222]. Donat que les respostes immunitàries de tipus Th1 estan implicades sobretot en la defensa contra patògens intracel·lulars com són els virus [223], aquests resultats indicarien certa immunodeficiència associada a l'exercici intens realitzat. Tot i així, per tal de confirmar aquesta hipòtesi caldria realitzar nous estudis a través de la inducció d'una infecció vírica en animals entrenats amb el protocol usat en aquesta tesi. Per altra banda, després de l'estimulació amb ConA i PWM, els limfòcits de melsa van incrementar la secreció d'IL-6 (**Article 2**). La IL-6 és una citocina proinflamatòria associada a la resposta Th2 [222] i també és una miocina secretada per cèl·lules del múscul esquelètic, sobretot en resposta a l'exercici [224]. S'ha descrit que la seva concentració plasmàtica augmenta després de l'exercici [225] i, en cas d'exercici excessiu pot provocar una disminució en el rendiment esportiu [226,227]. En aquest sentit, l'increment d'aquesta citocina podria justificar, almenys parcialment, la reducció del rendiment, observat en els animals d'estudi a les cinc setmanes d'entrenament respecte al màxim aconseguit a la segona setmana (**Articles 1 i 2**).

L'estimulació amb PWM en cèl·lules obtingudes després de cinc setmanes d'entrenament va mostrar una reducció en la síntesi d'IL-10 (**Article 2**). Aquesta és una citocina antiinflamatòria [228] i la disminució després de l'estimulació amb PWM, podria indicar una reducció de la seva síntesi per part dels limfòcits B [229] i, per tant, una disminució del seu paper immunoregulator en el control de la inflamació [230–232]. Malgrat tot, la IL-10 promou la proliferació i funció dels limfòcits B [231,233], i la seva disminució després de l'estimulació amb PWM no es relacionaria amb

l'augment de producció d'IgG i IgM observada *in vitro*, encara que aquests darrers resultats s'han obtingut en cèl·lules no estimulades.

Immediatament després de la prova d'extenuació final, la funcionalitat dels limfòcits esplènics dels animals entrenats durant cinc setmanes, va canviar significativament respecte al que es va observar abans de la realització d'aquesta prova. Concretament, la capacitat proliferativa i la producció d'IL-10 dels limfòcits va augmentar i es va normalitzar la secreció d'IFN- $\gamma$  i d'IL-6 (**Article 2**). Aquests resultats suggereixen que l'estrès induït per l'exercici extenuant, a través de la secreció d'hormones com adrenalina, noradrenalina i cortisol, pot tenir una influència sobre la funcionalitat dels limfòcits esplènics. Encara que s'ha associat un increment de catecolamines a un augment de la producció d'IL-6 [234], s'ha descrit que l'addició de cortisol *in vitro* no modifica la secreció d'IL-6 i incrementa la secreció d'IL-10 en limfòcits [235], de forma similar als resultats obtinguts en el present estudi.

Pel que fa a la capacitat de proliferació dels limfòcits obtinguts després de la prova d'extenuació final, es va observar un augment puntual, sense mantenir-se 24 h més tard (**Article 2**). Encara que falta consens en l'efecte de l'exercici sobre la proliferació de limfòcits estimulats, en general, l'exercici intens s'associa a una disminució de la capacitat proliferativa [72,100,126,236–239], si bé alguns estudis mostren l'augment de la capacitat proliferativa de limfòcits en funció del tipus d'exercici realitzat [95,121,123,148,240,241]. Els resultats obtinguts poden ser causa de l'increment d'hormones d'estrès, encara que les catecolamines i els glucocorticoides s'han associat a un efecte immunosupressor [153].

Una dada interessant obtinguda en els animals entrenats durant cinc setmanes, fa referència als anticossos presents en sang i la capacitat de síntesi d'immunoglobulines *in vitro*. L'entrenament durant cinc setmanes va provocar un increment de la concentració sèrica d'IgG que es va mantenir després de la prova d'extenuació final (**Article 2**). Aquest increment podria ser degut a un augment de la capacitat de síntesi de les cèl·lules productores d'IgG, tal com s'ha observat *in vitro* en cèl·lules



procedents d'animals entrenats (**Article 2**), i/o a un increment de la vida mitjana d'aquesta immunoglobulina, tal com s'ha descrit en ratolins després d'un exercici voluntari [242]. Aquests resultats coincideixen parcialment amb els resultats descrits per Mckune i col·laboradors en humans [136], que van observar un increment de la IgG sèrica després de la realització d'una ultramarató. Per altra banda, l'estudi *in vitro* mostra un increment d'IgG i IgM després de l'entrenament, efecte que desapareix en cèl·lules obtingudes després de l'extenuació final (**Article 2**). Això podria ser degut als efectes immunosupressors de les catecolamines i glucocorticoides alliberats en el moment de l'exercici extenuant [243]. De fet, la majoria d'estudis que avaluen l'efecte de l'exercici sobre immunoglobulines se centren en els anticossos mucosals, com els que es troben a la saliva. Així, està ben establert que l'exercici extenuant redueix la concentració d'IgA salival [244,245], fet que compromet la primera línia de defensa de l'organisme. En aquest sentit, estudis preliminars no inclosos en la present tesi van permetre quantificar la concentració d'IgA en glàndules salivals i en rentat intestinal dels animals després de cinc setmanes d'entrenament. Els resultats obtinguts van mostrar que l'entrenament disminuïa la concentració d'IgA en glàndules salivals i a les 24 h de l'extenuació final es reduïa la concentració d'IgA intestinal [246].

Una altra funció immunitària estudiada en la melsa ha estat la capacitat citotòxica de les cèl·lules NK (**Article 1**). La realització d'exercici intens durant cinc setmanes, però no durant dues setmanes, va disminuir la proporció de cèl·lules NK esplèniques (**Articles 1 i 2**); però, en canvi, quan es va analitzar la seva funció citotòxica es va observar que aquesta funció augmentava de forma relativa (**Article 1**). La majoria dels estudis referents a cèl·lules NK i exercici físic fan referència al compartiment sanguini i descriuen una davallada en el seu nombre [100]. Tot i que l'augment d'activitat observat en el present treball concorda parcialment amb resultats descrits [247], s'ha reconegut que l'activitat citotòxica de les cèl·lules NK pot variar en funció dels diferents règims d'entrenament seguits i dels moments d'obtenció de mostra [114]. En tot cas, l'increment de l'activitat NK observat en el present treball no correspon a la

immunodeficiència esperada després d'un exercici intens i extenuant. Aquest fet podria indicar un mecanisme de compensació de la disminució de cèl·lules NK i suggerir que la mobilització i/o apoptosi d'aquest tipus cel·lular comportés que les cèl·lules NK que queden a la melsa tinguin elevada activitat citotòxica, com podrien ser les cèl·lules NK CD56<sup>dim</sup> [248]. Tot i així, nous estudis enfocats en intensificar o allargar l'entrenament hauran de confirmar el paper de les cèl·lules NK en la immunodeficiència esperada després d'un exercici extenuant.

Per tal d'aprofundir en la influència de l'entrenament de cinc setmanes sobre el sistema immunitari innat, es va caracteritzar la funcionalitat dels macròfags peritoneals mitjançant la secreció de citocines després de l'estimulació amb lipopolisacàrid (**Article 1**).

Tal com s'ha comentat a l'**Article 1**, existeixen dues subpoblacions principals de macròfags amb diferents funcions: els macròfags activats clàssicament o inflamatoris (M1) i els macròfags activats de forma alternativa o antiinflamatoris (M2) [41]. Els macròfags M1 secreten elevades quantitats de citocines proinflamatòries, com TNF- $\alpha$ , IL-1, IL-12 i IL-23, i presenten una elevada activitat antimicrobiana [41,249]. L'entrenament durant cinc setmanes va comportar una disminució de la secreció de TNF- $\alpha$  i d'IL-12, el que suggereix una atenuació parcial de la funcionalitat dels macròfags M1 que podria derivar en l'elevada susceptibilitat a infeccions descrita després de la realització d'un exercici intens [250,251]. Tot i així, la realització d'una prova d'extenuació final després de l'entrenament de cinc setmanes, va restablir la secreció d'aquestes citocines. Això podria ser degut a canvis associats a aquesta prova com pot ser la secreció de catecolamines o cortisol i/o l'estrès oxidatiu present en els macròfags (**Article 4**). Els macròfags, de forma similar als limfòcits, presenten receptors adrenèrgics i també per a glucocorticoides que poden influir sobre la seva funcionalitat [85,252].

Per altra banda, els macròfags obtinguts d'animals entrenats durant cinc setmanes van mostrar un augment en la secreció d'IL-6, que va incrementar encara més després de l'extenuació final (**Article 1**), resultats

similars als obtinguts amb esplenòcits (**Article 2**). La secreció augmentada d'IL-6 per part dels macròfags contribuiria, juntament amb l'elevada producció d'aquesta citocina per part de limfòcits (**Article 2**) i de les fibres musculars [224], a un augment d'IL-6 en plasma que, si bé no es va analitzar en aquesta tesi, és característic després de la realització d'exercici [225] i pot associar-se a una reducció del rendiment esportiu [226,227].

Els macròfags peritoneals dels animals entrenats durant cinc setmanes i sotmesos a una prova d'extenuació van presentar una davallada de la secreció d'IL-10. Aquesta citocina és capaç d'inactivar el factor nuclear  $\kappa B$  i, conseqüentment, disminuir la producció d'espècies reactives d'oxigen (ROS) [253]. Donat que en les mateixes condicions que disminueix la producció d'IL-10 es va veure que els macròfags augmentaven la síntesi de ROS (**Article 4**), es pot suggerir que la davallada d'IL-10 i la producció de ROS per part dels macròfags podrien ser dos biomarcadors de l'estat inflamatori associat a l'exercici físic extenuant.

Per altra banda, relacionat amb la funcionalitat dels macròfags, es va estudiar la capacitat fagocítica dels monòcits en sang (**Article 1**). Després de cinc setmanes d'entrenament es va detectar una reducció de la capacitat fagocítica dels monòcits sanguinis, el que faria pensar en un efecte associat a l'entrenament intens i perllongat. Tanmateix, la capacitat fagocítica es va recuperar en fer la prova d'extenuació final i fins i tot va augmentar en el període de recuperació (**Article 1**). Tal com es comenta a l'**Article 1**, s'han trobat resultats similars en macròfags broncoalveolars de ratolí després de fer un exercici extenuant agut i l'augment de la capacitat fagocítica s'ha associat, en part, a un increment en el nombre de receptors *scavenger* per part dels macròfags [254]. Per altra banda, els canvis observats en les citocines alliberades pels macròfags peritoneals (**Article 1**), permeten també suggerir altres mecanismes relacionats amb aquests mediadors que poden influir sobre la funció fagocítica. Concretament, els canvis en la capacitat fagocítica dels monòcits sanguinis en els tres grups d'exercici estudiats, segueixen el mateix patró que la secreció d'IL-12 per part de macròfags peritoneals. A més a més s'ha trobat una correlació positiva entre la secreció d'IL-10 de macròfags i l'activitat fagocítica de monòcits (**Article 1**). Com que IL-10 i IL-12 poden

augmentar la capacitat fagocítica [41,255,256], es pot suggerir que els canvis funcionals presents en els monòcits sanguinis deriven de les citocines sintetitzades durant l'entrenament intens i l'exercici físic extenuant.

Tal i com s'ha comentat, la secreció de ROS per part dels macròfags peritoneals també es va alterar en fer la prova d'extenuació final després de cinc setmanes d'entrenament (**Article 4**). De fet, l'exercici físic extenuant s'ha associat a un augment de l'estrès oxidatiu de l'organisme (revisat a [153]) que en part, podria ser degut a un increment en la producció de ROS per part de macròfags. Aquest estrès oxidatiu, podria produir-se durant les darreres extenuacions al llarg de l'estudi, i podria ser parcialment responsable de la reducció en el rendiment esportiu observat en els animals entrenats durant cinc setmanes (**Articles 1 i 2**) tal com ha postulat Myburgh [166].

En resum, l'entrenament intens i extenuant de cinc setmanes ha induït canvis tant en el sistema immunitari innat com en l'adaptatiu. En concret, l'entrenament ha induït una menor activitat fagocítica i una disminució de la secreció de citocines proinflamatòries per part de macròfags, canvis que s'han revertit en el període de recuperació. A més en finalitzar l'entrenament, l'extenuació final i 24 h més tard, la proporció de cèl·lules NK s'ha reduït però ha augmentat la seva activitat citotòxica. Per altra banda, l'entrenament, però no l'extenuació final, ha reduït capacitat de proliferació dels limfòcits esplènics així com la secreció d'IFN- $\gamma$ , i ha incrementat la síntesi d'IgG. Tenint en compte tots aquests resultats, l'entrenament durant cinc setmanes utilitzat en la present tesi, tot i que és capaç de provocar alteracions en el sistema immunitari, sembla no ser suficient per tal d'induir una immunodeficiència completa. Per això, futurs experiments haurien d'extremar les condicions d'intensitat i durada de l'exercici per tal de veure més afectació sobre el sistema immunitari i quantificar de forma més aplicada la incidència i intensitat d'infeccions.

Una vegada establerts els efectes de l'entrenament intens i extenuant sobre el sistema immunitari, el segon objectiu d'aquesta tesi es va centrar

en establir l'efecte immunomodulador i antioxidant de l'hesperidina, flavonoide (flavanona) present en la taronja i altres cítrics [182]. Les intervencions nutricionals resulten de gran interès pels seus efectes beneficiosos sobre l'organisme. En concret, una dieta rica en components bioactius, com per exemple els polifenols, s'ha relacionat amb una menor incidència a patir certs tipus de malalties, com el càncer, diabetis, malalties cardiovasculars o neurodegeneratives [156]. A més, les intervencions nutricionals poden ser de gran utilitat per prevenir els efectes perjudicials d'un exercici físic excessiu [166,257].

Per tal d'assolir aquest segon objectiu, en primer lloc, es va avaluar la influència de la suplementació oral de 100 i 200 mg/kg d'hesperidina tres vegades la setmana durant quatre setmanes sobre el sistema immunitari intestinal i la microbiota intestinal (**Article 3**). L'hesperidina es va administrar per sonda oral, ja que estudis previs en què s'avaluava la influència d'aquesta flavanona afegida a la dieta van demostrar un menor efecte [192,258]. Les dosis d'hesperidina emprades en aquesta tesi es basen en un estudi previ, en el qual es va demostrar el seu efecte immunomodulador sobre el sistema immunitari intestinal [192]. L'estudi de la microbiota intestinal portat a terme a l'**Article 3**, revela que la suplementació amb hesperidina va induir un augment en el nombre de bacteris presents en el cec i, d'entre els grups bacterians estudiats, l'augment es va atribuir a un increment en el creixement d'*Streptococcus* spp., *Lactobacillus/Enterococcus*, *Staphylococcus* spp., *Bacteroides/Prevotella*, *Bifidobacterium* i *Escherichia coli*, mentre que el nombre de *Clostridium coccoides/Eubacterium rectale* i *Clostridium histolyticum/Clostridium perfringens* es va mantenir després de l'administració de la flavanona. De forma més significativa, en estudiar les proporcions de grups bacterians, es va observar que l'administració de la dosi superior d'hesperidina augmentava la proporció del grup *Lactobacillus/Enterococcus* a la microbiota cecal. Cal remarcar que, tal com s'ha comentat a l'**Article 3**, els bacteris del gènere *Lactobacillus* s'associen a efectes beneficiosos sobre l'organisme [259–262] i es consideren bacteris probiòtics [185]. Per tant, aquest efecte de l'hesperidina permetria considerar-la com a prebiòtic. A més a més, es va observar que la suplementació d'hesperidina comportava un augment del contingut d'IgA a l'intestí prim i del nombre de bacteris units a IgA en el

cec (**Article 3**). Aquests resultats van demostrar l'efecte beneficiós d'aquesta flavanona sobre el sistema immunitari intestinal. Com s'ha comentat anteriorment, un dels efectes de l'exercici intens sobre el sistema immunitari consisteix en la disminució de la IgA mucosal [244,245], en aquest sentit, l'administració d'hesperidina podria ser beneficiosa per tal de contrarestar aquest efecte.

La influència de l'hesperidina sobre la immunitat intestinal també es va observar en establir la composició limfocítica de ganglis limfàtics mesentèrics, on les dues dosis emprades van augmentar la proporció de limfòcits T davant de la dels limfòcits B, si bé, quan es va estudiar la funcionalitat d'aquestes cèl·lules a través de l'activitat proliferativa i la secreció de citocines, no es va observar cap efecte significatiu (**Article 3**). L'administració d'hesperidina tampoc va influir en l'expressió gènica de molècules implicades en la secreció d'IgA intestinal ni en la concentració d'immunoglobulines sèriques (**Article 3**).

Els efectes antioxidants establerts per l'hesperidina [263], van portar a avaluar la influència de la flavanona sobre el model d'exercici intens i extenuant de cinc setmanes. L'estudi va incloure la suplementació amb 200 mg/kg d'hesperidina, tres vegades la setmana, durant les 5 setmanes que durava l'entrenament. Aquesta dosi va ser establerta pels resultats obtinguts en l'**Article 3**, on es va veure que la major part dels efectes es produïen amb la dosi superior. L'administració d'hesperidina durant l'entrenament va produir una millora del rendiment esportiu dels animals, sobretot durant les tres primeres setmanes, quan va millorar la distància màxima aconseguida respecte als animals que no van rebre suplement (**Article 4**). Aquest resultat coincideix amb l'obtingut amb suplement amb altres flavonoides com la quercetina [264,265], la catequina [266] o l'epigallocatequina-3-galat [267].

Per altra banda, la suplementació amb hesperidina va demostrar els seus efectes antioxidants i va evitar l'increment de la producció de ROS per part dels macròfags peritoneals observat immediatament després de l'extenuació final (**Article 4**). Aquests efectes antioxidants podrien justificar la millora del rendiment esportiu ja que, tal i com s'ha comentat anteriorment i també es menciona a l'**Article 4**, s'ha relacionat un augment de la producció de ROS amb la fatiga muscular [166]. A més, es

va aprofundir en l'estrès oxidatiu induït per l'exercici físic intens durant cinc setmanes i en l'efecte de l'hesperidina mitjançant la determinació de l'activitat d'enzims antioxidants endògens (superòxid dismutasa o SOD, catalasa i glutatió peroxidasa) en el fetge i en dos teixits limfoides afectats per l'exercici físic com són el timus i la melsa (**Article 4**). L'extenuació final en els animals entrenats durant cinc setmanes va disminuir l'activitat SOD en timus i melsa, i la de la catalasa en melsa i fetge (**Article 4**), el que indicaria un esgotament dels enzims antioxidants presents en aquests teixits després de l'extenuació, si bé es van recuperar ràpidament un dia després (amb l'excepció de la catalasa hepàtica). La suplementació amb hesperidina durant l'entrenament (i fins i tot en algun cas en animals sedentaris) va provocar que les activitats antioxidants determinades fossin inferiors a les observades en els animals sense suplement (**Article 4**). Aquests resultats van ser sorprenents en observar que en molts estudis realitzats l'administració de polifenols augmentava la concentració o l'activitat d'enzims antioxidants [199,200]. Un fet que podria explicar aquesta diferència, és que en el nostre estudi vam utilitzar una dosi d'hesperidina més elevada i, en conseqüència, podria ser que la pròpia hesperidina fos capaç de contrarestar directament les ROS produïdes, i així evitar l'acumulació de ROS, i per tant, no caldria incrementar l'activitat dels enzims antioxidants. En aquest sentit, s'ha descrit que l'efecte antioxidant de l'hesperidina podria ser degut a un efecte directe contrarestant les ROS sense modificar la defensa antioxidant endògena [268].

Encara que, són necessaris futurs estudis centrats en establir la influència de l'hesperidina sobre les alteracions de la immunitat innata i adaptativa després de l'entrenament de cinc setmanes descrites en els **Articles 1 i 2**, els resultats de la tesi suggereixen el paper de l'hesperidina com a nutricèutic per tal d'evitar l'estrès oxidatiu derivat de l'exercici físic intens i extenuant i millorar, en conseqüència, el rendiment esportiu. A més, els efectes observats de l'hesperidina sobre la IgA mucosal i la microbiota poden ser beneficiosos en situacions fisiològiques i en cas d'estrès com l'induït per un exercici intens.



## CONCLUSIONS





The results obtained from the current thesis led us to conclude that:

- The training of male and female Wistar rats for two weeks with an intensive and exhausting exercise does not modify the considered functions associated with innate immunity.
- The training of female Wistar rats for five weeks with an intensive and exhausting exercise entails better exercise performance during the first two weeks. However, at the fifth week, the performance gets worse. At the end of this training, there are alterations in immune functions associated with both innate and acquired immunity.
- Blood monocytes collected after the five-week intensive training show a lower phagocytic activity. Similarly, in this condition, peritoneal macrophages decrease in their ability to secrete pro-inflammatory cytokines. Nevertheless, in the recovery period, the lowered phagocytic activity and the reduced levels of pro-inflammatory cytokines increase.
- Spleen NK cell proportion decreases after the five-week intensive training, the additional exhaustion test and in the recovery period. However, their cytotoxic activity is higher than that in sedentary animals.
- The proportion of the main lymphocyte subsets in blood and spleen does not vary after two weeks of intensive exercise but it is altered after the five-week intensive training, as well as immediately after the final exhaustion test and in the recovery period.
- Thymus is affected by the final exhaustion exercise both in the two-week and the five-week trainings, with a lower relative weight and a decrease in the proportion of the most mature lymphocytes.
- The leukocytosis observed immediately after the final exhaustion test is due to an increase in the counts of monocytes and lymphocytes, mainly T cells. Such an increase could be partially due to the mobilization of the Th subset from the thymus.

- Training for five weeks with an intensive exercise modifies spleen lymphocyte functionality, decreasing T-cell proliferative capacity and the secretion of interferon  $\gamma$ . However, these alterations are overcome in cells obtained after the final exhaustion test.
- The five-week intensive training increases the ability to synthesise IgG, a function that it is lost after the final exhaustion exercise.
- The administration of 200 mg/kg of hesperidin three times per week for four weeks to Lewis rats increases the total bacteria number in the caecum, due to, among others, an increase in the *Lactobacillus* group. Hesperidin also enhances the number of bacteria coated with IgA in the caecal content and the amount of intestinal IgA.
- An exhaustion test in five-week intensively trained Wistar rats decreases SOD activity in the thymus and spleen, and catalase activity in the spleen and liver. Hesperidin supplementation maintains antioxidant activities in these tissues.
- An exhaustion test in five-week intensively trained Wistar rats increases reactive oxygen species (ROS) production from peritoneal macrophages. The administration of 200 mg/kg of hesperidin to Wistar rats, three times per week, during the five-week intensive training prevents higher ROS production and produces a better exercise performance.



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