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Exercise-induced anxiety impairs local and systemic inflammatory response and glucose metabolism in C57BL/6J mice

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ABSTRACT

Introduction: The complex physiological and psychological responses to regular exercise are yet to be fully elucidated. Exercise strongly modulates the immune system, inducing a plethora of dynamic responses involving the innate immune cell function and inflammatory processes that contribute to both potential health benefits and harmful side effects. Indeed, the relationship between physical exercise, stress, immunity, and metabolism serves as a paramount model of neuroimmunoendocrine interaction. Thus, the objective of this study was to conduct a comprehensive analysis of both systemic and local immunophysiological responses together with behavioral responses to a protocol of anxiety-inducing exercise.

Material and methods: C57BL/6J mice were randomly allocated into sedentary or exercised groups, where the anxiety-inducing exercise protocol was based on a 14-day consecutive program of swimming in water at 38 °C. Anxiety-like behavior was corroborated through the elevated plus maze test. Systemic biomarkers of the stress response were assessed using ELISA technique and the expression of systemic inflammatory cytokines with Bio-Plex system. Phagocytic/microbicide activity, the expression of M1/M2 phenotype markers (CD11c, iNOS, CD206, ARG-1) and cytokines of the inflammatory response (MCP-1, IL-8, IL-6, TNF-α, TGF-β, IL-10) of peritoneal macrophages were determined via flow cytometry. Adipose tissue macrophage infiltration was studied through fluorescence immunohistochemistry.

Results: Anxiety-like behavior, elevated circulating glucose concentrations, systemic stress and inflammatory responses, together with increased oxidative stress and inflammatory profile of peritoneal macrophages, and macrophage infiltration in white adipose tissue were observed in exercised animals.

Conclusions: A protocol of exercise that induces anxiety is associated with a neuroimmunoendocrine dysregulation affecting the feedback between the inflammatory and the stress responses, together with detrimental metabolic effects in glucose modulation. Systemic inflammatory alterations are accompanied by detrimental inflammatory responses in tissue macrophage populations. Altogether, these results show that exercise associated with anxiety, stress, pro-inflammatory responses, and hyperglycaemia represents a model of 'dangerous exercise'.

1. Introduction

Regular physical exercise exerts diverse effects on different physiological systems. The intricate relationship between exercise and the body's response mechanisms, including metabolism and inflammation, remains an area of ongoing investigation in scientific research.

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Understanding how exercise may influence these physiological processes could provide valuable insights into optimizing the benefits of physical activity while mitigating potential drawbacks.

It is well-known that physical exercise strongly modulates the immune system, inducing a plethora of dynamic responses including innate immune cell function and inflammatory processes that contribute to health benefits, but also with potential inflammatory and metabolic side effects (Ortega, 2003, 2016). Physical exercise constitutes a model of physiological stress, where the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis play a pivotal role in modulating this response (Ortega, 1994; Ortega et al., 2010). Indeed, the relationship between physical exercise, stress, immunity, and metabolism serves as a paramount model of neuroimmunoendocrine interaction (Pedersen and Nieman, 1998) in which a proper regulation of the interplay between pro-inflammatory cytokines and activation of the HPA axis is crucial for achieving optimal exercise-induced responses (Ortega, 2016). In this context, macrophages, major components of the immune response, are key modulators of metabolic dysregulation and of the initiation, progression and resolution of inflammation (Chawla et al., 2011; Gálvez et al., 2023; Lee and Lee, 2014). Bearing this in mind, exercise can have a dual effect by stimulating or inhibiting macrophage infiltration into adipose tissue (Gálvez et al., 2023). The reduction in infiltration is recognized as one of the anti-inflammatory mechanisms contributing to the benefits and effectiveness of exercise (Gleeson et al., 2011; Kawanishi et al., 2010; Suzuki, 2019). Conversely, increased infiltration could contribute to exacerbate detrimental inflammatory and metabolic responses.

In this context, exercise should be able to exert 'bioregulatory effects', that is, to reduce or prevent any excessive effect of inflammatory mediators while stimulating, or at least not impairing, the innate defenses against pathogens (i.e. phagocytosis and microbicidal activity), as well as an optimal balance between the pro- and anti-inflammatory responses of monocyte and macrophage populations. These immunophysiological and immunometabolic adaptations are mediated via immunoneuroendocrine interactions, with stress hormones and mediators released by the HPA axis and the SNS playing a pivotal role (Ortega, 2016). On the contrary, exercise can also elicit a danger-stress and inflammatory response that may become dysregulated and detrimental to health, which has been coined 'dangerous exercise' (Cooper et al., 2007). However, the comprehensive implications of dangerous exercise on the complex interactions among the stress/immune responses and the behavioral responses are yet to be elucidated.

While the variability in exercise intensity underscores the diverse physiological and psychological responses that exercise can elicit, there has been limited research examining the potential role of exercise-induced anxiety as a determining factor of exercise outcomes. While stress has been defined as the physiological reaction of an organism to a real or perceived threat (stressor) that challenges its homeostasis (Chrousos, 2009; Selye, 1936), anxiety could be defined as the individual's psychological reaction to stress or potential stress (Daviu et al., 2019). Thus, while it is well established that exercise is a form of stress, the impact of physical activity on anxiety-related behavior in mice remains poorly understood. Current research findings span from reduced anxiety (Binder et al., 2004; Salam et al., 2009), absence of discernible effects on anxiety (Pietropaolo et al., 2006), to heightened anxiety observed in rodents after physical exercise (Fuss et al., 2010; Leasure and Jones, 2008). Although behavioral alterations induced by exercise have been extensively studied, available reports are remarkably controversial, where the intensity of physical activity, the environmental conditions of housing, or whether the exercise regimen is voluntary or imposed may have contributed to these inconsistencies. Particularly, regarding the effect of exercise-induced anxiety on immune and metabolic responses, the scarce evidence points to a relationship between anxiety levels and the characteristics of the immune response after exercise (Venezia et al., 2020).

Although some models of regular exercise/stress in water have been demonstrated to reduce, or at least not modify, anxiety in rodents, together with improvements in the innate and inflammatory responses (De la Fuente et al., 2011), recent pilot studies conducted in our laboratory showed that the protocol of swimming exercise herein evaluated promotes anxiety-like behavior in mice (Navarro et al., 2023). In this context, our hypothesis is that anxiety may serve as an important factor in determining the potentially harmful effects of exercise, particularly in the complex interaction between inflammatory, stress and glucose metabolism responses. In the present study, once corroborating that our swimming exercise protocol induced anxiety in the animals, we aimed to investigate the effects of exercise that causes stress/anxiety on the intricate interplay among the stress, metabolic, and inflammatory responses. In order to evaluate this, the systemic stress and inflammatory markers together with the innate immune and inflammatory response of the peritoneal macrophages, and the inflammatory response of macrophages infiltrating the adipose tissue were assessed.

2. Material and methods

2.1. Experimental design

A total of nineteen male (n = 10) and female (n = 9) C57BL/6J mice were sourced from the Animal Facility of the University of Extremadura. Mice were randomly divided (using OxMaR software for random number generator) into two groups, a sedentary group (n = 8, 3 males and 5 females) and an exercised group (n = 11, 7 males and 4 females). All animals were housed individually, under a reverse 12:12 h light-dark cycle, with lights turned off at 11:00 and turned on at 23:00, in a temperature- and humidity-controlled room (22 ± 1 °C; 60 ± 5%), where they were able to maintain olfactory and visual contact with each other to prevent potential negative effects of isolation. Mice were given *ad libitum* access to water and 30 g of standard laboratory rodent chow per week. At the age of 24 weeks, mice started the experimental exercise protocol for 2 weeks and then both sedentary and exercised groups were sacrificed, at 26 weeks of age. The behavioral assessment was conducted in both groups on the final day of the exercise protocol, prior to the last exercise session. For the sedentary group, the behavioral test was also conducted on the same day. Following a 12-h fasting period and 24 h of rest for the exercised group, blood samples, peritoneal fluid, and visceral white adipose tissue (WAT) were collected from anaesthetized animals. Experimenters were blind to group assignment during sample processing and data analysis. Fig. 1 illustrates the experimental design, detailing the various stages and procedures conducted throughout the study.

This investigation was approved by the Bioethics Committee for Animal Experimentation of the University of Extremadura (registry number 70/2018 for project IB18011), following the ARRIVE guidelines and national and european legislation for the protection of animals used for research, including the EU Directive 2010/63/EU for animal experiments.

2.2. Exercise protocol

The protocol of regular exercise began at approximately 24 weeks of age. Exercise protocol was a 14-day consecutive program based on swimming in water (animals are allowed to swim or rest in the water due to the characteristics of the vessel) at 38 °C, according to mice body temperature of 36–37 °C (Gordon, 2012). Animals are allowed to rest in the water due to the characteristics of the vessel as they have the option to stand on their hind legs at the bottom of the tank. The protocol was carried out in thermostatic water baths (40 × 30 × 20 cm) with controlled temperature, with gradually increasing times, starting at 10 min on the first day and reaching 30 min on the last days. The intervention was performed during the active period of the animals (dark phase from 11:00 a.m. to 11:00 p.m.), starting at approximately 12 p.m. Both sedentary and exercised mice were kept under the same conditions and performed the same behavioral tests. Animals were housed in the

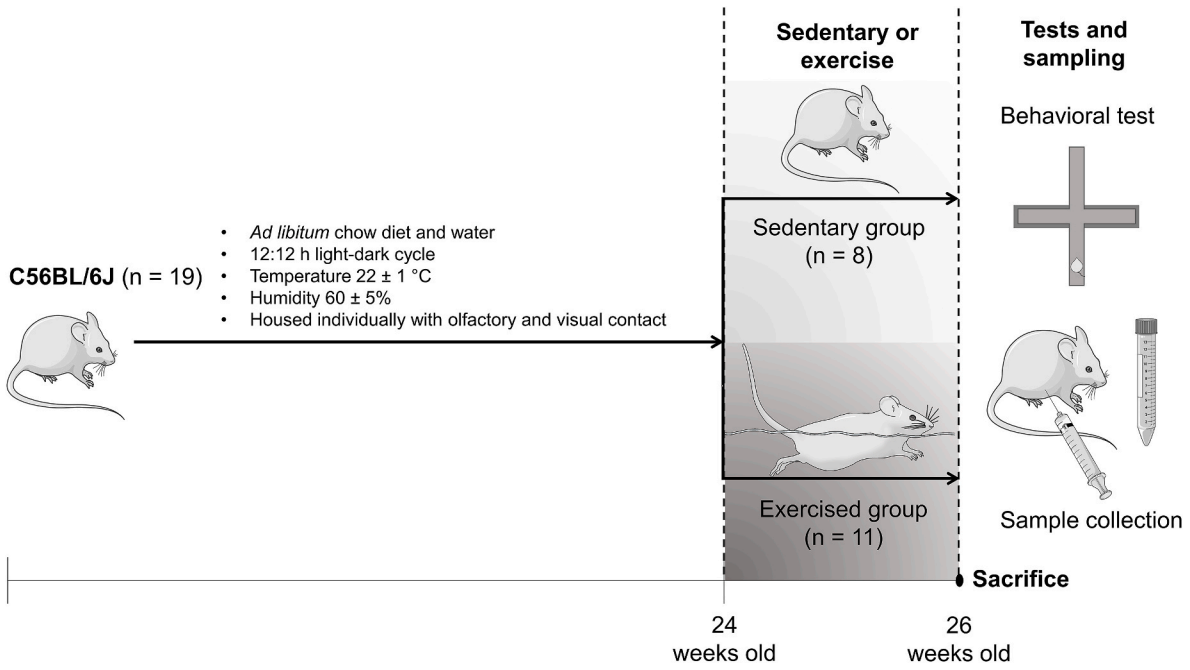

Fig. 1. Schematic representation of the experimental design. Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

same room, with identical lighting, temperature, and humidity controls, and were provided with the same food and water access throughout the entire study period. Animals were handled the previous week before the protocol of exercise in order to avoid additional handling stress. Previous pilot studies in our laboratory showed that this model of exercise induces anxiety in rodents (Navarro et al., 2021, 2023). In order to corroborate this, the behavioral test was performed on the last day of the exercise protocol, just before this last session, to avoid the influence of the acute effects of the exercise. Samples were obtained 24 h after the last training session also to avoid the assessment of the acute effects of exercise. It is important to note that this model of swimming exercise in swimming pools is different from the classical model of stress in rodents, where they are forced to swim inside a cylindrical water tank (the rodent forced swim test) (Can et al., 2012; Commons et al., 2017; Petit-Demouliere et al., 2005); while the latter represents a purely stress-inducing scenario, our model can be considered a protocol of exercise-induced stress (De la Fuente et al., 2011; Forner et al., 1995).

2.3. Elevated plus maze test

This test is commonly used to assess anxiety-like behavior. It is based on rodent's aversion to height and open spaces, as well as on its thigmotaxis behavior (a tendency to move along walls). The device consists of two types of arms, one with high opaque walls, representing an enclosed space, and the other without walls, allowing the animal to perceive the environment and height. The time and frequency the mouse enters and spends on each arm determine the anxiety-like behavior of the animal (Walf and Frye, 2007). Thus, when the animal feels threatened or stressed, it would seek protection and hiding, which, in the case of the elevated plus maze test, can be found in the closed arms. The most interaction and presence of the animal in this part of the device would represent anxiety-like behavior. Conversely, open spaces (such as open arms) are only explored when the animal's state of anxiety is low, thus the less interaction and time spent there would mean the animal presents anxiety-like behavior.

The elevated plus maze was arranged in a "+" shape measuring 50 cm in length and 10 cm in width, with two open arms facing each other and two closed arms (with walls 40 cm in height) of the same dimensions as the open arms. It is constructed of rigid black plastic material, and it is elevated 62 cm above the ground on a metal support with four legs.

The test was conducted with the room's lights off, and the sole source of illumination was a red light bulb focused on the apparatus. It began by placing the animal in the central area of the apparatus, facing a closed arm. Behavior on the elevated plus maze test was recorded using a video camera during the 5-min test.

2.4. Hole-board test

Anxiety-like behavior was also assessed using the hole-board test, following a protocol similar to that used by Viveros et al. (Viveros et al., 2001). The hole-board apparatus was an open square structure, 45 cm in height, with a 60 cm square base. The walls were matte black, and the base was marked with white lines dividing the surface into 36 squares, each measuring 10 cm per side. In the center, four evenly spaced holes, each 3.8 cm in diameter, held red circular objects. To start the test, the animal was placed in one of the apparatus corners, and behavior was recorded in video for 5 min under red lighting.

The parameter evaluating the anxiety-like behavior in this test was the total locomotion, recorded as the number of squares crossed by each animal.

2.5. Biological samples collection

Body weight was monitored weekly. Biological samples were obtained from live, anaesthetized animals. Mice were anaesthetized with isoflurane (starting dose 3–5% isoflurane, maintenance dose 1.5–3% isoflurane) after 12 h fasting period. Whole blood was collected via cardiac puncture. Fasting blood glucose concentration and lipid profile including total cholesterol, high-density lipoprotein cholesterol (HDL-C), calculated low-density lipoprotein cholesterol (cLDL-C) and triglycerides (TG) were measured in whole blood (LUX®, Microcaya, Bilbao, Spain). For the peritoneal cells extraction, 4 ml of phosphate-buffered saline (PBS) medium were injected into the peritoneal cavity, followed by aspiration of the fluid containing the cellular suspension, which was then deposited into polypropylene tubes. Visceral WAT was carefully collected and then embedded in optimal cutting temperature

(OCT) compound and snap-frozen in liquid nitrogen. Until further analysis, samples were stored at −80 °C.

2.6. Quantification of plasma concentrations of biomarkers of the stress response

Enzyme-Linked ImmunoSorbent Assay (ELISA) kits (Cusabio, Houston, USA and MyBioSource, San Diego, USA) were used to assess plasma concentrations of noradrenaline, corticosterone, and eHsp72 in the experimental groups.

For the analysis, plasma samples and standards (utilized in creating the standard curve) underwent incubation within a 48-well plate coated with a specific antibody targeting the protein of interest, which binds to the protein within the sample. This process eliminated any unbound material through subsequent washes. Additionally, a horseradish peroxidase (HRP) conjugate with the specific antibody was introduced into each well. Following incubation and removal of excess conjugate via washing, a chromogenic substrate solution (TMB) was introduced into the wells. This substrate solution catalytically reacted with the HRP conjugate, resulting in the formation of a chromogenic product. Subsequently, a "stop" solution was added to terminate the substrate reaction, and the absorbance was quantified at a wavelength of 450 nm utilizing an ELISA plate reader (Tecan, Männedorf, Switzerland). The absorbance signal was directly or inversely proportional to the amount of protein present in the sample, depending on the kit used. Protein concentration was determined by interpolation on the standard curve generated using values from standard samples of known concentration, performed manually and using MyAssays software (https://www.myassays.com/).

2.7. Assessment of phagocytic activity and oxidative burst, and the inflammatory profile of peritoneal macrophages

It is generally accepted that peritoneal macrophages are the most representative macrophages among all macrophage populations in the body, making them ideal for assessing the activation status of this cell type in the body. Additionally, they exhibit a rapid and robust response to inflammatory stimuli, allowing for the investigation of their inflammatory profile in a controlled environment. Thus, they are a good model for evaluating the general inflammatory and stress status of these cells but also for studying the microbicidal and phagocytic capacity.

The flow cytometry analysis was used to evaluate peritoneal macrophages' phagocytic and microbicide capacity (or oxidative burst) against opsonized bacteria. Using this technique, the capacity of macrophages to phagocytize bacteria and generate superoxide anion (O_2^-, indicative of oxygen-dependent microbicidal activity) can be accurately determined by the mean fluorescence intensity (mfi) of actively phagocytic cells, as measured by FITC (fluorescein isothiocyanate) to identify the phagocytized bacteria (phagocytic activity) and by hydroethidine (HE), to detect intracellular superoxide anion production by NADPH oxidase, respectively.

First, *Escherichia coli* (*E. coli*) was obtained at an optical density of 1.6 (O.D.), fixed in PFA (1%) (PanReac AppliChem, Barcelona, Spain), washed and filtered (0.22 μm diameter filter) with PBS. Subsequently, the bacteria were stained with FITC (fluorescein isothiocyanate) at a final concentration of 1 μg/ml for 30 min at 37 °C in darkness and agitation, and then washed twice with PBS. Once stained, the bacteria were opsonized by incubating them with serum for 2 h at 37 °C in darkness and agitation (800 μl of bacteria in PBS with 200 μl of serum).

Next, 200 μl of peritoneal exudate from each mouse were incubated for 1 h at 37 °C in darkness and agitation with: 50 μl of opsonized E.coli-FITC bacteria, Hoechst 33342 (10 μg/ml), 7-Aminoactinomycin D (7AAD 1 μg/ml), 250 μl of PBS, and 2% fetal bovine serum (FBS). After 30 min of incubation, HE was added, completing the incubation for another 30 min. A control was performed using 100 μl of peritoneal exudate along with Hoechst 33342 (10 μg/ml), 7AAD (1 μg/ml), 400 μl PBS, and 2% FBS. Finally, the sample was analyzed by flow cytometry (MACSQuant WYB, Miltenyi Bio-tec GmbH, Germany) with 3 lasers (405 nm, 488 nm, 561 nm), and the results were processed and analyzed using the "FlowJo data analysis" software. Results were expressed as the mfi shown by macrophages that have ingested bacteria (phagocytic activity) and that have produced O_2^- (microbicide activity).

For the evaluation of the inflammatory profile of peritoneal macrophages and their activity, the expression of CD11c and CD206 on the cell membrane, as well as the intracellular expression of iNOS, ARG-1, and the cytokines IL-8, IL-6, MCP-1, TNF-α, TGF-β, and IL-10 in these cells were assessed. Aliquots of 200 μl of cell suspension (1×10^7 cells/ml) obtained from peritoneal lavage were used. The aliquots were incubated with CD11c-PE, CD206-FITC, iNOS-Alexa430, ARG1-PE, IL-8-FIT, IL-6-PE, MCP-1-PE, TNF-α-FITC, TGF-β, and IL-10-APC antibodies. Antibody concentrations were determined by titration. Subsequently, they underwent incubation, in agitation and in darkness to enable eventual analysis and data collection using flow cytometry on the Cytoflex cytometer (Beckman Coulter, Indianapolis, United States).

2.8. Determination of systemic inflammatory cytokines concentrations

The Bio-Plex® 200 system (BioRad, Hercules, USA) was used to determine plasma concentrations of inflammatory cytokines (IL-1β, IL-10, IL-6, and TNF-α), using a high-sensitivity kit (ProcartaPlex Immunoassay kit, Invitrogen, Waltham, USA).

2.9. Assessment of macrophage infiltration in WAT

For WAT visualization, the tissue was cut into 12–15 μm sections in a cryostat (LEICA, CM 1950; Leica Biosystems, Illinois, United States) at −30 °C and mounted on Superfrost® Plus microscope slides (Thermo Fisher Scientific, Braunschweig, Germany). Samples were stored at −20 °C until immunostaining procedure. A hydrophobic barrier pen (ImmEdge Hydrophobic Barrier PAP Pen H-4000, Vector Laboratories, California, United States) was used to encircle the sections.

Slides containing the adipose tissue samples were washed with PBS + Triton X-100 (Merck KGAa, Darmstadt, Germany) and then fixed with 4% paraformaldehyde (PanReac AppliChem, Barcelona, Spain) for 5 min. After a series of washes with PBS + Triton X-100 and with PBS + gelatin (PanReac AppliChem, Barcelona, Spain) + Triton X-100, non-specific binding sites were blocked with PBS + gelatin + Triton X-100 + lysine (Merck KGAa, Darmstadt, Germany) for 1 h. Finally, the antibodies were added at concentrations determined after titration: F4/80 (Alexa Fluor® 488 Anti-mouse F4/80 Antibody, BioLegend, San Diego, United States) and CD206 (Alexa Flu-or® 594 anti-mouse CD206 MMR Antibody, BioLegend, San Diego, United States). The samples were then incubated overnight in a humid chamber in darkness. The next day, slides were washed again with PBS + Triton X-100 and with PBS + gelatin + Triton X-100. DAPI 2 μM (Invitrogen, Darmstadt, Germany) was added as a nuclear stain. After 15 min of incubation, the slides were washed with PBS. Coverslips and Mowiol® 40–88 (Sigma-Aldrich Merck KGAa, Darmstadt, Germany) were used for mounting the samples. The samples were stored at 4 °C in a humid chamber in darkness until they were visualized under the fluorescence microscope for the counting of infiltrated macrophages in the adipose tissue.

Immunostained adipose tissue sections were observed under a conventional transmitted light and fluorescence microscope (Nikon ECLIPSE 80i, Nikon, Tokyo, Japan), obtaining digital images with a camera attached to the microscope (Nikon Digital Camera DXM 1200F, Nikon, Tokyo, Japan). The images of the antibodies at different wavelengths were overlaid into a single image and optimized using Adobe PhotoShop v.CS4 software (Adobe, San Jose, United States). For cell counting, 10 randomly chosen fields of view were selected using a 40x objective. F4/80+ cells are macrophages, while F4/80+CD206+ cells are considered M2 macrophages, and F4/80+CD206- cells are considered M1 type macrophages (Fujisaka et al., 2009; Gálvez et al., 2023; Nawaz et al., 2017). Cells were counted using ImageJ image analysis

software.

2.10. Statistical analysis

The mean ± standard error of the mean (SEM) was used to express values, with the normal distribution of variables confirmed by the Kolmogorov-Smirnov test. Student's t-test was used to compare pairs of groups, whether the samples were paired or non-paired. Statistical significance was determined by the probability value "p" (p-value), with thresholds set at $p < 0.05$, $p < 0.01$, and $p < 0.001$, each indicating increasing levels of significance. For each comparison, the *t*-test values are presented as t(df), where "t" represents the statistic test value and "df" indicates degrees of freedom.

3. Results

3.1. Exercise protocol does not cause weight reduction and increases fasting glucose concentrations

In this study, no significant differences were observed between male and female subjects, indicating that the sex of the animals did not substantially influence the results. Then, all the results are presented as the mean of both sexes.

Table 1 shows the results regarding body and metabolic parameters in sedentary and exercised animals. Notwithstanding a reduction ($p < 0.05$) in food intake (just during exercise period), body weight did not change between sedentary and exercised mice, despite exercise performance.

There were no changes in the lipid profile of sedentary and exercised animals, although higher fasting glucose concentrations ($p < 0.01$) were found in animals after the exercise protocol.

3.2. Exercise protocol promotes anxiety-like behavior in the elevated plus maze test and the hole-board test

Animal behavior was analyzed through the elevated plus maze test and the hole-board test and found that exercised mice showed anxiety-like behavior.

Considering rodents' thigmotaxis behavior, if they interact less in the open arms of the test and spend less time in them, they would be exhibiting anxiety-like behavior. Similarly, if the number of entries and time spent in closed arms increases, due to their tendency to move along the walls when they want to feel protected, it would mean that their anxiety levels are high. Then, as shown in Fig. 2, the results demonstrated that exercised animals exhibited a significantly lower percentage of time spent in the open arms ($p < 0.01$) compared to sedentary animals (Fig. 2A), indicating a heightened anxiety state. In contrast, exercised animals spent a greater percentage of time in the closed arms ($p < 0.05$), remaining in this sheltered area for nearly 90% of the observation period (Fig. 2B). This behavior reflects an increased anxiety level in the exercised group, as evidenced by their preference for the closed arms over the open arms. Additionally, the time spent in the central platform was significantly less for exercised animals ($p < 0.01$), further supporting the observation that they favoured the closed arms (Fig. 2C).

Fig. 3 shows the total locomotion covered by each group in the hole-board test. The exercised group exhibited statistically significant reduced total locomotion ($p < 0.05$) compared to the sedentary group. Locomotor activity is inversely related to anxiety levels, suggesting that animals exhibit anxiety-like behavior as a result of the exercise protocol.

Table 1
Body weight, food intake, glycaemic and lipid profile in sedentary and exercised mice.

	Sedentary	Exercise	*t*-test
Body weight (g)	23.8 ± 0.7	23.8 ± 0.8	$t(17) = 0.002$, $p = 0.99$
Food intake during exercise period (g/day)	3.5 ± 0.6	3.2 ± 0.1[a]	$t(34) = 0.68$, $p = 0.5$
Glucose (mg/dL)	173 ± 51	260.6 ± 18.8[b]	$t(16) = 3.3$, $p = 0.005$
Cholesterol (mg/dL)			
- Total Cholesterol	<100	<100	–
- HDL Cholesterol	50.5 ± 17.3	62 ± 3.9	$t(12) = 1.52$, $p = 0.15$
- LDLc Cholesterol	32 ± 19.2	22.3 ± 4.5	$t(16) = 1.21$, $p = 0.24$
- Triglycerides (mg/dL)	<50	73.3 ± 8.8	–

Each value represents the mean ± SEM of 8 sedentary animals (3 males and 5 females) and 11 exercised animals (7 males and 4 females). The t and degrees of freedom (df) values are provided as t(df). HDL – high-density lipoproteins. LDL – low-density lipoproteins. LCLc, LDL calculated using the Friedewald equation (LDL = Total cholesterol – (Triglycerides/5) – HDL).

[a] $p < 0.05$ compared to the sedentary group.

[b] $p < 0.01$ compared to the sedentary group.

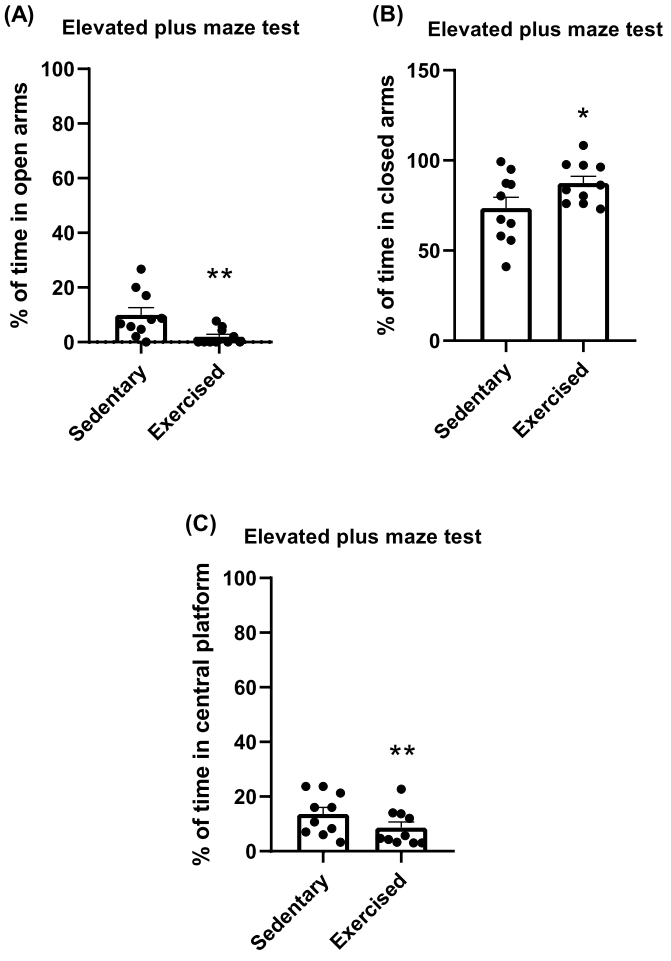

Fig. 2. Effect of the protocol of exercise on anxiety-like behavior. The figure shows the results regarding the open arms of the elevated plus maze test. Percentage of time in open (A), closed (B) arms and in central platform (C). Percentage of time is represented in relation to the total time (5 min) spent. Each column represents the mean ± SEM of the values obtained from 11 animals from each group (7 males and 4 females). *$p < 0.05$, **$p < 0.01$, compared to the sedentary group. The t and degrees of freedom (df) values are provided as t(df): % of time in open arms, $t(18) = 2.81$; % of time in closed arms, $t(18) = 2$; % of time in central platform, $t(18) = 1.6$.

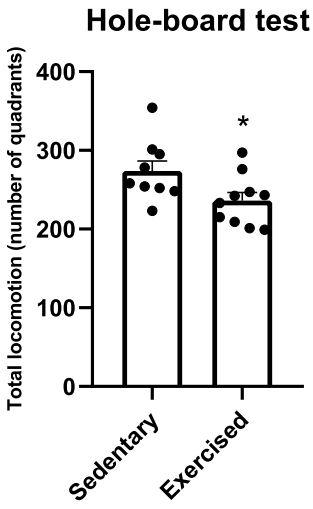

Fig. 3. Effect of the protocol of exercise on anxiety-like behavior in the hole-board test. Total locomotion is represented as the mean of total number of quadrants covered by each animal. Each column represents the mean ± SEM of the values obtained from 11 animals from each group (7 males and 4 females). *p < 0.05, compared to the sedentary group. The t and degrees of freedom (df) values are provided as t(df): t(17) = 2.3.

3.3. Exercise protocol induces systemic stress and inflammatory responses

Assessment of systemic biomarkers of the stress response revealed that exercised animals presented higher concentrations of corticosterone ($p < 0.05$) and lower concentrations of noradrenaline ($p < 0.05$), than sedentary mice (Fig. 4). Regarding eHsp72, we did not find statistically significant differences between both groups (Table 2).

The effects of an exercise-induced stress protocol are depicted in Table 3 and Fig. 4, representing plasma concentrations of systemic inflammatory cytokines, related to the inflammatory response. While there were no differences in IL-10, IL-6, and TNF-α concentrations between both groups of animals, an increase in IL-1β concentrations ($p < 0.05$) was found in exercised animals compared to sedentary ones, showing that the exercise protocol could induce an inflammatory response in this group of animals.

Then, the exercise protocol could provoke a systemic stress and inflammatory response in animals, as shown by elevated corticosterone levels and an increase in pro-inflammatory IL-1β concentrations in this group of exercised animals.

3.4. Exercise protocol boosts activation of peritoneal macrophages: oxidative stress and inflammatory profile

Fig. 5 shows the results corresponding to phagocytosis and superoxide anion production (measured by the mfi) of peritoneal macrophages in sedentary and exercised animals. No statistical differences were found between sedentary and exercised mice in the phagocytic activity (Fig. 5A). On the other hand, macrophages from exercised animals showed higher oxidative stress ($p < 0.05$) than the sedentary ones (Fig. 5B).

Regarding the inflammatory profile of the peritoneal macrophages, there are two types of macrophage phenotypes, pro-inflammatory M1 expressing CD11c and iNOS, and anti-inflammatory M2 expressing CD206 and ARG-1. Table 4 and Fig. 6 below illustrate the percentage of those cells showing each phenotype.

The results indicate that the exercise protocol induced an imbalance towards a pro-inflammatory state, represented by a reduced percentage of cells with anti-inflammatory M2 phenotype ($p < 0.05$ for CD206+ and ARG-1+CD206+; $p < 0.01$ for ARG-1+) in this group of exercised animals in comparison with the sedentary ones. Additionally, ratios of

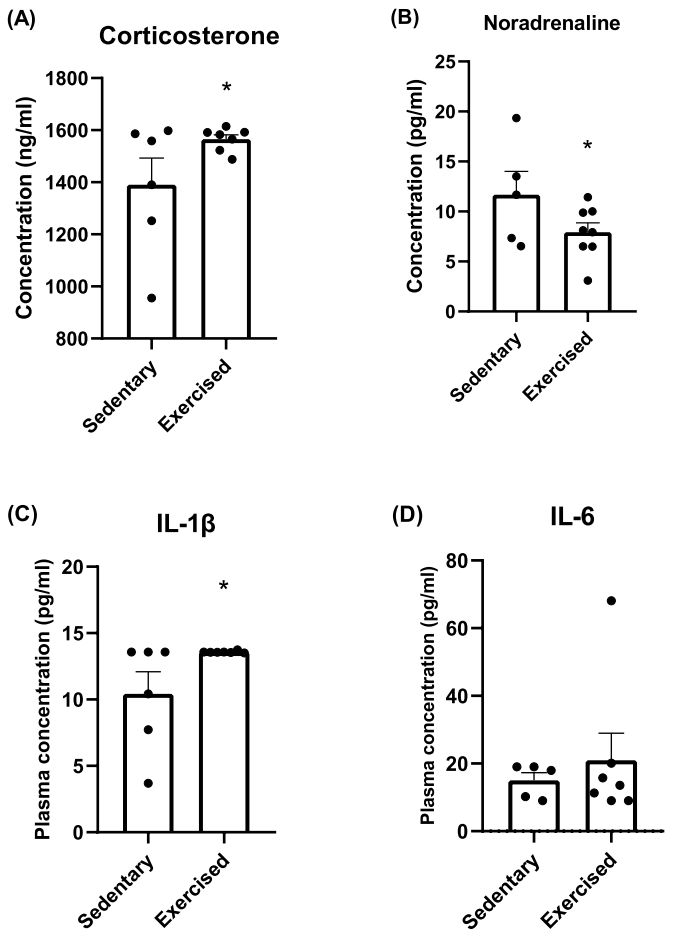

Fig. 4. Effect of the protocol of exercise on systemic biomarkers of the stress response (A and B) and systemic inflammatory cytokines (C and D). Each value represents the mean ± SEM of the values obtained from 5 sedentary animals (2 males and 3 females) and 7 exercised animals (4 males and 3 females). *p ≤ 0.05, compared to the sedentary group. The t and degrees of freedom (df) values are provided as t(df): for corticosterone t(11) = 1.81; for noradrenaline t (11) = 1.75; for IL-1β t(11) = 2.07; for IL-6 t(10) = 0.6.

Table 2
Effect of the protocol of exercise on the plasma concentration of the systemic biomarker of the stress response eHsp72.

	Sedentary	Exercise	*t*-test
eHsp72 (ng/ml)	1.68 ± 1.4	1.1 ± 0.3	$t(9) = 0.84$, $p = 0.42$

Each value represents the mean ± SEM of the values obtained from 5 sedentary animals (2 males and 3 females) and 7 exercised animals (4 males and 3 females). Results are expressed in ng/ml. The t and degrees of freedom (df) values are provided as t(df).

Table 3
Effect of the protocol of exercise on the plasma concentrations of the inflammatory cytokines IL-10 and TNF- α.

	Sedentary	Exercise	*t*-test
IL-10 (pg/mL)	22.5 ± 5.6	23.6 ± 10.5	$t(10) = 0.085$, $p = 0.46$
TNF-α (pg/mL)	14.3 ± 4.1	12.4 ± 3.8	$t(10) = 0.34$, $p = 0.37$

Each value represents the mean ± SEM of 5 sedentary animals (2 males and 3 females) and 7 exercised animals (4 males and 3 females). Results are expressed in pg/ml. The t and degrees of freedom (df) values are provided as t(df).

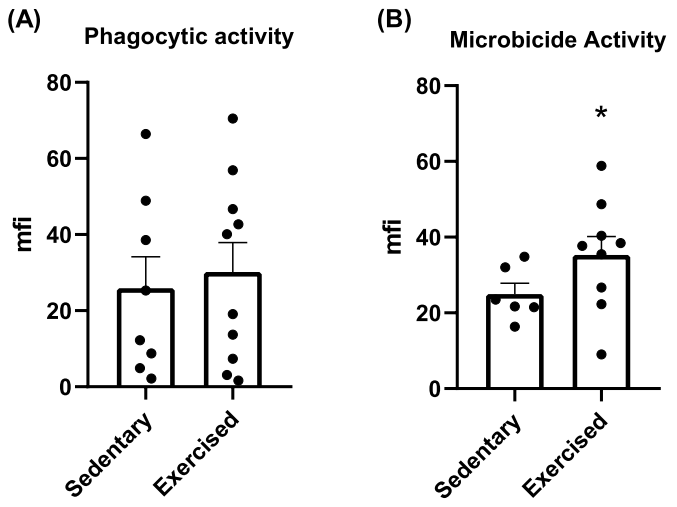

Fig. 5. Effect of the protocol of exercise on the phagocytic (A) and microbicide (B) activities of the peritoneal macrophages, expressed as the mean fluorescence intensity (mfi). Each column represents the mean ± SEM of the values obtained from 8 sedentary animals (3 males and 5 females) and 11 exercised animals (7 males and 4 females). *p < 0.05, compared to the sedentary group. The t and degrees of freedom (df) values are provided as t(df): phagocytic activity, t(16) = 0.38; microbicide activity, t(13) = 1.6.

Table 4
Effect of the protocol of exercise on the inflammatory phenotypic profile of peritoneal macrophages.

Phenotypic profile (% of cells)		Sedentary	Exercised	*t*-test
M1 (pro-inflammatory)	$CD11c^+$	58.3 ± 9	73.2 ± 6	$t(16) = 1.4$, $p = 0.09$
	$iNOS^+$	7.1 ± 2	7.1 ± 2	$t(17) = 0.02$, $p = 0.5$
	$iNOS^+$ $CD11c^+$	11.1 ± 4	13.9 ± 2	$t(16) = 0.72$, $p = 0.24$
M2 (anti-inflammatory)	CD206+	17.8 ± 5	9.1 ± 2[a]	$t(17) = 1.8$, $p = 0.04$
	$ARG\text{-}1^+$	40.3 ± 1	11.5 ± 2[b]	$t(16) = 3.3$, $p = 0.002$
	$ARG\text{-}1^+$+$CD206^+$	16.1 ± 5	6 ± 2[a]	$t(17) = 2.18$, $p = 0.02$

Each value represents the mean ± SEM of the determinations obtained from 8 sedentary animals (3 males and 5 females) and 11 exercised animals (7 males and 4 females). The t and degrees of freedom (df) values are provided as t(df).
[a] p < 0.05 compared to the sedentary group.
[b] p < 0.01, compared to the sedentary group.

pro-inflammatory/anti-inflammatory markers were higher (p < 0.05 for CD11c+/CD206+ and p < 0.01 for iNOS+/ARG-1+) in the exercised group compared to the sedentary group, corroborating that this exercise protocol that induces anxiety provokes a pro-inflammatory state of peritoneal macrophages.

Finally, concerning the expression of cytokines in peritoneal macrophages, a reduction in the expression of the anti-inflammatory IL-10 (p < 0.01) in the exercised group was found (Fig. 6), confirming the pro-inflammatory response in the peritoneal macrophages due to the exercise protocol. There were no differences in the expression of the rest of cytokines (Table 5).

3.5. Exercise inducing anxiety increases macrophage infiltration in white adipose tissue

The frequency and distribution of infiltrated macrophages in the WAT, as well as the presence of crown-like structures (CLS), were assessed in each group of animals. Cells were identified based on

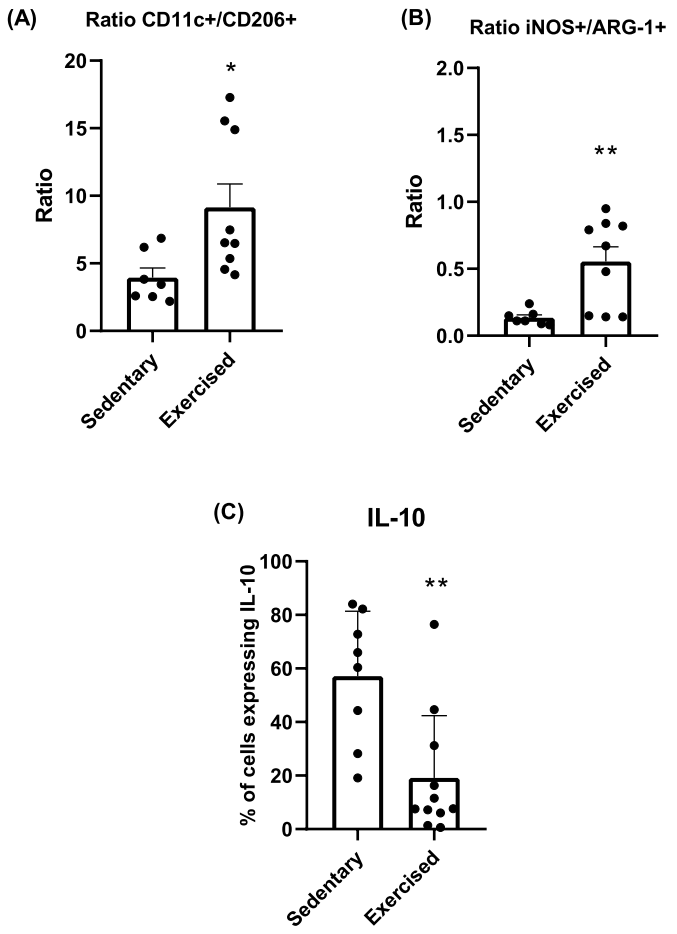

Fig. 6. Effect of the protocol of exercise on the ratio of the inflammatory phenotypic profile (A and B) and the expression of the anti-inflammatory cytokine IL-10 (C) in peritoneal macrophages. Each value represents the mean ± SEM of the determinations obtained from 8 sedentary animals (3 males and 5 females) and 11 exercised animals (7 males and 4 females). *p < 0.05, **p < 0.01, compared to the sedentary group. The t and degrees of freedom (df) values are provided as t(df): for ratio CD11c+/CD206+ t(14) = 2.5; for ratio iNOS+/ARG-1+ t(14) = 3.3; for expression of IL-10 t(17) = 3.5.

Table 5
Effect of the protocol of exercise on the expression of inflammatory response cytokines in peritoneal macrophages.

Cytokines	Sedentary	Exercised	*t*-test
MCP-1	41.4 ± 12	37 ± 8	$t(17) = 0.32$, $p = 0.38$
IL-8	18.9 ± 6	26.7 ± 10	$t(17) = 0.63$, $p = 0.27$
IL-6	32.1 ± 9	36.6 ± 9	$t(17) = 0.34$, $p = 0.37$
TNF-α	24.7 ± 5	28.4 ± 5	$t(17) = 0.3$, $p = 0.19$
TGF-β	26.5 ± 9	28.7 ± 5	$t(17) = 0.23$, $p = 0.41$

Each value represents the mean ± SEM of the determinations obtained from 8 sedentary animals (3 males and 5 females) and 11 exercised animals (7 males and 4 females). The t and degrees of freedom (df) values are provided as t(df).

immunoreactivity for both F4/80 (macrophages) and CD206 (M2 macrophages) antibodies. Data analysis considered both representative histological images (Fig. 7) and objective statistical data acquired from those images (Table 6).

Following the exercise protocol, there was an elevated number of infiltrated macrophages (p < 0.05) compared to the sedentary group, but no differences were found in the number of infiltrated M2 macrophages, suggesting that exercise entails a greater infiltration of macrophages and therefore a heightened state of inflammation. Exercised animals also showed an increase in CLS in the tissue with respect to

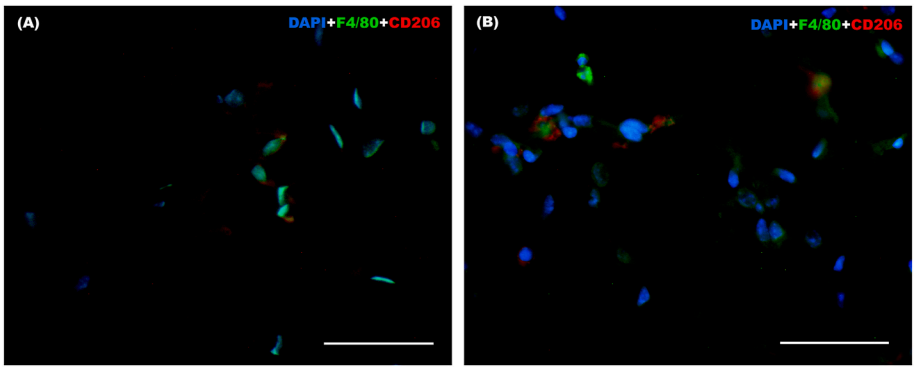

Fig. 7. Representative images of F4/80+ (green) cells (macrophages) and F4/80+CD206+ (red) cells (M2 macrophages) in white adipose tissue. (A) sedentary; (B) exercised. Nuclei were stained with DAPI (blue). Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 6
Effect of the protocol of exercise on the number of CLS, infiltrated macrophages, M2 and % of M2 macrophages found in the adipose tissue of sedentary and exercised mice.

	Sedentary	Exercised	*t*-test
CLS	0.2 ± 0.2	2 ± 1.4	$t(11) = 1.4$, $p = 0.12$
Infiltrated macrophages (number)	17 ± 2.8	32.5 ± 4.4[a]	$t(10) = 2.3$, $p = 0.03$
Infiltrated M2 macrophages (number)	8.7 ± 3	11 ± 2.3	$t(11) = 0.6$, $p = 0.28$
% M2 macrophages	38.4 ± 8.1	33 ± 5	$t(11) = 0.62$, $p = 0.28$

Each value represents the mean ± SEM of the determinations in 10 random fields at 40x magnification using fluorescence microscopy, performed in 5 sedentary animals (3 males and 2 females) and 8 exercised animals (4 males and 4 females). The t and degrees of freedom (df) values are provided as t(df).

[a] $p < 0.05$ compared to the sedentary group.

sedentary animals, although without statistically significant differences, supporting the WAT inflammation found in the group of exercised animals.

4. Discussion

The findings of this study highlight the significant impact of a protocol of exercise-induced anxiety on the intricate neuro-immunoendocrine interactions. First, we validated that our exercise model induced anxiety in mice by employing the elevated plus maze test and the hole-board test. The first of these widely used behavioral assays evaluates anxiety-like behavior by assessing the preference of mice for open versus enclosed arms, providing robust evidence of the anxiety-inducing effects of our exercise protocol. Additionally, results from the hole-board test also reinforce the anxiety-like behavior in our animals after the protocol of exercise, since locomotor activity is inversely related to anxiety levels (Himanshu et al., 2020). Results clearly showed that our model of swimming exercise caused anxiety in the animals, as previously shown in recent pilot studies in our laboratory (Navarro et al., 2023).

It is well-known that exercise is a form of stress that activates various physiological pathways in order to maintain homeostasis during physical exercise, including the release of the stress hormone corticosterone (Ortega, 1994, 2003). Moreover, during the immune response, pro-inflammatory cytokines such as IL-1β activate the stress system through stimulation of the HPA axis, resulting in the release of corticosterone to prevent an excessive immune response and protect the organism from an overshoot of pro-inflammatory cytokines (Besedovsky and Rey, 2007; Elenkov, 2008). Optimal regulation of the interactions between the inflammatory and stress responses in the absence of pathogen challenge is crucial for the effects of exercise on the innate response (Ortega, 2016).

In the present work, exercised mice presented a significant increase in the systemic concentrations of the potent pro-inflammatory cytokine IL-1β in comparison to sedentary animals. This IL-1β upregulation suggests a heightened inflammatory state in response to the exercise protocol, indicating a sterile inflammation due to the potential "danger signal" caused by anxiety induced by the exercise. Moreover, exercised animals also presented increased systemic corticosterone levels compared to sedentary animals. These elevated concentrations of corticosterone are unable to inhibit further IL-1β release, reflecting a potential dysregulation in the negative feedback between corticosterone and IL-1β. This alteration can perpetuate a stress and pro-inflammatory state where the normal regulatory mechanisms fail to effectively modulate the inflammatory response. Therefore, our results suggest that when exercise is accompanied by anxiety, there is an immunoneuroendocrine dysregulation consisting of potential disruption in the feedback mechanisms between inflammatory and neuroendocrine/stress responses. The paradoxical decrease in NA could be compatible with a decrease in the concentration of pro-inflammatory cytokines, such as IL-1β in the present study, since NA has an inhibitory effect on the release of IL-1β by macrophages (Elenkov et al., 2008). Then, physiologically decreased NA levels may allow for an increased inflammatory response. Lower NA levels also align with the decreased levels of eHsp72, as NA is a signal that induces the release of eHsp72 by immune cells (Giraldo et al., 2010).

Macrophages play a crucial role in the immune defense of the body against infections (Lee and Lee, 2014), with roles including pathogen detection and elimination, the production of cytokines and chemokines that orchestrate inflammatory responses, and the maintenance of homeostasis (Hirayama et al., 2018). Results in the present study clearly showed that exercise produced a pro-inflammatory state in peritoneal macrophages, mainly manifested by a decrease in the percentage of cells with an anti-inflammatory M2 phenotype (including the markers CD206, ARG-1, and its combination), an increase in the ratios of pro-inflammatory M1/anti-inflammatory M2 phenotype markers (CD11c+/CD206+ and iNOS+/ARG-1+) and a reduced expression of the anti-inflammatory cytokine IL-10. These findings are consistent with the aforementioned systemic pro-inflammatory effects of the exercise protocol, thus reinforcing its impact in immune function and inflammation at the organism level. Given that the inflammatory response is an inherent part of the innate response, an exercise-induced increased microbicidal capacity of peritoneal macrophages might be expected, a phenomenon that has been previously documented in various exercise modalities and in mice strains of different ages (De la Fuente et al., 2011). The microbicidal capacity of macrophages refers to their ability to produce microbicidal substances such as superoxide anion to destroy

or eliminate pathogenic microorganisms, significantly contributing to the host's immune response in the presence of infectious agents (Sasada and Johnston, 1980). Indeed, findings from the present study revealed stimulation of superoxide anion production, which could be interpreted as an enhanced oxygen-dependent microbicidal capacity following phagocytosis. However, given the lack of stimulation of the phagocytic capacity of these cells, it seems more plausible to interpret this elevation in reactive oxygen species (ROS) levels in the absence of phagocytic stimulation as indicative of oxidative stress dysregulation within these cells. This phenomenon seems consistent with the increased pro-inflammatory state of macrophages after the exercise protocol, reflecting their activation state. However, when research conducted on mice using a similar water tank and also involving daily baths in warm water over a 2-week period, but not inducing anxiety (as assessed by the plus maze test and other behavioral tests), beneficial effects in the macrophage-mediated inflammatory response, innate capacity against pathogens and oxidative stress were found (De la Fuente et al., 2011). This observation underscores that the association between exercise and anxiety can elicit different effects on stress and innate/inflammatory responses compared to situations where exercise is not associated with anxiety, therefore suggesting that individuals experiencing anxiety during exercise may be at increased risk for neuroimmunoendocrine dysregulation.

Assessing the local response of macrophages in the adipose tissue is crucial, as it provides insights into the tissue-specific inflammatory processes that contribute to systemic stress, metabolic, and inflammatory dysregulation. After exercise, there was a significant increase in macrophage infiltration in the adipose tissue, together with a tendency towards greater CLS formation, indicating greater metabolic activity and a pro-inflammatory state of the adipose tissue that leads to increased recruitment of active immune cells such as macrophages. The infiltration of macrophages into adipose tissue, particularly when forming CLS, is a hallmark of inflammation and is associated with metabolic alterations (Cinti et al., 2005). However, it could be that the infiltrated macrophages had an anti-inflammatory profile, so we aimed to verify that the increase in the number of infiltrating macrophages (F4/80+) was not due to a significant increase in just M2 macrophages. This was confirmed by finding no significant differences when assessing the effect of exercise on CD206+ macrophages. Determination of CD206+ cells has been accepted for the evaluation of the pro-/anti-inflammatory profile of macrophages in adipose tissue (Fujisaka et al., 2009; Gálvez et al., 2023). These results strongly suggest that the evaluated anxiety-inducing exercise protocol induced a pro-inflammatory response in the adipose tissue that would contribute, through the induction of insulin resistance (Ye, 2013), to the increased fasting blood glucose concentrations and the systemic inflammatory responses in the exercised animals.

These elevated glucose levels, in absence of weight gain or lipid profile changes, suggest a direct impact of the anxiety-inducing exercise on glucose metabolism. Regular, moderate-intensity physical activity is known to enhance insulin sensitivity and promote glucose uptake, which typically leads to lower fasting glucose levels (Colberg et al., 2016; Perseghin et al., 1996). However, in our experimental group, the elevated glucose concentrations indicate a disruption in glucose homeostasis, potentially due to the neuroimmunoendocrine dysregulation caused by the exercise protocol, and particularly to the increased pro-inflammatory state of the adipose tissue. Also, chronic exposure to high levels of corticosterone can lead to glucocorticoid resistance, avoiding its inhibitory capacity of the release of IL-1β, suppressing the anti-inflammatory pathways and promoting proinflammatory ones (Elenkov and Chrousos, 2002; Knezevic et al., 2023). In fact, the stress response triggered by exercise, as reflected by the release of corticosterone, may contribute to the promotion of gluconeogenesis and insulin resistance, impairing glucose utilization in peripheral tissues, thus leading to hyperglycaemia (Preiser et al., 2016). In turn, the elevated systemic glucose levels in these mice may exacerbate immune activation, as hyperglycemia can trigger the activation of immune cells like macrophages therefore increasing the production of pro-inflammatory cytokines (Xiu et al., 2014). This elevation of IL-1β can also contribute to the anxiety of our animals. In fact, IL-1β activates the HPA axis which has been suggested to mediate anxiety. For instance, IL-1 receptor KO mice have shown decrease anxiety-like behavior (Koo and Duman, 2009). Thus, the interrelated effects of corticosterone overexposure, glucose metabolism and immune inflammation can create a feedback loop driving neuroimmunoendocrine dysregulation, where inappropriate exercise can exacerbate inflammation and the stress/anxiety response.

To the best of our knowledge, this is the first study assessing the effects of a model of non-forced swimming in rodents that causes anxiety on the complex interplay between the systemic and macrophage-mediated inflammatory response, and the stress, and metabolic responses. Taking all these results into account, neuroimmunoendocrine anomalies in the systemic inflammatory/stress feedback together with hyperactivity of the local pro-inflammatory factors and hyperglycaemia reflect the adverse effects of regular exercise when it leads to anxiety-like behavior. Therefore, this anxiety-inducing exercise modality could serve as a robust model of 'dangerous exercise' within the immunometabolic setting mediated by the inflammatory responses of macrophages (Gálvez et al., 2022), particularly concerning its impact on the inflammatory response and its manifestation in animals' hyperglycaemia. This confirms that not all exercise modalities can achieve anti-inflammatory responses and improve homeostasis. In fact, inappropriate exercise may also trigger pro-inflammatory and metabolic deleterious responses (Martín-Cordero et al., 2011, 2013). This highlights the importance of customizing each exercise modality to each individual's physiological and psychological state. These findings emphasize the need for personalized exercise prescriptions that maximize benefits while minimizing risks, with a particular focus on avoiding exercise modalities that provoke anxiety.

5. Limitations of the study

Despite the valuable insights our findings provide into the significant impact of exercise-induced stress and anxiety on complex neuroimmunoendocrine interactions, the relatively small sample size for each sex may have limited our ability to detect sex-specific differences in response to the exercise protocol.

6. Conclusions

Exercise that induces anxiety is associated with a neuroimmunoendocrine dysregulation affecting the feedback between the inflammatory and the stress responses and leading to detrimental metabolic effects in glucose modulation. Systemic inflammatory alterations are accompanied by detrimental inflammatory responses in tissue macrophage populations, which can also explain the changes in glucose metabolism. Altogether, these results confirm that exercise associated with anxiety, stress, and pro-inflammatory responses represents a model of 'dangerous exercise', leading to deleterious effects, such as hyperglycaemia, crucially linked to adverse events and chronic disease.

CRediT authorship contribution statement

I. Gálvez: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **M.C. Navarro:** Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **S. Torres-Piles:** Investigation, Methodology. **L. Martín-Cordero:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **M. D. Hinchado:** Formal analysis, Investigation, Methodology, Supervision. **E. Ortega:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Visualization,

Writing – original draft, Writing – review & editing.

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Declaration of competing interest

There are conflict of interest among all authors.

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Data availability

Data will be made available on request.

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