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Original Research

Aerobic Running Training Attenuates Lipid and Protein Oxidation and Modulates Membrane Dynamics in Metabolically Active Tissues

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Abstract

Background: Chronic aerobic exercise is known to modulate oxidative stress, yet comparative analyses across multiple tissues remain limited. This study aimed to evaluate the effects of different training durations on oxidative damage and membrane fluidity in metabolically active tissues, including skeletal muscle, heart, and brain. **Methods**: Forty male Sprague–Dawley rats were randomly assigned to four groups (n = 10/group): control (CON), and aerobic treadmill training for 1 week (1W), 4 weeks (4W), and 12 weeks (12W). Training consisted of four 60-minute sessions per week, alternating intensities at 35% and 80% of maximal velocity. Quadriceps skeletal muscle, heart, and brain were collected. Oxidative damage was assessed via malondialdehyde and 4-hydroxyalkenals (MDA + 4-HDA) and protein carbonyl content. Membrane fluidity was evaluated in plasma and mitochondrial membranes using fluorescence spectroscopy. Statistical analyses were performed using one-way analysis of variance followed by Tukey post-hoc tests. **Results**: Aerobic training significantly reduced MDA + 4-HDA levels in skeletal muscle and heart compared with control values, with a downward trend in brain tissue that did not reach statistical significance. Protein carbonyls decreased significantly in skeletal muscle at 4W and 12W, but remained unchanged in heart and brain (despite lower mean values observed in all training groups compared with the control). Plasma membrane fluidity declined significantly in all tissues, especially at 4W and 12W, indicating structural remodeling. Mitochondrial membrane fluidity values were lower in heart and skeletal muscle across the different training groups, with statistically significant differences observed only and remarkably in skeletal muscle compared with control values; meanwhile, values remained stable in brain tissue. These findings reveal tissue-specific biochemical and biophysical adaptations to aerobic training. **Conclusions**: Chronic aerobic treadmill training induces protective adaptations against oxidative stress in skeletal muscle, heart, and brain. The reduction in lipid peroxidation and protein oxidation, along with changes in membrane fluidity, reflects enhanced cellular resilience and structural integrity. These results support the role of sustained aerobic exercise as a non-pharmacological strategy to mitigate oxidative damage and promote tissue health. The rat model used provides translational relevance for understanding exercise-induced protection mechanisms.

Keywords: exercise; lipid peroxidation; protein carbonylation; membrane fluidity; mitochondria; oxidative stress; endurance training

1. Introduction

Lipid peroxidation (LPO) is a well-established mechanism of cellular damage, particularly affecting polyunsaturated fatty acids within biological membranes. This oxidative process leads to the formation of various degradation products, including reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA), which serve as biomarkers of oxidative damage to lipids and contribute to structural and functional alterations in membranes [1,2]. Among the tissues most vulnerable to LPO are skeletal muscle, cardiac muscle, and brain, due to their high metabolic activity and lipid content [3].

Regular physical exercise is widely recognized for its health-promoting effects, including improved cardiovascular function, enhanced metabolic efficiency, and increased antioxidant capacity [4]. However, the physiological adaptations induced by chronic exercise are complex and tissue-specific, particularly in relation to oxidative stress. Although acute bouts of exercise may transiently increase reactive oxygen species (ROS) production, long-term training has been shown to reduce oxidative damage in various tissues [5,6].

Membrane fluidity, a critical determinant of cellular function, is highly sensitive to oxidative modifications. Changes in lipid composition and protein cross-linking due to ROS can lead to increased membrane rigidity, impairing processes such as ion transport, receptor signaling, and mitochondrial respiration [7]. Notably, strenuous exercise has been associated with a significant accumulation of carbonylated proteins in the plasma, suggesting elevated oxidative damage. This may contribute to increased rigidity in cellular membranes [8]. The extent to which chronic aerobic training modulates membrane fluidity and oxidative damage remains an area of active investigation, with implications for understanding exercise-induced cellular resilience.

In this context, the quantification of lipid peroxidation products such as MDA and 4-HDA (MDA + 4-HDA) provides a reliable index of oxidative damage to tissue lipids.

Additionally, the measurement of protein carbonyl content serves as a marker of oxidative modification of proteins, which can compromise enzymatic activity and structural integrity [9,10]. These biochemical indicators, combined with assessments of membrane fluidity (both in the plasma membrane and mitochondrial membrane) offer a comprehensive view of the oxidative status and functional adaptability of tissues subjected to chronic exercise.

Previous studies have demonstrated that endurance training can attenuate LPO and improve cellular resilience in skeletal muscle and heart [11,12]. Similarly, adaptations in brain tissue have been observed, although the central nervous system exhibits lower antioxidant capacity and higher susceptibility to oxidative injury [13]. Despite these findings, comparative analyses across multiple tissues under standardized training protocols remain limited, particularly those integrating biochemical and biophysical markers of oxidative stress.

1.1 Rationale

Understanding the tissue-specific oxidative adaptations induced by chronic aerobic training is essential for elucidating the mechanisms underlying exercise-mediated cellular protection. By integrating biochemical markers of lipid and protein oxidation with biophysical assessments of membrane fluidity, this study provides novel insights into the systemic impact of long-term physical activity. These findings may contribute to the development of targeted exercise interventions aimed at enhancing cellular resilience and preventing oxidative damage in vulnerable tissues.

1.2 Aims

The present study aimed to evaluate the effects of chronic aerobic treadmill training on lipid peroxidation, protein oxidation, and membrane fluidity in the quadriceps skeletal muscle, heart, and brain tissues of male Sprague–Dawley rats. Specifically, we investigated whether different durations of training (1, 4, and 12 weeks) modulate oxidative damage markers (MDA + 4-HDA levels, protein carbonyl content) and membrane dynamics in both plasma and mitochondrial membranes.

It was hypothesized that chronic aerobic treadmill training would attenuate lipid and protein oxidation and promote structural adaptations in plasma and mitochondrial membranes of metabolically active tissues, including skeletal muscle, heart, and brain.

2. Materials and Methods

2.1 Animals

Forty male Sprague–Dawley rats (average weight: 342.15 ± 22.74 g; age: 10 ± 0.5 weeks) were obtained from Harlan Interfauna Ibérica (Barcelona, Spain). Only male rats were used in this study to avoid any hormonal variability in exercise response mediated by the ovarian cycle. Animals were housed in pairs under controlled temperature (22.0 ± 1.0 °C) and a 12:12 h light-dark cycle (dark phase starting at 8:00 a.m., and light phase at 8:00 p.m., in order to simulate their peak activity period), with *ad libitum* access to food and water (RMM rodent maintenance diet, Harlan Interfauna Ibérica [Barcelona, Spain]). Animal handling and experimental procedures were carried out in the morning in a thermoregulated isolated room simulating darkness, covering natural light entrances and using red light. During transportation between the animal facility and the experimental room, cages were placed inside opaque compartments to prevent exposure to ambient light. All procedures were conducted in accordance with the current legislation on the protection and use of animals for scientific purposes at the European (Directive 2010/63/EU), national (Spain: RD53/2013) and regional (Aragón: Law 1/2019) levels, and approved by the Clinical Research Ethics Committee of Aragón (ref. CP02/2010).

2.2 Experimental Design

Rats were randomly assigned to four groups, homogeneously, with n = 10/group [11,12,14]: control (CON), and training groups of 1, 4, and 12 weeks (1W, 4W, and 12W, respectively).

All rats of training groups underwent a maximal ergometry consisting of a progressive incremental treadmill test to exhaustion. CON group did not complete any ergometry. The purpose of this test was to assess physical capacity in the three training groups, with regard to determine the workload each rat would perform throughout its corresponding exercise duration (1, 4, or 12 weeks).

Training sessions were performed 4 days/week for 60 min/day, alternating 6 cycles of 2 min at 35% and 8 min at 80% of maximal velocity, determined from the initial maximal ergometry.

Animals were euthanized by CO_2 exposure, followed by decapitation. The gas was introduced into the induction chamber at a rate equivalent to 50% of the chamber's total volume per minute, supplemented by ambient air (4.5 L/min) [15]. Animals were continuously monitored throughout the procedure, and CO_2 administration was maintained for at least one minute after respiration ceased. Death was confirmed by cervical dislocation in accordance with ethical guidelines. Decapitation was subsequently performed to obtain brain tissue following completion of euthanasia. Trained rats were sacrificed 24 h after the last training session to avoid any potential acute effects of exercise, and CON rats the day after their housing, at the same time slot (between 10 a.m. and 12 p.m.). Tissue samples (quadriceps skeletal muscle, heart, and brain) were immediately extracted, rinsed in cold saline (4 °C), dried, and stored at –80 °C until analysis.

2.3 Exercise Equipment and Protocols

A single-lane treadmill was used for maximal ergometry assessments. However, training sessions were per-

formed on a five-lane treadmill (Panlab, Cornellà de Llobregat, Barcelona, Spain). Both types of treadmills were equipped with an electrical stimulation grid at the rear, and they were connected to a module that allowed for speed regulation and recording.

During the week prior to the maximal exercise test, animals underwent an adaptation protocol to the treadmill, consisting of low-intensity running at a constant speed (0.10–0.12 m/s) for 5 days, 1 h/day. This test was performed between 10 a.m. and 12 p.m., and it began at a speed of 0.15 m/s with a treadmill incline of 10°, increasing by 0.03 m/s every 3 min [16]. The test was terminated at maximal effort upon the appearance of unmistakable signs of fatigue, defined as rats' inability to continue running on the treadmill despite electrical stimulation [17]. An initial electrical stimulation intensity of 0.2 mA was applied (the minimum permitted by the ergometry device), progressively increasing to a maximum of 0.4 mA by the end of the test.

Rats from each training group (1W, 4W, and 12W) were divided into two subgroups of five rats each to carry out the training programs on the five lane treadmill. Physical capacity, defined by maximal velocity and assessed through a prior maximal ergometry test, served as the selection criterion. This produced two subgroups per training group: one comprising the five top-performing animals, and the other the five lowest-performing.

The training protocol for the 1W, 4W, and 12W groups consisted of exercise interventions lasting 1, 4, and 12 weeks, respectively. Animals completed four structured treadmill sessions per week (on Monday, Tuesday, Thursday, and Friday). Each exercise session lasted 60 min, with alternating intensity phases repeated over six consecutive 10-min cycles. Each cycle comprised a 2-min low-intensity phase at 35% of the maximal velocity reached during the ergometry test, followed by an 8-minute high-intensity phase at 80% of that velocity [18,19]. The treadmill incline was set at 10°, and all training sessions were conducted between 10 a.m. and 2 p.m. to ensure temporal consistency.

Given the extended duration of the training period and the expected physiological adaptation of the animals, 12W training group underwent a second acute ergometry test at the midpoint of the intervention. This reassessment allowed for a slight adjustment of the initially prescribed workloads, ensuring that the exercise intensity remained appropriate throughout the protocol.

2.4 Analytical Procedures

All chemical reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Panreac (Castellar del Vallès, Barcelona, Spain), except for the Bradford reagent used for protein quantification (Bio-Rad, Hercules, CA, USA), and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluenesulfonate (TMA-DPH), used to assess membrane fluidity (Molecular Probes, Eugene, OR, USA).

2.4.1 Lipid Peroxidation

MDA + 4-HDA concentration was determined using a colorimetric method based on its reaction at 45 °C with a chromogenic reagent, N-methyl-2-phenylindole, yielding a stable chromophore that exhibits a maximum absorbance at 586 nm in the presence of methanesulfonic acid [2]. Calibration was performed using 1,1,3,3-tetramethoxypropane as a standard, which releases MDA upon hydrolysis in a stoichiometric ratio, enabling accurate quantification [20]. Final concentrations of MDA + 4-HDA were normalized to total protein content and expressed as nmol/mg protein.

Tissue samples were homogenized in 0.02 M tris(hydroxymethyl)aminomethane (TRIS) buffer at a ratio of 1:10 (w/v) and subsequently centrifuged at 3000 $\times g$ for 10 min at 4 °C to obtain the supernatant. The aliquots from each sample were then mixed with 10.3 mM N-methyl-2-phenylindole, prepared in a 3:1 (v/v) mixture of acetonitrile and methanol. After vortexing, 15.4 M methanesulfonic acid were added, followed by incubation at 45 °C for 40 min. The samples were centrifuged again under the same conditions, and the resulting supernatants were allowed to equilibrate at room temperature for 10 min. Absorbance was then measured at 586 nm using a spectrophotometer (Beckman Coulter, Brea, CA, USA) to quantify the concentration of MDA + 4-HDA.

2.4.2 Protein Carbonylation

The analytical procedure for protein carbonyl groups quantification was based on the method described by Levine *et al*. [21], with minor modifications. This assay relies on the reaction between protein-bound carbonyls and 2,4-dinitrophenylhydrazine (DNPH), forming hydrazone derivatives that exhibit a characteristic absorbance peak between 360 and 390 nm. During the procedure, trichloroacetic acid (TCA) was used to precipitate proteins, followed by successive washes to remove unreacted DNPH. The final protein pellet was solubilized in guanidine hydrochloride, allowing spectrophotometric quantification (Beckman Coulter, Brea, CA, USA). Carbonyl content was calculated using the Beer–Lambert law and the molar extinction coefficient of DNPH ($\varepsilon = 22{,}000\ M^{-1}\cdot cm^{-1}$), with 6 M guanidine hydrochloride serving as the blank. Final results were normalized to total protein content and expressed as nmol/mg protein.

Tissue samples were homogenized in 0.5 M TRIS buffer (pH 7.4). These homogenates were mixed with 0.02 M TRIS buffer and 0.01 M DNPH in 2 N HCl. After vortexing, samples were incubated for 1 hour at 37 °C. Subsequently, cold 50% TCA were added, and samples were incubated on ice for 10 min. The precipitate was collected by centrifugation at 3000 $\times g$ for 10 min at 4 °C and washed three times with ethanol:ethyl acetate (1:1, v/v), centrifuging at 11,000 $\times g$ for 3 min at 4 °C after each wash. The final pellet was dissolved in 6 M guanidine-HCl (pH 2.0), vortexed, and incubated for 15 min at 37 °C. After a final cen-

trifugation at 12,000 $\times g$ for 10 min at 4 °C, the absorbance of the supernatant was measured at 375 nm.

2.4.3 Plasma and Mitochondrial Membrane Fluidity

Prior to membrane fluidity analysis, plasma and mitochondrial membranes were isolated. For quadriceps skeletal muscle and heart, the protocol described by Graham was followed [22], with slight modifications in brain. All procedures were based on differential centrifugation at varying speeds and durations.

Quadriceps and heart were weighed and finely minced on a Petri dish placed on ice. The tissue fragments were then transferred to a glass - Teflon homogenizer connected to a rotor (Heidolph RZR 2020, Schwabach, Germany) and homogenized at 1650 rpm in 0.02 M 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES) - 0.14 M KCl buffer (pH 7.4). All procedures were carried out under cold conditions to preserve sample integrity. Tissues were homogenized and centrifuged at 1000 $\times g$ for 10 min at 4 °C to remove cellular debris and nuclei. The supernatant was then centrifuged at 50,000 $\times g$ for 20 min at 4 °C. The resulting pellet, containing membrane fractions, was resuspended in HEPES buffer, homogenized, and centrifuged at 10,000 $\times g$ for 10 min at 4 °C. Subsequently: (1) the supernatant and buffy coat were centrifuged at 50,000 $\times g$ for 20 min at 4 °C to isolate plasma membranes; (2) the pellet was resuspended in HEPES and centrifuged at 10,000 $\times g$ for 10 min at 4 °C to isolate mitochondrial membranes. Both final pellets were resuspended in 0.05 M TRIS buffer (pH 7.4), aliquoted, and stored at –80 °C until analysis.

For brain tissue, samples were homogenized in 0.32 M sucrose at 4 °C and centrifuged at 1000 $\times g$ for 10 min at 4 °C. The supernatant was centrifuged at 30,000 $\times g$ for 20 min at 4 °C. The pellet was resuspended in ultrapure water [23,24], homogenized, and centrifuged at 8000 $\times g$ for 20 min at 4 °C. Then: (1) the supernatant and buffy coat were centrifuged at 48,000 $\times g$ for 20 min at 4 °C to obtain plasma membranes; (2) the pellet was resuspended and centrifuged again at 8000 $\times g$ for 20 min at 4 °C to isolate mitochondrial membranes. Both membranes were resuspended in 0.05 M TRIS buffer (pH 7.4), aliquoted, and stored at –80 °C until use.

Fluidity of plasma and mitochondrial membranes isolated from all three tissues was evaluated using fluorescence spectroscopy (PerkinElmer, Waltham, MA, USA) with the probe TMA-DPH [7]. This amphipathic molecule integrates into the phospholipid bilayer and emits fluorescence upon excitation, thereby serving as an indirect indicator of lipid dynamics [7]. For each assay, 0.5 mg/mL of membrane protein (quantified via the Bradford method) was incubated with 25 µM TMA-DPH in 0.05 M TRIS buffer (pH 7.4) to a final volume of 3 mL. Samples were vigorously mixed and incubated at 37 °C for 30 min with continuous agitation. Prior to measurement, tubes were equilibrated in a water bath at room temperature and gently agitated to resuspend the membranes. Fluorescence readings were taken at 360 nm excitation and 430 nm emission wavelengths. Polarization (P) was calculated using the equation:

$$P = \frac{IV_V - GIV_H}{IV_V + GIV_H}$$

where IV_V and IV_H represent the intensities of vertically and horizontally polarized emitted light, respectively, and G is the instrumental correction factor. Each sample was measured 30 times, and membrane fluidity was expressed as the inverse of polarization (1/P) [7,25].

2.4.4 Protein Quantification

All results related to oxidative damage markers assessed in this study (including MDA + 4-HDA concentrations, protein carbonylation, and membrane fluidity) were normalized to the protein concentration of each sample to ensure comparability across experimental conditions.

Protein concentration was determined using the Bradford assay [26], based on the binding of Coomassie Brilliant Blue G-250 to basic amino acid residues, producing a shift in absorbance measured at 595 nm. Sample absorbance values were interpolated against a bovine serum albumin (BSA) standard curve, and results were expressed in mg/mL.

2.5 Statistical Analysis

Data normality was assessed using the Shapiro-Wilk test. After confirming the assumption of normal distribution, descriptive statistics (mean ± standard error [SE]) and a one-way between-groups ANOVA were performed to evaluate MDA + 4-HDA levels, protein carbonyl content, plasma membrane fluidity, and mitochondrial membrane fluidity in quadriceps skeletal muscle, heart, and brain of control and exercised rats (1W, 4W, and 12W). Homogeneity of variances was verified using Levene's test. Effect sizes were calculated using partial eta squared (η^2) and interpreted according to established benchmarks: trivial ($\eta^2 < 0.01$), small ($0.01 \leq \eta^2 < 0.06$), medium ($0.06 \leq \eta^2 < 0.14$), and large ($\eta^2 \geq 0.14$). Observed statistical power was calculated to ensure values exceeded 0.80, thereby minimizing the risk of type II error. Post-hoc pairwise comparisons were conducted using Tukey's test to adjust for multiple testing. Statistical significance was set at $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. All analyses were performed using SPSS v.30 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism v.10.6.1 (GraphPad, Boston, USA) software.

3. Results

Descriptive analysis revealed distinct patterns in biochemical markers across quadriceps skeletal muscle, heart, and brain tissues in response to different durations of ex-

Table 1. Descriptive statistics (mean ± SE) and one-way ANOVA outcomes for MDA + 4-HDA levels, protein carbonyls, plasma membrane fluidity, and mitochondrial membrane fluidity in skeletal muscle, heart, and brain of control and exercised rats (1, 4, and 12 weeks of aerobic training).

Marker	Tissue	CON	1W	4W	12W	p-value (ANOVA)	η^2 partial	1-β
	SM	0.1707 ± 0.0165	0.1030 ± 0.0125	0.1056 ± 0.0052	0.1152 ± 0.0082	<0.0001***	0.619	0.997
MDA (nmol/mg protein)	H	0.4336 ± 0.0238	0.3399 ± 0.0161	0.3636 ± 0.0120	0.3566 ± 0.0214	0.0084**	0.425	0.550
	B	0.4499 ± 0.0347	0.3721 ± 0.0308	0.4185 ± 0.0329	0.3968 ± 0.0333	0.072	0.523	0.549
	SM	3.9074 ± 0.5133	2.8267 ± 0.3972	2.1935 ± 0.1484	2.3482 ± 0.2540	0.021*	0.365	0.762
CARB (nmol/mg protein)	H	8.0937 ± 0.2626	6.1625 ± 0.9797	6.4004 ± 0.9779	6.2146 ± 0.9304	0.241	0.358	0.282
	B	13.7608 ± 1.3097	10.8230 ± 1.9992	8.8934 ± 1.5829	9.8647 ± 2.1513	0.384	0.164	0.142
	SM	3.3028 ± 0.0327	3.1367 ± 0.0510	3.0974 ± 0.0475	3.1679 ± 0.0452	0.001**	0.477	0.964
PlMF (1/P)	H	3.0833 ± 0.0479	2.9596 ± 0.0298	2.9058 ± 0.0165	2.9168 ± 0.0223	0.002**	0.446	0.939
	B	2.8586 ± 0.0329	2.7490 ± 0.0226	2.7471 ± 0.0166	2.7274 ± 0.0242	<0.001***	0.569	0.996
	SM	3.2719 ± 0.0390	3.0821 ± 0.0277	3.0313 ± 0.0179	3.0601 ± 0.0406	<0.001***	0.728	1.000
MitoMF (1/P)	H	3.0419 ± 0.0385	2.9921 ± 0.0321	2.9738 ± 0.0241	2.9240 ± 0.0231	0.042*	0.284	0.660
	B	3.3953 ± 0.0596	3.5349 ± 0.0579	3.5152 ± 0.0772	3.4429 ± 0.0887	0.775	0.044	0.112

Values in 'CON', '1W', '4W' and '12W' columns are expressed as mean ± standard error (SE). CON, control group; 1W, 1 week-training group; 4W, 4 weeks-training group; 12W, 12 weeks-training group; MDA, malondialdehyde and 4-hydroxyalkenals; CARB, protein carbonyls content; PlMF, plasma membrane fluidity; MitoMF, mitochondrial membrane fluidity; SM, skeletal muscle; H, heart; B, brain. p significance values: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Partial eta squared (η^2), and observed power (1-β) correspond to one-way ANOVA.

ercise (1, 4 and 12 weeks), compared to control rats. In addition, one-way between-groups ANOVA revealed significant effects of exercise duration on several biochemical markers. All these data can be observed in Table 1.

3.1 Quadriceps Skeletal Muscle

3.1.1 MDA + 4-HDA

Lipid peroxidation assessed via MDA + 4-HDA levels, was significantly reduced in skeletal muscle following exercise. Control values (0.17 ± 0.02 nmol/mg protein) dropped to 0.10 ± 0.01 at 1 week, and remained low at 4 and 12 weeks. ANOVA confirmed a strong time effect ($p < 0.0001$), with a large effect size ($\eta^2 = 0.619$) and high statistical power (1-$\beta = 0.997$). Tukey post-hoc comparisons showed significant differences between CON and 1W (95% CI: 0.024–0.110; adjusted p value = 0.0009), CON and 4W (95% CI: 0.021–0.107; adjusted p value = 0.0015), and CON and 12W (95% CI: 0.011–0.099; adjusted p value = 0.0092). The pairwise comparisons can be observed in Fig. 1a.

3.1.2 Protein Carbonyls

Protein oxidation in quadriceps skeletal muscle, measured via carbonyl content, decreased significantly following exercise. CON rats showed levels of 3.91 ± 0.51 nmol/mg protein, which dropped to 2.19 ± 0.15 at 4 weeks post-training. ANOVA indicated a significant effect of time post-exercise ($p = 0.021$), with a large effect size ($\eta^2 = 0.365$) and statistical power close to 0.8 (1-β = 0.762). Tukey post-hoc comparisons showed significant differences between CON and 4W (95% CI: 0.398–3.029; adjusted p value = 0.0066), and between CON and 12W (95% CI: 0.210–2.908; adjusted p value = 0.0184). See Fig. 1b for details.

3.1.3 Plasma Membrane Fluidity

In quadriceps skeletal muscle, plasma membrane fluidity was significantly altered at different time points following the cessation of exercise. CON group exhibited the highest fluidity values (3.30 ± 0.03 as 1/P), which decreased notably at 1W (3.14 ± 0.05), and reached a minimum at 4W (3.10 ± 0.05), following by a slight recovery at 12W (3.17 ± 0.05). One-way ANOVA confirmed a statistically significant effect of time post-exercise ($p = 0.001$), with a large effect size ($\eta^2 = 0.477$) and high statistical power (1-$\beta = 0.964$). Post-hoc analyses revealed significant differences between CON and 4W (95% CI: 0.037–0.373; adjusted p value = 0.0116). See Fig. 1c for pairwise comparisons.

3.1.4 Mitochondrial Membrane Fluidity

Mitochondrial membrane fluidity in skeletal muscle also decreased significantly post-exercise. CON rats showed values of 3.27 ± 0.04 as 1/P, which dropped to 3.03 ± 0.02 at 4 weeks, with a slight recovery at 12 weeks (3.06 ± 0.04). ANOVA confirmed a strong time effect ($p < 0.001$), with a large effect size ($\eta^2 = 0.728$) and robust statistical power (1-$\beta = 1.000$). Tukey post-hoc comparisons showed significant differences between CON and 1W (95% CI: 0.068–0.311; adjusted p value = 0.0009), CON and 4W (95% CI: 0.119–0.361; adjusted p value < 0.001), and between CON and 12W (95% CI: 0.087–0.336; adjusted p value = 0.0003). See Fig. 1d for details.

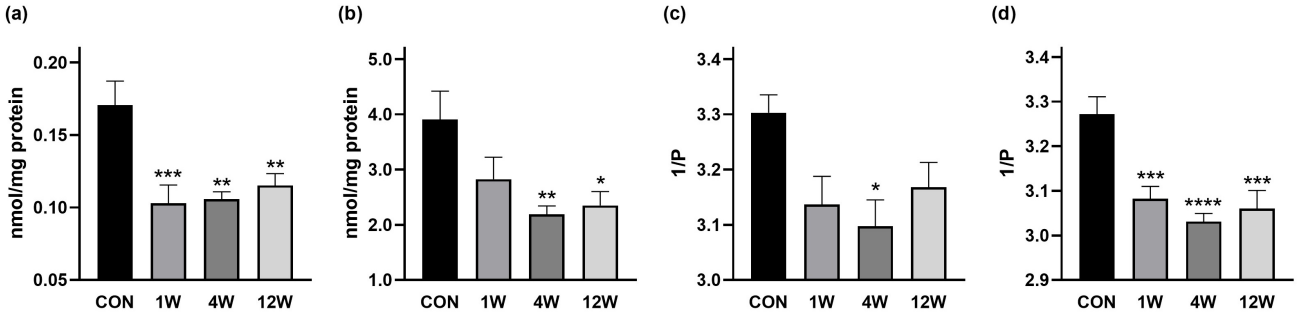

Fig. 1. Quadriceps skeletal muscle. (a) Concentration of malondialdehyde and 4-hydroxyalkenals (MDA + 4-HDA); results expressed as nmol/mg protein. (b) Content of protein carbonyl groups; results expressed as nmol/mg protein. (c) Membrane fluidity of tissue plasma membranes; results expressed as the inverse of polarization (1/P). (d) Membrane fluidity of tissue mitochondrial membranes; results expressed as the inverse of polarization (1/P). CON, control group; 1W, 1 week-training group; 4W, 4 weeks-training group; 12W, 12 weeks-training group. $^{*}p < 0.05$ vs. CON; $^{**}p < 0.01$ vs. CON; $^{***}p < 0.001$ vs. CON; $^{****}p < 0.0001$ vs. CON.

3.2 Heart

3.2.1 MDA + 4-HDA

MDA + 4-HDA levels in cardiac tissue decreased significantly after exercise, from 0.43 ± 0.02 nmol/mg protein in controls to 0.34 ± 0.02 at 1 week training group. ANOVA showed a significant time effect (p = 0.0084), with a large effect size (η^2 = 0.425), but suboptimal statistical power (1-β = 0.550). Post-hoc Tukey comparisons showed significant differences between CON and 1W (95% CI: 0.021–0.166; adjusted p value = 0.0074), and CON versus 12W (95% CI: 0.004–0.149; adjusted p value = 0.0342). See Fig. 2a for details.

3.2.2 Protein Carbonyls

Regarding protein oxidation in the heart, a reduction in the mean levels of protein carbonyl content was observed in all three training groups compared to the control group. However, none of these changes reached statistical significance. ANOVA and Tukey post-hoc comparisons revealed no statistically significant differences between CON and 1W, 4W and 12W training groups. See Fig. 2b.

3.2.3 Plasma Membrane Fluidity

In cardiac tissue, plasma membrane fluidity progressively declined after exercise cessation. Control values were 3.08 ± 0.05 as 1/P, decreasing to 2.92 ± 0.02 at 12 weeks of training. This reduction was statistically significant (p = 0.002), with a large effect size (η^2 = 0.446) and robust statistical power (1-β = 0.939). Significant pairwise differences were found between CON and 1W (95% CI: 0.003–0.243; adjusted p value = 0.0412), CON and 4W (95% CI: 0.057–0.297; adjusted p value = 0.0017), and between CON and 12W (95% CI: 0.043–0.289; adjusted p value = 0.0046). See Fig. 2c for details.

3.2.4 Mitochondrial Membrane Fluidity

Cardiac mitochondrial membrane fluidity showed a modest but significant decline, from 3.04 ± 0.04 as 1/P in controls to 2.92 ± 0.02 at 12 weeks of training. ANOVA confirmed a time effect (p = 0.042), with a moderate to large effect size (η^2 = 0.284) and suboptimal statistical power (1-β = 0.660), indicating a reasonable probability of detecting true differences, albeit below the conventional threshold of 0.80. No significant pairwise differences were detected. See Fig. 2d.

3.3 Brain

3.3.1 MDA + 4-HDA

MDA + 4-HDA levels in brain tissue remained relatively unchanged across all training durations, although all trained groups exhibited lower means than control. ANOVA did not reveal significant differences (p = 0.072; η^2 = 0.523; 1-β = 0.549). No post-hoc comparisons reached statistical significance. See Fig. 3a.

3.3.2 Protein Carbonyls

Carbonyl levels in the brain were highest at baseline (13.76 ± 1.31 nmol/mg protein). However, no significant changes were detected across post-training periods. Neither the one-way ANOVA (p = 0.384, η^2 = 0.164, 1-β = 0.142), nor the pairwise Tukey post-hoc comparisons revealed statistically significant differences. See Fig. 3b.

3.3.3 Plasma Membrane Fluidity

Brain tissue exhibited lower baseline plasma membrane fluidity (2.86 ± 0.03 as 1/P) compared to skeletal muscle and heart tissues, and showed a consistent decline across all post-training periods, reaching 2.73 ± 0.02 at 12 weeks. ANOVA revealed highly significant differences (p < 0.001), with a large effect size (η^2 = 0.569) and high statistical power (1-β = 0.996). Tukey post-hoc comparisons showed significant differences between CON and 1W (95% CI: 0.016–0.203; adjusted p value = 0.0163), CON and 4W (95% CI: 0.018–0.205; adjusted p value = 0.0142), and between CON and 12W (95% CI: 0.035–0.227; adjusted p value = 0.0041). See Fig. 3c for details.

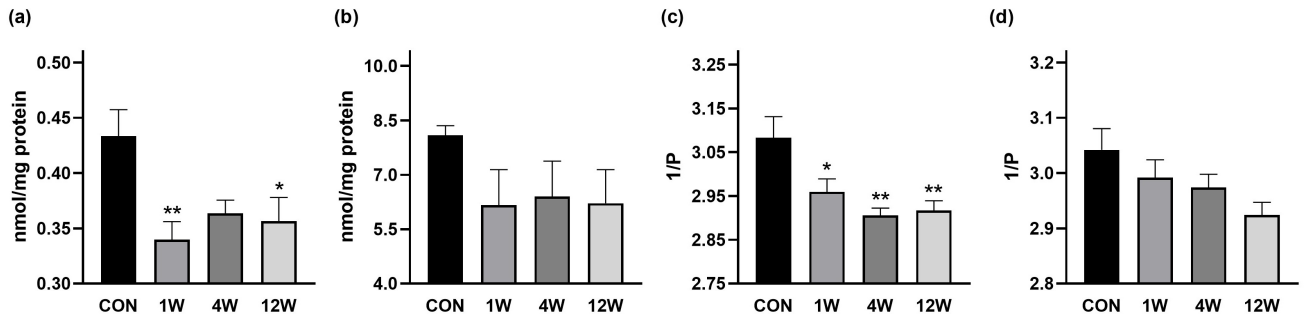

Fig. 2. Heart. (a) Concentration of malondialdehyde and 4-hydroxyalkenals (MDA + 4-HDA); results expressed as nmol/mg protein. (b) Content of protein carbonyl groups; results expressed as nmol/mg protein. (c) Membrane fluidity of tissue plasma membranes; results expressed as the inverse of polarization (1/P). (d) Membrane fluidity of tissue mitochondrial membranes; results expressed as the inverse of polarization (1/P). CON, control group; 1W, 1 week-training group; 4W, 4 weeks-training group; 12W, 12 weeks-training group. $^{*}p < 0.05$ vs. CON; $^{**}p < 0.01$ vs. CON.

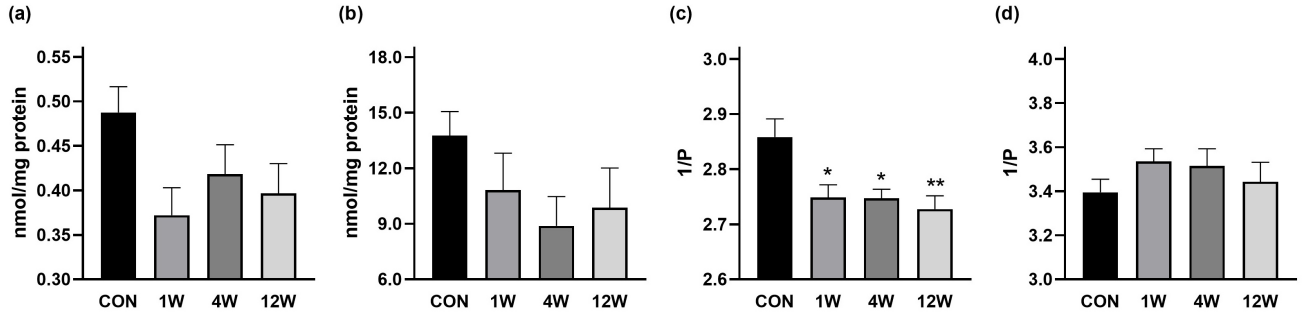

Fig. 3. Brain. (a) Concentration of malondialdehyde and 4-hydroxyalkenals (MDA + 4-HDA); results expressed as nmol/mg protein. (b) Content of protein carbonyl groups; results expressed as nmol/mg protein. (c) Membrane fluidity of tissue plasma membranes; results expressed as the inverse of polarization (1/P). (d) Membrane fluidity of tissue mitochondrial membranes; results expressed as the inverse of polarization (1/P). CON, control group; 1W, 1 week-training group; 4W, 4 weeks-training group; 12W, 12 weeks-training group. $^{*}p < 0.05$ vs. CON; $^{**}p < 0.01$ vs. CON.

3.3.4 Mitochondrial Membrane Fluidity

Brain mitochondrial membrane fluidity remained stable across all post-training periods. No significant differences were detected ($p = 0.775$; $\eta^2 = 0.044$; 1-$\beta = 0.112$), and no pairwise comparisons reached significance. See Fig. 3d.

4. Discussion

The present study aimed to evaluate the effects of chronic aerobic training on oxidative stress and membrane fluidity in skeletal muscle, heart, and brain tissues. Specifically, we investigated whether different durations of aerobic exercise could attenuate lipid peroxidation and protein oxidation, as measured by MDA + 4-HDA and protein carbonyl concentrations, respectively, and modulate the biophysical properties of plasma and mitochondrial membranes. Our findings reveal tissue-specific adaptations, with skeletal muscle showing the most pronounced response, followed by the heart and brain. These results underscore the systemic impact of aerobic training on cellular oxidative balance and membrane dynamics.

The data reveal different tissue-specific patterns of adaptation. Skeletal muscle showed the most pronounced changes, including significant reductions in lipid peroxidation and protein oxidation, together with marked decreases in plasma and mitochondrial membrane fluidity. Cardiac tissue exhibited moderate improvements, characterized by reduced lipid peroxidation and plasma membrane remodeling, while mitochondrial fluidity changes were less evident. In contrast, brain tissue displayed stable oxidative markers but a consistent and significant decline in plasma membrane fluidity across all training durations.

In skeletal muscle, all training protocols significantly reduced lipid peroxidation, as evidenced by decreased MDA + 4-HDA levels. Protein oxidation, assessed via carbonyl group concentration, was significantly reduced in the 4W and 12W training groups. These biochemical improvements were accompanied by a consistent decrease in plasma and mitochondrial membrane fluidity, suggesting a structural reorganization of membrane lipids. This is supported by Ni *et al.* [27], and Noland [28], who explain that chronic exercise induces changes in membrane lipid composition, favoring the incorporation of saturated and less

peroxidizable fatty acids. Other studies in rats have shown that endurance training promotes the incorporation of saturated fatty acids and reduces polyunsaturated fatty acids in muscle membranes, thereby decreasing susceptibility to peroxidation [29,30]. These changes reduce membrane fluidity and enhance structural integrity, representing an adaptive response to oxidative stress. These studies have shown that exercise alters lipid composition and reduces oxidative markers, supporting our findings. Clinically, these adaptations may enhance metabolic efficiency and reduce the risk of insulin resistance and sarcopenia, particularly in aging populations.

Cardiac tissue exhibited similar trends, with significant reductions in MDA + 4-HDA but not in protein carbonyls. Plasma membrane fluidity decreased significantly, while mitochondrial membrane fluidity showed a non-significant downward trend. These results suggest partial adaptation of cardiac membranes, possibly due to differential lipid remodeling or compartment-specific oxidative environments. High oxidative capacity and distinct lipid metabolism in cardiac tissue have been highlighted by several studies [28,31], which may lead to compartment-specific responses. The heart's mitochondria are particularly efficient in handling oxidative stress, which could explain the selective adaptation observed in plasma versus mitochondrial membranes. A study in infarcted rats showed that aerobic training enhances glutathione peroxidase activity and reduces lipid hydroperoxides in cardiac tissue [32], indicating that even under pathological conditions, exercise confers antioxidant benefits. The selective modulation observed in our study may reflect the heart's intrinsic resistance to oxidative stress and its reliance on fatty acid metabolism.

In the brain, both lipid peroxidation and protein oxidation markers showed a downward trend, although statistical significance was not reached. However, plasma membrane fluidity was significantly reduced in all training groups, while mitochondrial membrane fluidity remained unchanged. This selective modulation may reflect the brain's unique lipid composition and tightly regulated oxidative balance. According to a recent study, the brain is rich in polyunsaturated fatty acids, making it highly susceptible to lipid peroxidation [33]. However, it also possesses robust antioxidant defenses and tightly regulated redox mechanisms, which may buffer against significant changes in mitochondrial membrane fluidity despite systemic oxidative challenges. Another study in rats subjected to exhausting exercise showed transient increases in lipid peroxidation markers and decreased membrane fluidity, which were mitigated by prior training [29]. Furthermore, Delezie and Handschin [34] discussed the endocrine crosstalk between skeletal muscle and the brain, suggesting that exercise-induced myokines may influence neuroplasticity and vascularization. The observed changes in membrane fluidity could therefore have implications for synaptic function and neuroprotection, particularly in aging and neurodegenerative conditions.

The reduction in MDA + 4-HDA and protein carbonyls across tissues highlights the antioxidant effects of chronic aerobic training. Lipid peroxidation typically disrupts membrane integrity and increases fluidity due to the formation of reactive aldehydes and polar groups. However, the observed decrease in membrane fluidity across tissues suggests a compensatory incorporation of saturated or less peroxidizable lipids, enhancing membrane stability under oxidative stress. This interpretation is supported by findings that aerobic training alters lipid composition and reduces membrane susceptibility to peroxidation. In skeletal muscle, endurance training has been shown to upregulate lipid metabolism genes and increase the expression of fatty acid transporters such as fatty acid transporter 4 (FATP4), contributing to enhanced fatty acid oxidation and membrane remodeling [35,36]. These adaptations may represent a protective strategy to maintain cellular homeostasis under repeated oxidative challenges [37].

The integration of biochemical markers (MDA + 4-HDA, carbonyls) and biophysical assessments (membrane fluidity) provides a comprehensive view of exercise-induced adaptations. Organ-specific physiological responses to exercise may be mediated by perfusion and metabolic shifts [38], which may explain the differential responses observed in our study. Improved redox homeostasis and membrane resilience may enhance cellular function, metabolic efficiency, and resistance to oxidative damage, contributing to the systemic benefits of regular physical activity. The rat model used in this study provides translational relevance, as similar oxidative and structural responses have been observed in human tissues following aerobic training [27].

Clinical relevance is evident throughout these findings. Reduced lipid peroxidation and protein oxidation in skeletal muscle and heart may lower the risk of metabolic and cardiovascular diseases. In the brain, modulation of membrane fluidity may support cognitive function and delay neurodegeneration. These tissue-specific patterns highlight the complexity of exercise-induced oxidative adaptations and their potential clinical relevance. In skeletal muscle, the pronounced reduction in lipid and protein oxidation, coupled with decreased membrane fluidity, suggests enhanced structural resilience and metabolic efficiency-adaptations that may mitigate sarcopenia and insulin resistance in aging populations [4,5]. Cardiac responses, although less marked, indicate partial remodeling that could contribute to improved myocardial tolerance to oxidative stress, consistent with evidence from infarcted rat models and human endurance training studies [31,32]. In the brain, the selective modulation of plasma membrane fluidity without significant changes in global lipid peroxidation may reflect compartment-specific antioxidant strategies and lipid architecture [13,33]. From a translational perspective, these

findings reinforce the role of sustained aerobic exercise as a non-pharmacological strategy to preserve cellular integrity and mitigate oxidative damage in metabolically active tissues. The observed biochemical and biophysical adaptations (particularly in skeletal muscle and cardiac tissue) may contribute to improved metabolic efficiency, enhanced antioxidant capacity, and reduced vulnerability to oxidative stress, which are critical for preventing sarcopenia, cardiovascular dysfunction, and metabolic disorders in aging populations [28,31,32]. In the central nervous system, modulation of membrane fluidity could support synaptic stability and neuroprotection, with potential implications for delaying cognitive decline and neurodegenerative processes [13,33,34]. Future research integrating multi-omic profiling and functional assessments will be essential to translate these mechanistic insights into targeted exercise interventions for clinical populations [35,36].

5. Limitations and Future Directions

While the study offers valuable insights, it does not directly assess the molecular mechanisms underlying membrane remodeling or oxidative stress attenuation. Future research should incorporate lipidomic and proteomic analyses to characterize membrane composition and protein-lipid interactions. Functional assays of mitochondrial activity and membrane permeability could elucidate the physiological consequences of altered fluidity. Longitudinal studies with extended training durations and diverse exercise modalities may further clarify the temporal and mechanistic aspects of these adaptations. Additionally, exploring the role of specific lipid species and antioxidant enzymes could enhance our understanding of tissue-specific responses to aerobic training.

This study has three scope-related limitations that warrant acknowledgment but do not detract from the robustness of the observed tissue-specific adaptations. First, only male rats were included to avoid estrous-cycle variability (see Materials and Methods), which restricts generalizability; future designs should incorporate both sexes and control for cycle phases [4,5,13]. Second, mechanistic inferences regarding membrane lipid remodeling and antioxidant/proteostatic regulation remain indirect, as lipidomic and proteomic profiling were beyond the present study's remit; multi-omic evidence suggests these pathways may underpin our biophysical and biochemical findings, and should be directly tested in follow-up work [28–30,35,36]. Third, in brain tissue, intrinsic lipid composition and robust antioxidant control may buffer global lipid peroxidation indices and decouple them from fluidity changes; functional assays (neurophysiology, cognition, mitochondrial bioenergetics) are recommended to clarify translational relevance [13,33].

6. Conclusions

Chronic aerobic treadmill training induces tissue-specific protective adaptations against oxidative stress in skeletal muscle, heart, and brain. These changes (reduced lipid peroxidation and protein oxidation, particularly in skeletal muscle and cardiac tissue, together with altered membrane fluidity) reflect enhanced antioxidant capacity and structural resilience. From a translational perspective, these findings support the role of sustained aerobic exercise as a non-pharmacological strategy to preserve cellular mechanisms, promote functional integrity in key tissues, and mitigate oxidative damage, with implications for metabolic, cardiovascular, and neurodegenerative health.

Abbreviations

LPO, lipid peroxidation; MDA, malondialdehyde; 4-HDA, 4-hydroxyalkenals; ROS, reactive oxygen species; CON, control group; 1W, 1 week-training group; 4W, 4 weeks-training group; 12W, 12 weeks-training group; O_2, oxygen; CO_2, carbon dioxide; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-p-toluenesulfonate; TRIS, tris(hydroxymethyl)aminomethane; DNPH, 2,4-dinitrophenylhydrazine; TCA, trichloroacetic acid; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; KCl, potassium chloride; P, polarization; 1/P, inverse of polarization; IV_V, intensity of vertically polarized emitted light; IV_H, intensity of horizontally polarized emitted light; G, instrumental correction factor; BSA, bovine serum albumin; SE, standard error; η^2, partial eta squared; p, statistical significance level; CI, confidence interval; FATP4, fatty acid transporter 4.

Availability of Data and Materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

EP conceptualized and designed the research study. EP and CB performed the research and acquired the data. CB provided advice on animals handling. PJB and AVB-C provided help on the statistical data analysis. EP, CB and PJB interpretated and analyzed the data. EP and PJB wrote the manuscript. CB and AVB-C reviewed critically the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The research protocol was approved by the Clinical Research Ethics Committee of Aragón (CP02/2010).

All animal procedures were conducted in full compliance with the relevant European (Directive 2010/63/EU), national (Spain: RD 53/2013), and regional (Aragón: Law 1/2019) regulations governing the protection and use of animals for scientific purposes.

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Conflict of Interest

The authors declare no conflict of interest.

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