

PhD programme in Human Movement and Sport Sciences – XXXVIII Cycle

# **EXERCISE-INDUCED SYSTEMIC ADAPTATIONS: ROLE OF REDOX PERTURBATION IN THE MOLECULAR CROSS-TALK MEDIATED BY EXTRACELLULAR VESICLES (EVs)**

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## INTRODUCTION

### 1. Exercise-induced adaptations: implications for skeletal muscle function and human health

Acting simultaneously on multiple systems, organs and tissues [1], regular and moderate physical activity provides many health benefits, extending lifespan and preventing a broad range of chronic diseases such as cardiovascular diseases, type 2 diabetes, obesity, malignancies (e.g. colon and breast cancer) and arthritis [2]. According to the World Health Organization, adults should engage in 150–300 minutes per week of moderate-intensity aerobic physical activity, or 75–150 minutes of vigorous-intensity activity, complemented by muscle-strengthening exercises involving all major muscle groups on at least two days per week [3]. Indeed, all forms of physical activity are generally associated with better quality of life but for maximal beneficial effects a minimum duration and intensity is usually prescribed [4]. Exercise is commonly distinguished into two major modalities: endurance and resistance training. Endurance or aerobic activities, such as running, cycling or swimming, rely predominantly on oxygen-dependent metabolic pathways and require continuous, rhythmic muscle contractions primarily involving slow-twitch oxidative myofibers in skeletal muscles [5,6]. In contrast, resistance exercise encompasses short, high-intensity efforts like lifting weights or sprints, where force production relies predominantly on rapid, anaerobic energy sources, such as creatine phosphate and anaerobic glycolysis, and primarily recruits fast-twitch glycolytic myofibers [7]. Consequently, a single bout of each exercise modality generates distinct mechanical, metabolic and redox signaling events that, when repeated over time, give rise to highly coordinated adaptations across the entire organism, including the cardiovascular and immune system, skeletal muscle, adipose tissue, liver, pancreas, gut, and brain (Fig.1) [8].

A key adaptive endpoint associated with both regular endurance and resistance exercise training is the improvement of cardiorespiratory fitness and function which is evidenced by an increase in the maximum volume of oxygen ( $VO_{2max}$ ) [9] linking to cardiac hypertrophy [10] and mitochondrial biogenesis [11], increased blood flow [12] and capillary growth [13], augmented red blood cell volume and haemoglobin mass [14]. Among all tissues responsive to exercise, skeletal muscle stands out as both a primary target and a fundamental driver of these adaptations. Skeletal muscle adaptations to training involve a coordinated set of changes in form and function, which contribute to enhance its capacity to optimize energy production and sustain prolonged exercise [15]. Endurance exercise promotes a greater reliance on lipid oxidation at submaximal intensities and increases the maximal capacity for carbohydrate oxidation during high-intensity efforts [16]. These functional shifts are supported by higher expression of proteins involved in oxygen delivery [17], antioxidant defense [18], glucose transport [19], glycolysis [20], lactate handling [21], and fatty acid metabolism

[22]. A central feature of this adaptation is the expansion and qualitative improvement of the mitochondrial network, driven by increased mitochondrial biogenesis, enhanced respiratory capacity [23], and refined mitochondrial dynamics supported by fusion–fission processes [24], autophagy and mitophagy [25]. Exercise also acutely enhances insulin-independent glucose uptake and sensitizes muscle to insulin [26], while chronic training increases the abundance of key glucose-handling proteins such as glucose transporter type 4 (GLUT4) and hexokinase II [27,28], together with microvascular remodeling [29]. At the molecular level, acute exercise activates a broad network of signaling pathways, including AMP-activated protein kinase (AMPK), calcium/calmodulin-dependent protein kinases (CaMKs), mitogen-activated protein kinases (MAPKs), sirtuin 1 (SIRT1), mechanistic target of rapamycin (mTOR) and protein kinase A (PKA), and induces extensive changes in protein phosphorylation [30]. These signals converge on transcriptional regulators such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha PGC-1 $\alpha$ , which integrates upstream cues to coordinate exercise-responsive gene expression [31]. Overall, the post-translational and transcriptional responses to exercise are highly dynamic and depend on the exercise modality and the tissue involved.

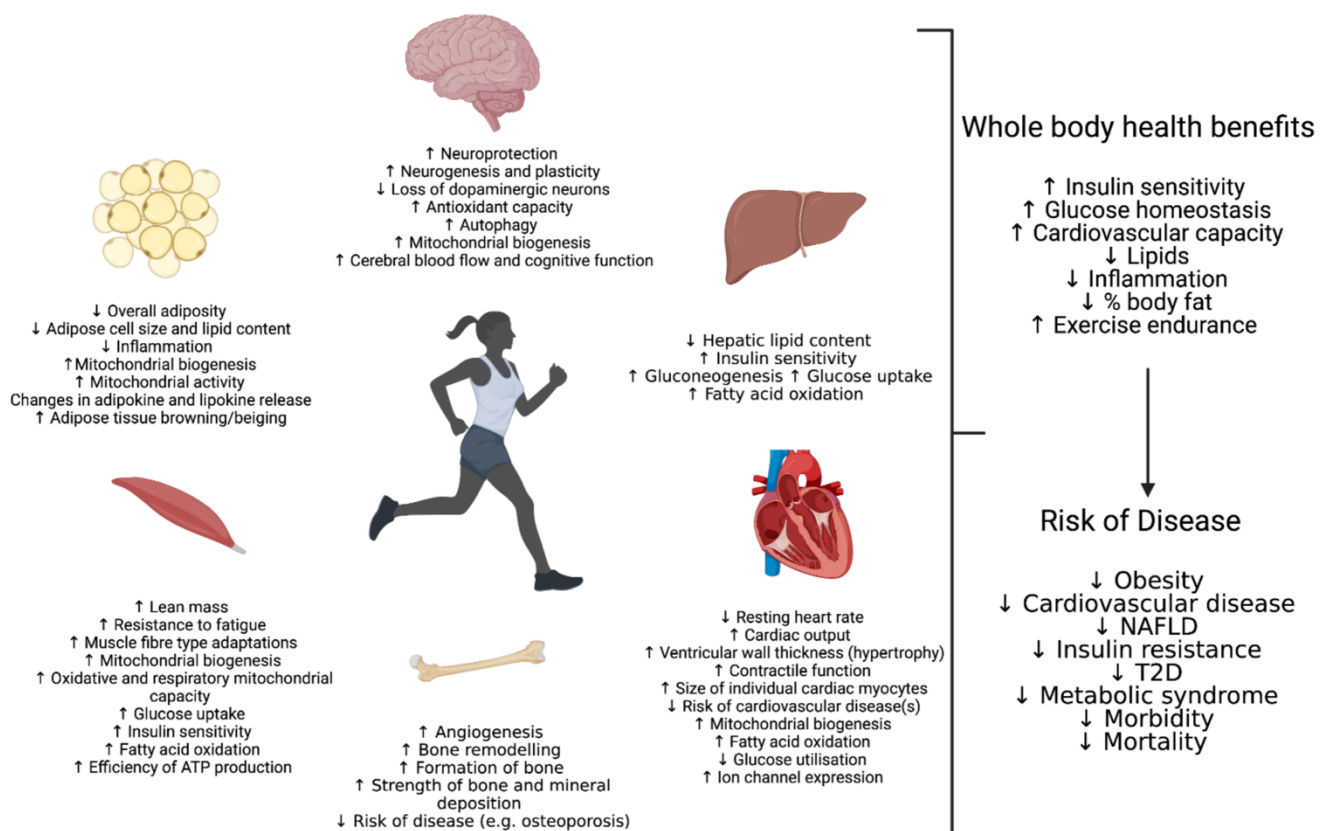


**Fig.1** Physical activity elicits widespread, coordinated adaptations across multiple organs and tissues, including the brain, skeletal muscle, adipose tissue, liver, heart, and bone. Exercise enhances mitochondrial biogenesis and function, improves insulin sensitivity, glucose homeostasis, and lipid metabolism, and promotes favorable structural and functional remodeling of tissues. Collectively, these adaptations increase cardiorespiratory fitness, endurance, and metabolic flexibility, while reducing inflammation, adiposity, and the risk of chronic diseases such as obesity, cardiovascular disease, NAFLD, type 2 diabetes, and metabolic syndrome [32].

## **2. Redox homeostasis and signaling in exercise biology**

Due to its capacity to induce transient and tightly regulated modification of cellular homeostasis, physical exercise is widely regarded as a controlled physiological stressor [33] and redox biology plays a central role in coordinating these response, with skeletal muscle acting as a primary site where redox-sensitive pathways translate transient perturbations into long-term adaptations [34]. Reactive oxygen species (ROS) can increase by approximately 86% in human skeletal muscle during moderate-intensity cycling [35], being simultaneously produced from mitochondrial and nonmitochondrial sources, such as the mitochondrial electron transport chain (ETC) [36], NADPH oxidases (NOXs/DUOXs) [37], xanthine oxidase (XO) [38], phospholipase A<sub>2</sub> [39] and uncoupled nitric oxide synthase. During regular exercise, ROS generation typically remains within an hormetic window, whereby low-to-moderate oxidative challenges, defined as oxidative eustress, are buffered by a group of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), thioredoxin (Trx), thioredoxin reductase (TrxR), and peroxiredoxin (Prx) [40], and elicit beneficial cellular responses. However, when ROS production becomes chronically elevated and exceeds the capacity of antioxidant defenses to maintain redox balance, the system shifts toward oxidative distress, leading to DNA damage, inflammation, neuronal degeneration, muscle damage until cell death [41]. ROS, especially hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), plays an essential role in redox signaling, which affects physiological processes, leading to changes in signaling outputs, enzyme activity, gene expression, and membrane and genome integrity [42]. Through reversible oxidative modifications of key cysteine residues [43], exercise-induced ROS interact with Kelch-like ECH-associated protein 1 (KEAP1), disrupting its association with the nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which subsequently binds antioxidant response elements (AREs) to induce genes involved in detoxification and ROS scavenging [44]. In addition, these redox signals activate the AMPK–SIRT1–PGC-1 $\alpha$  axis, enhancing the expression of mitochondrial genes and proteins required for fatty acid oxidation, the tricarboxylic acid cycle, and the electron transport chain [45]. Redox perturbations also influence MAPKs, including p38 MAPK, extracellular signal-regulated kinases 1 and 2 (ERK1/2) and c-Jun N-terminal kinase (JNK), as well as transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), which are transiently activated during acute exercise and promote the expression of genes

involved in inflammatory signaling, angiogenesis, glycolysis and substrate transport [46,47]. Finally, ROS contribute to the regulation of proteostasis and mitochondrial quality control by modulating autophagy and mitophagy, the unfolded protein response, and key post-translational modifications [48,49].

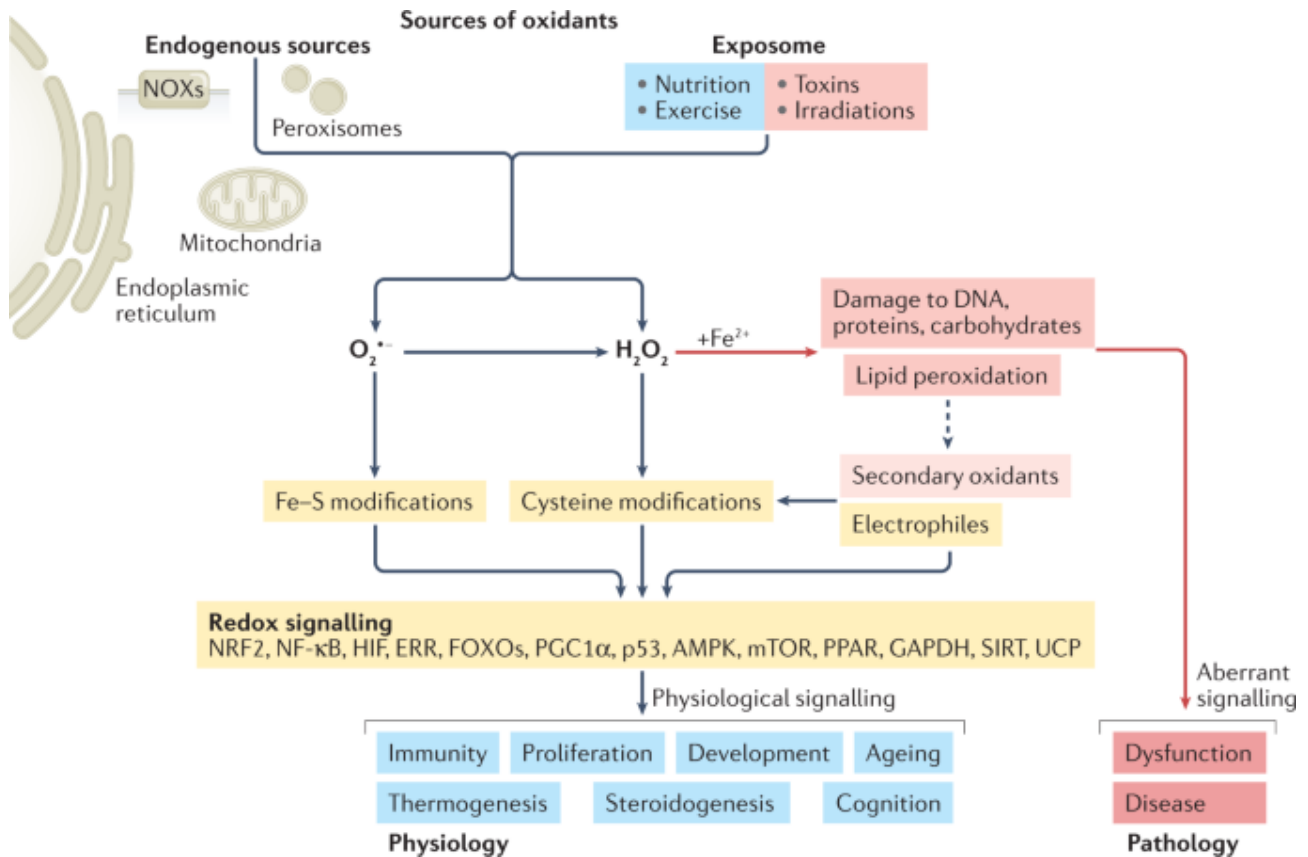


**Fig.2** Endogenous and exogenous sources of oxidants, including mitochondria, peroxisomes, NADPH oxidases, nutrition, exercise, toxins, and irradiation, contribute to the generation of reactive oxygen species such as SOD and  $H_2O_2$ . At controlled levels, these species function as signaling molecules through reversible redox modifications of cysteine residues and iron–sulfur centers, regulating key molecular pathways involved in metabolism, immunity, development, thermogenesis, cognition, and aging. When oxidant production overwhelms cellular defenses, lipid peroxidation and damage to DNA, proteins, and carbohydrates lead to aberrant signaling, cellular dysfunction, and the development of disease [50].

### 3. Extracellular vesicles (EVs): an overview

The beneficial effects of physical activity arise not only from tissue-specific signaling events but also from the coordinated exchange of information between organs. This systemic integration is mediated, in part, by the exercise-induced release of enzymes, cytokines, chemokines, hormones, and growth factors peptides, metabolites, and nucleic acids from skeletal muscle and other tissues, collectively called exerkines [51]. While exerkines were initially characterized as soluble molecules released into the bloodstream during physical activity, it is now evident that many exercise-responsive signals are

instead secreted and transported within extracellular vesicles (EVs) [52], which provide protection from extracellular degradation, allow the simultaneous transfer of multiple bioactive components (proteins, nucleic acids, lipids, and metabolites), and enhance the specificity of interactions with target cells [53,54]. Importantly, EVs act also as an autonomous communication network, acting in an autocrine, paracrine, or endocrine fashion across different tissues to promote systemic adaptations to exercise [55,56].

The International Society for Extracellular Vesicles (ISEV) recommends the use of the generic term “EVs” to refer to “particles that are released from cells, are delimited by a lipid bilayer, and cannot replicate on their own (i.e., do not contain a functional nucleus)”, due to the lack of specific markers for each EV subtype and the overlap in size between them [57]. Exosomes (30-150 nm), microvesicles (MVs, 100-1000 nm) and apoptotic bodies (1000-5000 nm) are traditionally considered the three main EV subtypes, to which exomeres (<50 nm), migrasomes (500-3000 nm) and oncosomes (1000-10000 nm) have recently been added as newly discovered members [58–60]. Exosomes originate primarily from the endolysosomal system, undergoing a complex and tightly orchestrated biogenesis pathway which begins with the invagination and inward budding of the endosomal membrane into the intraluminal space, leading to the formation of early endosomes [61]. These structures serve as central sorting hubs where cargo, depending on cellular needs, is directed either for recycling back to the plasma membrane, for degradation via lysosomes, or for further maturation into late endosomes, also referred to as multivesicular bodies (MVBs) [62]. During this process, the endosome undergoes a dynamic remodelling of its membrane composition to enable membrane curvature and intraluminal vesicles (ILVs) formation, while Rab5, a key marker of early endosomes, is replaced by Rab7, a key regulator of late endosome maturation and trafficking [63,64]. As MVBs mature, a highly specific selection of ILVs-enclosed cargo occurs, regulated by multiple molecular mechanisms both endosomal sorting complex required for transport (ESCRT)-dependent and independent. The sequential recruitment and activation of ESCRT complexes (0, I, II, III), assisted by several accessory proteins and ALG-2–Interacting Protein X (ALIX) as a multifunctional adaptor protein integrated into the so-called syndecan-syntenin-ALIX pathway, cluster cargo proteins into ILVs, actively steering them toward the exosomal route rather than the lysosomal degradation [65,66]. Alternative exosome biogenesis process relies on tetraspanins, a family of transmembrane proteins including CD9, CD63 and CD81, and ceramide-driven lipid raft microdomains, particularly enriched in sphingolipids, cholesterol, and key proteins such as flotillins, caveolins and glycosylphosphatidylinositol (GPI)-anchored proteins, facilitating ILVs formation in the absence of ESCRT-III and redirecting them away from the degradative pathway [67,68]. Also, Rab31, a member of the Rab family of small GTPases, acts as a critical switch controlling the fate of ILVs: it further

stabilizes secretion-competent MVBs by antagonizing Rab7-driven degradation and cooperating with the syndecan-syntenin-ALIX axis, promoting selective cargo sorting [69]. Once matured, MVBs are anterograde transported along microtubules to the cell periphery, where cortactin-mediated actin remodelling allows access to exocytic sites [70]; then, Rab GTPases (Rab27a/b, Rab11) orchestrate proper vesicle docking and tethering [71,72]. Finally, The MVBs-plasma membrane fusion event is mediated by the SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment Protein Receptor) complex wherein vesicle-associated membrane protein 7 (VAMP7) on MVBs interacts with target membrane SNAREs such as Syntaxin-4 and synaptosomal-associated protein of 23 KDa (SNAP-23) driving the merger of lipid bilayers and the subsequent release of exosomes [73].

MVs biogenesis process differs considerably from that of exosomes and is far less characterized in detail; however, certain mechanisms and regulatory elements appear to be shared between these two EVs subtypes. MVs are released into the extracellular space directly after the fission and outward budding of the plasma membrane [74], a process primarily driven by the cytoskeleton remodelling, occurring through increased intracellular  $\text{Ca}^{2+}$  levels, activation of cytoskeleton-degrading enzymes, such as calpain [75], and coordinated action of membrane lipid-regulating enzymes, such as flippase, floppase and scramblase [76]. Moreover, some ESCRT factors and accessory proteins [77–80], lipids, such as cholesterol and acid sphingomyelinase [81,82] and post-translational modification (PTMs) [83], are reported to be involved in MVs formation (Fig.3).

The genesis of apoptotic bodies is tightly linked to the progression of apoptosis, a highly regulated and controlled cell death programme [84]. During this process, the activation of caspases, often initiated by DNA damage or receptor-ligand signals [85], leads to cell shrinkage, chromatin condensation and cytoskeleton reorganization until extensive plasma membrane blebbing and nuclear fragmentation occur, giving rise to membrane-bound vesicles enriched in portions of the cytoplasm, organelles and nuclear fragments from the dying cells [86]. Long considered waste disposal and therefore removed by phagocytic cells to prevent a potential harm to the surrounding cells or tissues, apoptotic bodies are increasingly appreciated as functional entities actively involved in extracellular signalling [87].

The primary function of EVs, particularly exosomes and microvesicles, is to facilitate the transfer of a wide range of bioactive molecules, including proteins, nucleic acids (DNA, RNA, and microRNA), lipids, and metabolites, from donor cells to recipient cells. When selectively packaged within EVs, this cargo reflects the physiological or pathological state of the cell of origin and affects the behaviour of recipient cells by modulating activities such as gene expression, signal transduction, and regulatory responses [88]. The presence of EVs in various biological fluids, such as blood, urine, saliva, and

cerebrospinal fluid, enables EV-mediated communication with nearby cells and distant tissues, thereby playing a pivotal role in local and systemic homeostasis [89].

Among EVs' numerous and diverse cellular and tissue sources, skeletal muscle (SkM) has recently been identified as an important contributor [90], representing a new and exciting paradigm for understanding how this tissue communicates with other organs, including the brain, adipose tissue, bone, liver, pancreas, vascular bed, skin, and muscle itself in health, ageing and disease [91]. Indeed, skeletal muscle's physiological or pathological states profoundly impact EV production, composition, and function, underscoring their dual role as mediators of adaptation and as indicators for disease detection. For example, regular exercise induces the release of SkM-EVs enriched with beneficial molecules that promote tissue regeneration [92], improve insulin sensitivity [93] and enhance systemic metabolic homeostasis [94], thereby contributing to overall well-being. Conversely, muscle atrophy, sarcopenia, or chronic diseases such as diabetes and muscular dystrophy lead to the release of altered SkM-EVs carrying aberrant molecules that exacerbate chronic inflammation [95], disrupt insulin signalling [96], and worsen systemic metabolic dysfunctions, potentially accelerating disease progression.

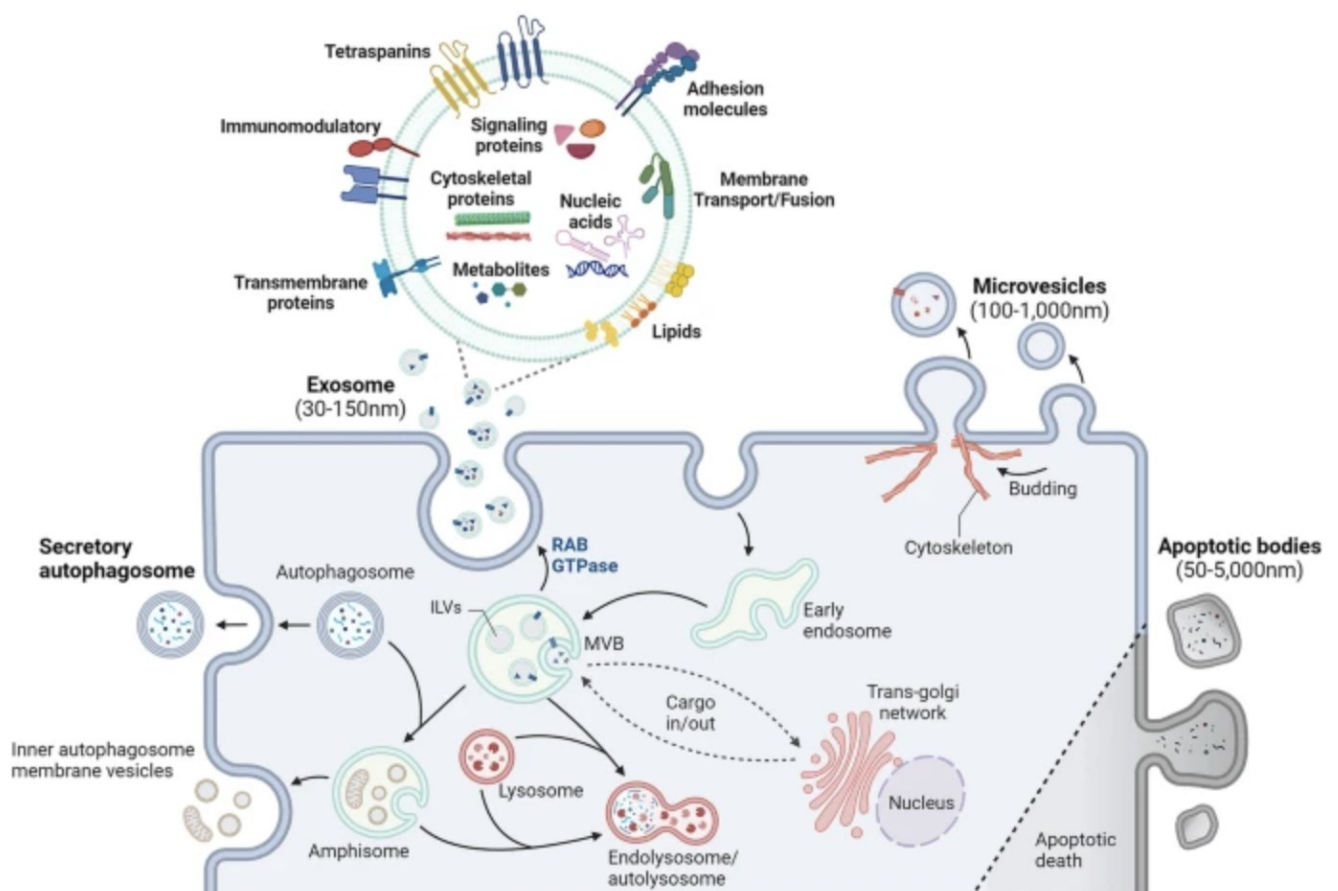


**Fig.3** EVs comprise exosomes, microvesicles, and apoptotic bodies, which differ in size, origin, and mechanisms of formation. Exosomes originate from the endosomal system through the formation of intraluminal vesicles within multivesicular bodies and are released upon fusion with the plasma membrane, whereas microvesicles are generated by direct outward budding of the plasma membrane and apoptotic bodies arise during programmed cell death. These vesicles carry a diverse cargo of lipids, proteins, nucleic acids, and metabolites, including signaling, adhesion, and immunomodulatory molecules, enabling intercellular communication and the regulation of physiological and pathological processes [97].

#### **4. Exercise and tissue cross-talk: EVs as redox-sensitive messengers**

Physical activity is increasingly recognized as a potent trigger for EV release into the circulation, with both acute and chronic exercise influencing the quantity and molecular characteristics of circulating vesicles. During a single bout of acute exercise, including cycling, running, and swimming, circulating EV levels rise rapidly, typically reaching their peak either during the activity itself or immediately after, before gradually returning to baseline within a few hours [92,98]. In contrast, long-term engagement in regular physical activity produces more sustained changes in EV biology: chronic training is associated with moderately elevated resting EV levels and with qualitative remodeling of EV cargo [99,100]. Vesicles released in trained individuals are enriched in molecules linked to metabolic regulation, immune modulation, antioxidant defense, and tissue repair, suggesting that habitual exercise promotes an adaptive pattern of EV-mediated communication that enhances cellular resilience and promotes tissue homeostasis [101]. An increasing body of evidence indicates that EV biogenesis, cargo composition, and biological activity are strongly influenced by the redox state of the releasing cell, especially during conditions that perturb cellular redox balance such as physical activity [102]. A hallmark of redox-sensitive EV communication is the selective enrichment of antioxidant enzymes, stress-response proteins and miRNAs which serve as regulators of redox homeostasis in recipient cells by modulating gene expression, buffering ROS accumulation, and influencing pathways linked to antioxidant defense. Among antioxidant enzymes, the superoxide dismutase family (SOD1 and SOD2) and CAT represent some of the most consistently reported EV cargo during and after exercise [103–105]. Additional redox-regulatory proteins—including PRDX1, TRX, glutathione reductase (GR), and glutathione-biosynthetic enzymes such as carnosine dipeptidase 2 (CNDP2), glutamate-cysteine ligase modifier subunit (GCLM) and microsomal glutathione S-transferase 2 (MGST2)—have also been identified [106,107]. Exercise-induced EVs are likewise enriched in redox-sensitive miRNAs that fine-tune oxidative stress responses at the post-transcriptional level. For instance, miR-124, miR-148a, and miR-146a, known modulators of NADPH oxidase isoforms (NOX2 and NOX4), show increased or exercise-dependent regulation within EVs, contributing to the suppression of excessive ROS production and inflammatory signaling [108–110]. Other miRNAs, such as miR-93, miR-142-5p and miR-128, influence the Nrf2/ARE axis

by targeting repressors of this pathway [111]. Finally, exercise-induced EVs also transport a range of stress-response proteins—including heat shock protein 70 (HSP70), HSP27, and MAP2K1—which contribute to proteostasis, enhance cellular resilience, and modulate signaling pathways activated during redox and metabolic stress [112]. However, when oxidative stress surpasses the physiological adaptive range, circulating EVs begin to exhibit a pro-oxidant signature, reflecting cellular injury rather than beneficial remodeling. Under conditions of excessive ROS accumulation, EVs become enriched in oxidized lipids [113], carbonylated proteins [114], and stress-associated miRNAs such as miR-21 and miR-34a [115–117], all of which contribute to the propagation of oxidative damage, inflammation, cellular senescence, and mitochondrial dysfunction in recipient cells [118–120]. In this respect, the molecular cargo of circulating EVs offers a promising, minimally invasive avenue for monitoring systemic redox balance, identifying early transitions toward oxidative dysfunction, and guiding individualized exercise prescription (Fig.4) [121].

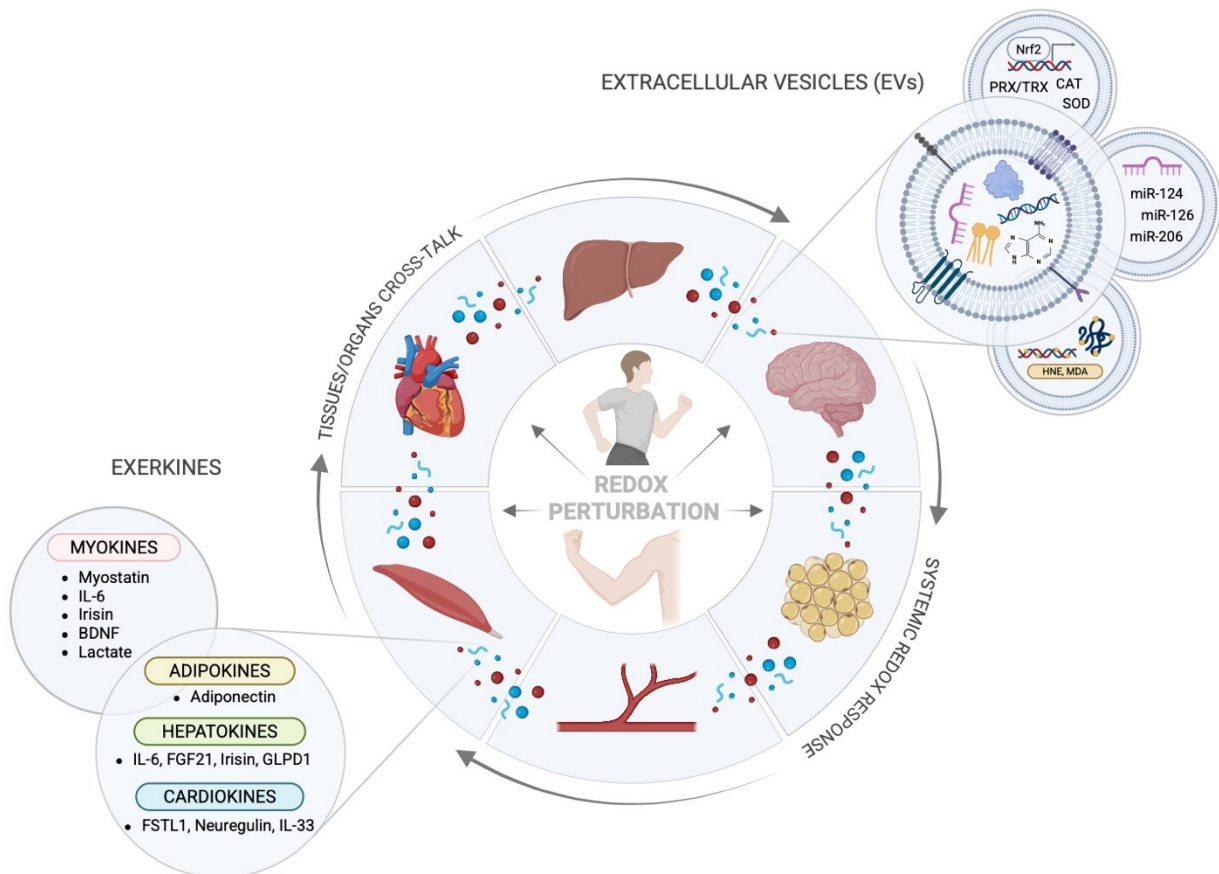


**Fig.4** Physical exercise induces transient redox perturbations that stimulate the release of extracellular vesicles (EVs) and soluble exerkines from multiple tissues, including skeletal muscle, adipose tissue, liver, heart, vasculature, and brain. These EVs act as carriers of redox-related bioactive cargo—such as antioxidant enzymes, redox-sensitive proteins, lipids, and regulatory RNAs (including microRNAs)—that enable tissue-to-tissue communication and coordinated systemic adaptation. Through EV-mediated cross-talk, exercise modulates antioxidant defenses, metabolic homeostasis, inflammation, and cellular resilience, contributing to whole-body redox balance and the beneficial health effects associated with regular physical activity [34].

## Aims of the PhD Project

### 1. Aim I

ROS play a complex role in exercise biology. High levels of ROS are linked to oxidative damage, aging, and chronic disease, but controlled ROS production is now seen as important for redox signaling and adaptation. Research over the past decades shows that exercise-induced ROS affect not only muscle health but also communication throughout the body, involving redox-sensitive pathways and circulating factors like exerkins and EVs. Indeed, EVs have emerged as important mediators of intercellular communication, due to their ability to transport bioactive cargo, such as miRNAs, that can influence gene expression, redox signaling, and long-term cellular adaptation. Increasing evidence indicates that EVs act not only as acute modulators of oxidative stress but also as epigenetic regulators capable of modulating antioxidant defenses and redox-sensitive pathways in recipient cells. To date, the available studies differ considerably in EV sources, isolation techniques, and analytical approaches, making it difficult to synthesize the real involvement of EVs in redox homeostasis. In our literature reviews we aimed to provide an integrated and updated perspective on the multifaceted role of exercise-induced ROS in cellular and systemic adaptation. In the systematic literature analysis **“Extracellular vesicles as epigenetic regulators of redox homeostasis: a systematic review and meta-analysis”** (Mas-Bargues et al., *Antioxidants*, 2025), finalized during my international experience at the MiniAging/Freshage Research Group, Department of Physiology, Faculty of Medicine, University of Valencia (Chair Prof. Consuelo Borras), our objective was to comprehensively evaluate the role of EV-derived miRNAs in the modulation of redox homeostasis, strengthening the evidence supporting their regulatory role. By systematically comparing and critically analyzing multiple studies, our results showed that treatment with EV-derived miRNAs consistently reduced ROS and malondialdehyde (MDA) levels and increased SOD activity, whereas GSH responses were variable but suggested a potential antioxidant benefit. However, despite these positive findings, substantial heterogeneity in EV isolation and characterization methods emerged, reinforcing the need for standardized procedures to enhance comparability and translational relevance. In the recently submitted narrative review **“Reactive Oxygen Species In Exercise Biology: From Adaptive Stress Response To Cell Signaling and Beyond”** (Sireno et al., 2026), which integrates my developing knowledge on the redox biology of exercise and the competencies on muscle extracellular vesicles acquired during my experimental work, the aim was to delineate the complex and multifaceted role of exercise-induced ROS as both mediators of tissue damage and essential signaling molecules orchestrating adaptive responses at the cellular, tissue, and systemic levels.

## 2. Aim II

Regular and moderate physical activity induces a plethora of physiological changes to which skeletal muscle tissue shows a great adaptive capacity. These adaptations span from metabolic to structural remodeling and depend on the activation of specific cell signalling pathways, such as those involving redox homeostasis. In this scenario,  $H_2O_2$  stands out, amongst ROS, as a key signalling molecule capable of stimulating muscle adaptive responses to exercise in a dose-dependent manner. In recent years, the growing body of evidence, regarding the prominent role of exercise-induced EVs in cell-cell communication, has provided a powerful tool to better investigate the molecular mechanisms underlying the complex, and not yet fully characterised, phenomenon of exercise adaptation. In our research proposal “**Small extracellular vesicles biogenesis and release in a murine model of proliferating and differentiated skeletal muscle cells: role of redox modulation** (Sireno *et al.*, *Free Radical Biology and Medicine*, 2025, *submission in progress*), we aimed to investigate the interplay between exercise-induced redox perturbations and EV biogenesis in skeletal muscle cells. Specifically, our objectives were to determine whether controlled redox changes modulate the release of EVs from skeletal muscle cells, both in terms of quantity and vesicle subtypes, and the molecular pathways governing their biogenesis, evaluating the transcriptional responses of gene involved in the vesiculation process. Gene expression analysis revealed that  $H_2O_2$  triggers a transcriptional reprogramming of multiple components of ESCRT machinery and tetraspanin family, suggesting an active redox control of sEVs biogenesis pathways. Consistently, nanoparticle tracking analysis (NTA) showed that  $H_2O_2$  modulates sEVs release in a cell state-dependent manner without altering vesicle size distribution. Western blotting confirmed the enrichment of canonical markers (ALIX, TSG101, CD81) in isolated vesicles, supporting their endosome-derived identity. These findings demonstrate that physiological redox cues regulate exosome-like production in skeletal muscle cells and identify  $H_2O_2$  as a key modulator of vesicular trafficking. By coupling cellular redox status to intercellular communication, this mechanism may contribute to the systemic adaptations elicited by exercise.

## 3. Aim III

This final aim of my PhD research represents a complementary experience for my scientific growth in exercise redox biology, where I had the chance to participate in collaborative projects running within the Unit of Biology and Genetics of Movement.

Oxidative stress is a critical factor impairing myoblast viability, proliferation, and regenerative capacity. Elevated ROS levels, such as those induced by  $H_2O_2$ , disturb cellular redox homeostasis, reduce glutathione availability, suppress antioxidant defenses, and ultimately compromise muscle repair. Natural compounds with antioxidant properties represent a promising strategy to

counteract these effects. *Moringa oleifera* leaf extract (MOLE), rich in glucosinolates, flavonoids, phenolic acids, and other bioactive molecules, has previously demonstrated antioxidative and cytoprotective potential in muscle cell models. However, its role in supporting proliferating myoblasts under oxidative insult remained to be clarified. In our original research work “**The effect of *Moringa oleifera* leaf extract on C2C12 myoblast proliferation and redox status under oxidative insult**” (Ceci et al., *Antioxidants*, 2024), we aimed to investigate whether MOLE can protect C2C12 myoblasts exposed to oxidative stress. This study demonstrated that MOLE, thanks to a complex mixture of antioxidant molecules including glucosinolates, flavonoids, and phenolic acids, exerts strong cytoprotective and antioxidant effects in myoblasts exposed to oxidative stress by restoring redox balance and preserving their capacity for proliferation and repair.

Oxidative stress is a major determinant of male infertility, as excessive ROS impair spermatogenesis, damage sperm DNA and lipids, disrupt mitochondrial function, and compromise steroidogenesis. While ROS play essential physiological roles in sperm maturation, capacitation, and intracellular signaling, their imbalance leads to oxidative distress and functional decline. Antioxidant systems, both endogenous and diet-derived, are critical to maintaining testicular redox homeostasis, yet their regulation is strongly influenced by environmental factors, lifestyle, and metabolic health. Among these, physical has emerged as a potential modulator of oxidative balance, capable of enhancing antioxidant defenses and improving sperm quality when appropriately dosed. However, the relationship between exercise intensity, redox control, and male reproductive function is complex and still not fully resolved. In this review “**Protective role of physical activity and antioxidant systems during spermatogenesis**” (Antinozzi et al., *Antioxidants*, 2025), we summarized current knowledge on the interplay between oxidative stress, antioxidant defenses, and physical activity in the regulation of spermatogenesis, reinforcing the importance of optimal training load, antioxidant capacity, and lifestyle factors in supporting male reproductive health.

## Systematic Review

# Extracellular Vesicles as Epigenetic Regulators of Redox Homeostasis: A Systematic Review and Meta-Analysis

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**Abstract:** Extracellular vesicles (EVs) are emerging as key regulators of cellular communication, with increasing evidence supporting their role in oxidative stress (OS) modulation. In particular, the miRNA cargo of EVs plays a crucial role in mitigating OS and promoting redox balance through both direct antioxidant effects and epigenetic regulation. This study aimed to evaluate the impact of EVs on OS markers, influenced by their miRNA-mediated effects and potential epigenetic modifications in target cells. A systematic literature search was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines to identify studies reporting the effects of EVs on OS parameters. A meta-analysis was performed on key OS biomarkers, including reactive oxygen species (ROS), superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA). The heterogeneity of EV isolation and characterization techniques was also analyzed. The included studies demonstrated that EVs exert significant antioxidant effects by reducing ROS levels, increasing SOD activity and GSH levels, and lowering MDA levels. These effects were largely attributed to EV-miRNAs, which induce epigenetic modifications that modulate redox-related signaling pathways. However, the variability in EV isolation methods and characterization approaches highlights the need for standardization to improve data comparability. Despite their therapeutic potential, this significant heterogeneity in EV research remains a barrier to translation. Moreover, further exploration of epigenetic mechanisms is essential to fully harness their benefits for OS-related diseases.

**Keywords:** extracellular vesicles; miRNAs; oxidative stress; ROS; epigenetics



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## 1. Introduction

Epigenetics is an evolving field of study that explores the molecular mechanisms influencing gene expression and heritable traits without altering the underlying DNA sequence. The term “epigenetics” refers to changes in gene activity that do not involve alterations to the DNA sequence itself. Instead, it involves modifications to the DNA structure or the proteins with which DNA interacts. Epigenetics primarily regulates gene expression, determining which genes are turned on or off in a particular cell at a given time. This regulation is crucial for the development, differentiation, and functioning of various

cell types within an organism. Three main epigenetic mechanisms exist: DNA methylation, histone modification, and non-coding RNA molecules [1].

DNA methylation involves adding methyl groups to the cytosine bases of DNA. This modification usually occurs at specific CpG island sequences (CGI; genomic regions that contain a high frequency of cytosine and guanine nucleotides connected by a phosphodiester bond) and is associated with gene silencing. When a gene is methylated, it is less likely to be transcribed and, therefore, less likely to be expressed [2]. Histones are proteins that package DNA into a compact structure called chromatin. Chemical modifications to histones, such as acetylation, methylation, phosphorylation, and ubiquitination, can alter the chromatin structure and influence gene expression. For example, acetylation of histones is generally associated with gene activation, while methylation can either activate or repress gene expression, depending on the specific context [3]. Non-coding RNAs, such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), play a role in post-transcriptional regulation. They can bind to messenger RNAs (mRNAs) and either promote their degradation or inhibit their translation into proteins. This, in turn, affects the amount of protein produced from a particular gene [4].

Extracellular vesicles (EVs) have emerged as key mediators of intercellular communication, transferring bioactive molecules such as proteins, lipids, and nucleic acids, including miRNAs and lncRNAs [5]. In recent years, growing evidence has highlighted the involvement of EV-derived miRNAs in redox biology, suggesting that these molecules can influence oxidative stress (OS) responses and epigenetic modifications in target cells [5].

Changes in the redox state play a crucial role in regulating various cellular processes, including cell growth, differentiation, apoptosis, and immune response. OS, resulting from an imbalance between reactive oxygen species (ROS) production and antioxidant defenses, is a key contributor to aging and various pathological conditions, including neurodegenerative diseases, cancer, and cardiovascular disorders [6]. Cells have evolved sophisticated mechanisms to counteract oxidative damage, including enzymatic (e.g., superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic antioxidant systems. Emerging research indicates that EVs can modulate these redox processes by delivering miRNAs that regulate key components of OS responses. Specifically, miRNAs carried by EVs have been shown to target mRNAs encoding redox-related enzymes, thereby influencing ROS levels, lipid peroxidation (e.g., malondialdehyde [MDA] production), and overall redox homeostasis [7].

The interplay between EV-mediated miRNA transfer and redox regulation also extends to epigenetic modifications, which influence gene expression without altering the DNA sequence. miRNAs are well recognized as epigenetic regulators, capable of modulating DNA methylation, histone modifications, and chromatin remodeling [8]. By limiting the expression of antioxidant genes and redox-sensitive transcription factors, EV-derived miRNAs may contribute to cellular adaptation to OS [9], with potential implications for disease progression and therapeutic strategies.

This systematic review aims to comprehensively analyze the current evidence supporting the role of EV-carried miRNAs in redox homeostasis. Specifically, we will explore their capacity to regulate post-transcriptional processes, enhance antioxidant defenses, and mitigate oxidative damage in recipient cells. Understanding these mechanisms may open new avenues for targeting OS-related diseases through EV-based strategies.

## 2. Materials and Methods

This systematic review was prepared in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines for systematic reviews [10].

The protocol of this study was developed a priori, peer-reviewed, registered, and published in the Open Science Framework (OSF; protocol ID: s2bc4).

### 2.1. Literature Search Strategy

A literature search was performed in PubMed and conducted according to standard systematic review methodology to ensure transparency and reproducibility. The following keywords and Boolean operators were used: (“extracellular vesicles” OR “exosomes” OR “microvesicles”) AND (“ROS” OR “redox” OR “oxidative stress”) AND (“epigenetics” OR “DNA methylation” OR “histone” OR “non-coding RNA”).

### 2.2. Study Selection: Inclusion and Exclusion Criteria

The inclusion criteria consisted of peer-reviewed original research studies published in the last 10 years (2015–2025), written in English, and available as free full-text publications. Keywords were required to appear in each article’s title and/or abstract. The exclusion criteria were as follows: non-primary or secondary research (including literature reviews, meta-analyses, letters to the editor, commentaries, preprints, poster/conference presentations or abstracts, dissertations, protocols, and ongoing project reports) published before 2015, not written in the English language, or lacking available full text.

Figure 1 illustrates the process of identifying and selecting eligible studies. Initially, 101 records were retrieved. Among these, 30 duplicates were removed. As a result, 71 unique records remained that were assessed for eligibility after screening. Of these, 43 were excluded because they were not original studies, and 6 more were excluded because they did not report oxidative stress-related or epigenetic-related data relevant to this study. Ultimately, 22 studies were included in the analysis [11–32].

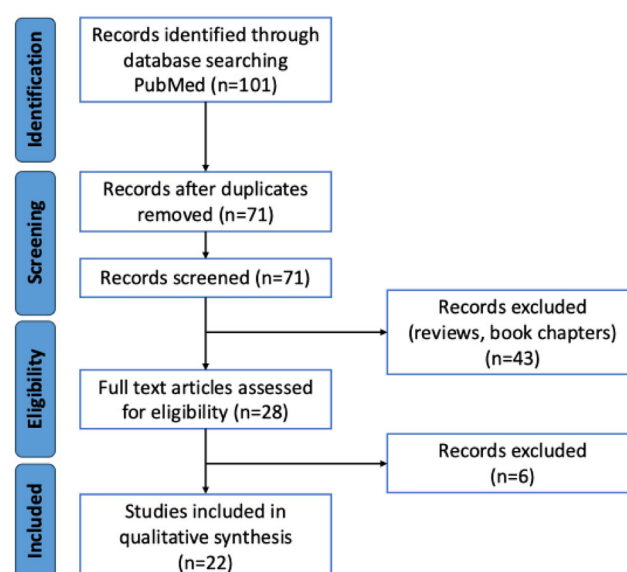


Figure 1. PRISMA flow diagram of the selected studies.

### 2.3. Risk of Bias Assessment

The quality of the included studies was assessed using the Risk of Bias in Non-Randomized Studies of Interventions (ROBINS-I) tool for both in vivo and in vitro studies [33]. Studies were categorized as low, moderate, or severe risk of bias based on selection bias, confounding variables, blinding, and outcome reporting.

The assessment of the risk of bias revealed that six studies were identified as low risk, making them the most reliable sources for the meta-analysis; fourteen studies had moderate risk, indicating that their results should be interpreted with some caution; and two studies

were classified as serious risk, raising concerns about their reliability. Sensitivity analysis should consider excluding these studies (see Table 1). Most studies exhibited confounding and selective reporting issues, emphasizing the need for standardized methodologies in EV research. Low-risk studies (e.g., Fafián-Labora et al. (2020) [14], Sanz-Ros et al. (2022) [21], Della Rocca et al. (2024) [28], and Gyorgy et al. (2025) [31]) were prioritized for conclusion. Serious-risk studies (Wang et al. (2019) [12] and Hussain et al. (2023) [24]) were carefully evaluated. Overall, while most studies support the beneficial effects of EVs, methodological variability remains a challenge, reinforcing the need for standardized protocols in future research.

**Table 1.** Risk of bias assessment of the selected studies.

| Study                                 | Confounding | Selection Bias | Classification of Interventions | Deviations from Intended Interventions | Missing Data | Measurement of Outcomes | Selective Reporting | Overall Risk |
|---------------------------------------|-------------|----------------|---------------------------------|----------------------------------------|--------------|-------------------------|---------------------|--------------|
| Pavanello et al. (2016) [11]          | Moderate    | Low            | Low                             | Moderate                               | Low          | Moderate                | Moderate            | Moderate     |
| Wang et al. (2019) [12]               | Serious     | Moderate       | Low                             | Serious                                | Moderate     | Moderate                | Serious             | Serious      |
| Xu et al. (2019) [13]                 | Moderate    | Moderate       | Moderate                        | Low                                    | Low          | Low                     | Moderate            | Moderate     |
| Fafián-Labora et al. (2020) [14]      | Low         | Low            | Low                             | Low                                    | Low          | Low                     | Low                 | Low          |
| El-Derany et al. (2021) [15]          | Low         | Low            | Low                             | Low                                    | Moderate     | Moderate                | Moderate            | Moderate     |
| Homme et al. (2021) [16]              | Moderate    | Moderate       | Low                             | Moderate                               | Low          | Low                     | Moderate            | Moderate     |
| Lopez de las Hazas et al. (2021) [17] | Moderate    | Low            | Low                             | Moderate                               | Low          | Moderate                | Moderate            | Moderate     |
| Mecocci et al. (2021) [18]            | Low         | Low            | Low                             | Low                                    | Low          | Low                     | Moderate            | Low          |
| Li et al. (2022) [19]                 | Moderate    | Moderate       | Low                             | Moderate                               | Moderate     | Moderate                | Moderate            | Moderate     |
| Qu et al. (2022) [20]                 | Moderate    | Low            | Low                             | Moderate                               | Low          | Moderate                | Moderate            | Moderate     |
| Sanz-Ros et al. (2022) [21]           | Low         | Low            | Low                             | Low                                    | Low          | Low                     | Low                 | Low          |
| Zhang et al. (2022) [22]              | Moderate    | Low            | Low                             | Moderate                               | Low          | Moderate                | Moderate            | Moderate     |
| Hu et al. (2023) [23]                 | Moderate    | Low            | Low                             | Moderate                               | Low          | Moderate                | Moderate            | Moderate     |
| Hussain et al. (2023) [24]            | Serious     | Moderate       | Moderate                        | Serious                                | Moderate     | Moderate                | Serious             | Serious      |
| Khamis et al. (2023) [25]             | Low         | Low            | Low                             | Low                                    | Low          | Low                     | Low                 | Low          |
| Papini et al. (2023) [26]             | Moderate    | Low            | Low                             | Moderate                               | Moderate     | Low                     | Moderate            | Moderate     |
| Xu et al. (2023) [27]                 | Low         | Low            | Moderate                        | Low                                    | Low          | Moderate                | Moderate            | Moderate     |
| Della Rocca et al. (2024) [28]        | Low         | Low            | Low                             | Moderate                               | Low          | Low                     | Low                 | Low          |
| Hassan et al. (2024) [29]             | Moderate    | Low            | Low                             | Moderate                               | Moderate     | Moderate                | Moderate            | Moderate     |
| Xu et al. (2024) [30]                 | Moderate    | Moderate       | Low                             | Low                                    | Low          | Moderate                | Moderate            | Moderate     |
| Gyorgy et al. (2025) [31]             | Low         | Low            | Low                             | Low                                    | Low          | Low                     | Low                 | Low          |
| Shinde et al. (2025) [32]             | Moderate    | Moderate       | Low                             | Moderate                               | Low          | Moderate                | Moderate            | Moderate     |

## 2.4. Analysis and Data Synthesis

The final selection of studies was reviewed in detail. The extracted information included the author, year of publication, experimental model, EV source, EV type, isolation technique, characterization methods, EV cargo content, and downstream effects on target cells, focusing on OS markers and epigenetic modifications.

A meta-analysis was conducted using a meta-R package (version 8.0-2) to assess differences in EV size and concentration between experimental and control groups [34]. The metacont function was used to analyze continuous data (ROS levels, SOD levels,

SOD activity, GSH levels, and MDA levels), specifying sample sizes, means, and standard deviations for both experimental and control groups.

The effect size was estimated using the Standardized Mean Difference (SMD), with Hedges'  $g$  as the selected method. This approach is preferred in studies with small sample sizes, as it corrects the bias in SMD estimation. Given the expected heterogeneity among studies, a random-effects model was applied, which assumes that differences between studies may arise from both within-study and between-study variability, thereby providing a more generalizable effect estimate [35].

Additionally, a prediction interval was calculated, providing an estimate of the range in which effects in future similar studies are expected to fall. This offers insights into the generalizability of the findings beyond the analyzed studies. A fixed-effects model was not computed, as it assumes homogeneity across studies, which was deemed inappropriate given the expected variability in methodologies and EV characteristics.

### 3. Results

#### 3.1. Study Results Synthesis

Figure 2 presents a series of donut charts summarizing the distribution of key characteristics across the studies included in this systematic review. Each panel represents a different categorical variable, with color-coded segments corresponding to the proportion of studies within each category. EVs were predominantly of animal (non-primate) origin rather than human origin. EVs were mostly isolated by ultracentrifugation (UC) or using commercial kits, although some papers used size-exclusion chromatography (SEC) or did not report any information about the isolation technique.



**Figure 2.** Summary of the reported data by the selected studies.

EVs were used for in vitro and in vivo experiments or cargo content analysis. Regarding EV cargo content, some studies did not analyze it. In contrast, most assessed their nucleic acid content (mRNA, miRNA, circRNA, and DNA), followed by the analysis of protein and metabolic cargo. Following isolation, EVs were characterized. The most commonly used characterization techniques were transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and Western Blotting (WB). Some studies also performed Flow Cytometry (FC), Enzyme-linked Immunosorbent Analysis (ELISA), or Dynamic Light Scattering (DLS). Importantly, none of these techniques is sufficient on its own. Thus, many papers offered a combination of two or even three techniques.

These data illustrate the diversity of methodological approaches and thematic foci within the reviewed literature, providing insights into potential gaps and areas of focus in the field.

### 3.2. EV Size and Concentration: NTA Data Summary

Figure 3 presents a double plot summarizing the nanoparticle tracking analysis (NTA) data reported across multiple studies, including the mean EV size (nm) and concentration (particles/mL). The left panel illustrates the mean EV size with 95% confidence intervals, revealing variability across different sources and isolation methods. The reported EV sizes range from approximately 90 to over 250 nm, with most studies clustering around 100–150 nm. Larger EVs were observed in studies focusing on skin-derived vesicles [14], plasma [17], and adipose-derived stem cell EVs [21]. In contrast, smaller vesicles were reported for umbilical cord-derived exosomes [30].

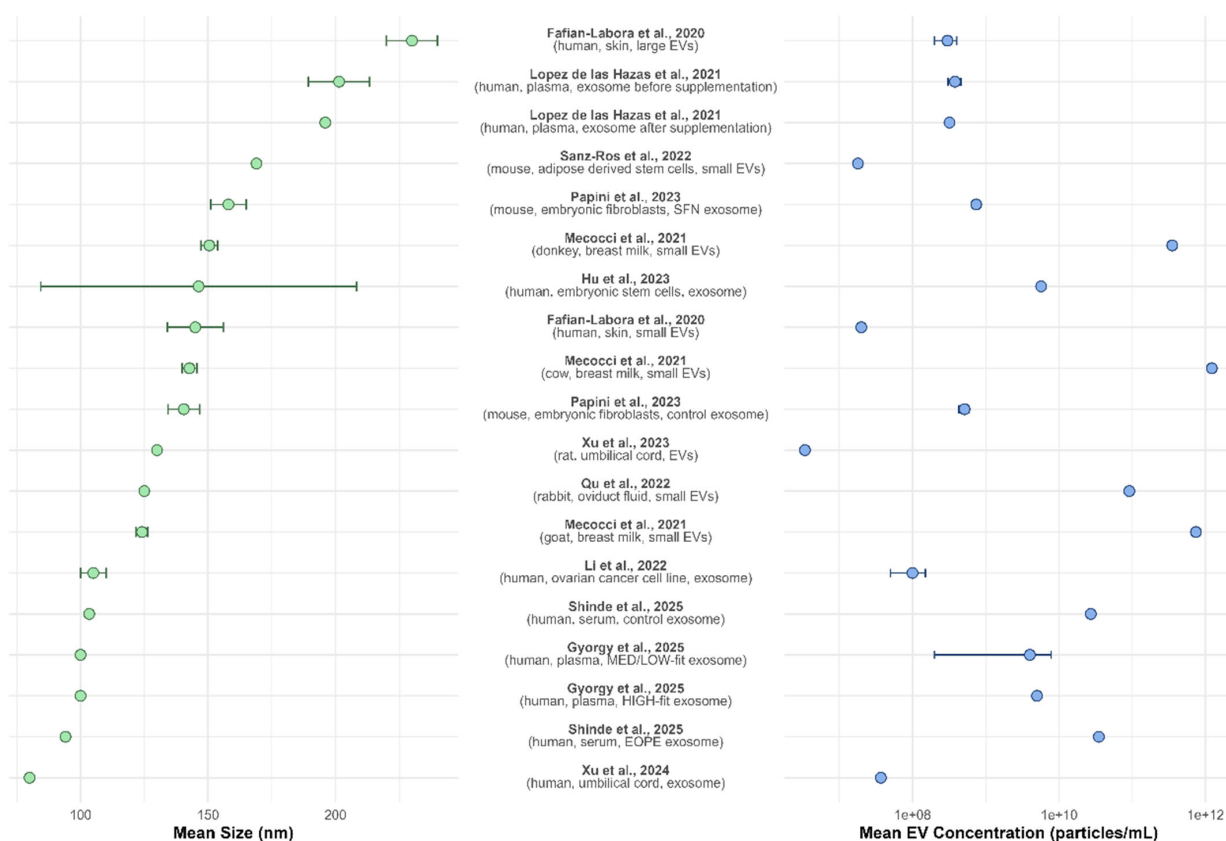


**Figure 3.** Summary of the NTA analysis of EVs across different studies. The left panel shows the distribution of the mean EV size (in nanometers), while the right panel represents the mean EV concentration (in particles/mL). Each point indicates the reported mean value for a given study, with error bars representing the range from mean  $\pm$  standard deviation.

The right panel of the figure displays EV concentration, which spans several orders of magnitude, from approximately  $10^7$  to  $10^{12}$  particles/mL. Notably, umbilical cord-derived exosomes [30], skin-derived vesicles [14], and adipose-derived stem cell EVs [21] exhibited the lowest concentrations. In contrast, higher EV concentrations were found in studies analyzing breast milk from different species [18]. EV concentration variation may reflect differences in sample type, isolation techniques, and experimental conditions across studies.

Together, these data highlight the heterogeneity in EV size and concentration among different biological sources and underscore the need for standardized isolation techniques and characterization methodologies in EV research.

3.3. Effect of EV Treatment on ROS Levels

The analysis of ROS levels (see Figure 4) shows a significant overall effect, with an SMD of  $-4.17$  (95% CI:  $-5.62, -2.72$ ),  $p < 0.0001$ , indicating that EV treatment significantly reduces ROS levels compared to the control group. Individual studies show consistent effects in favor of EV treatment, with all 95% confidence intervals falling within the negative range, reinforcing the robustness of the observed effect. The heterogeneity analysis revealed moderate-to-high variability among studies ( $I^2 = 67.1\%$ ,  $p = 0.0057$ ), suggesting that methodological or biological factors may contribute to the observed differences in effect sizes. Additionally, the prediction interval ( $-8.20, -0.14$ ) indicates that, although most future studies are likely to report a reduction in ROS with EV treatment, the effect may be smaller or even non-significant in some cases.

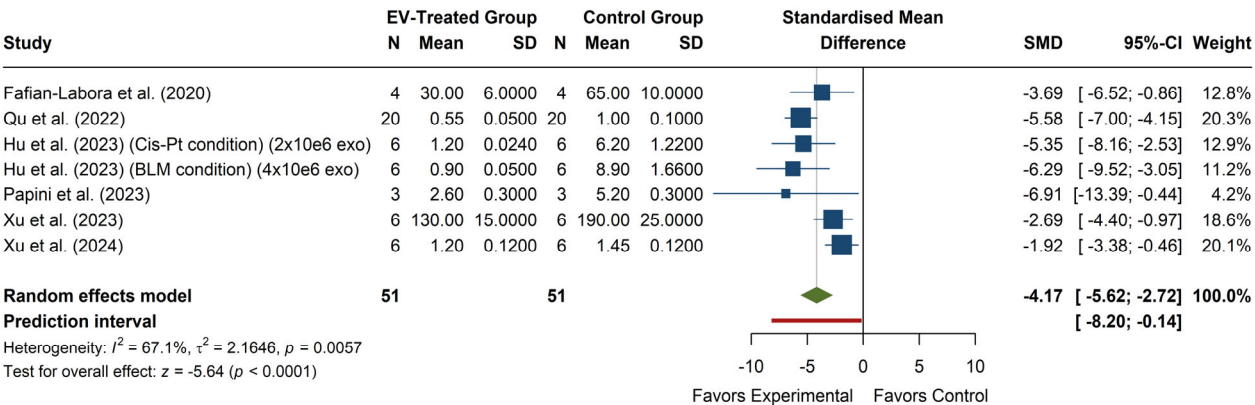


**Figure 4.** Forest plot of the effect of EVs on ROS levels. Color legend: Standardized Mean Difference of each article (blue), Average Standardized Mean Difference of all articles (green), Prediction Interval (red).

3.4. Effect of EV Treatment on SOD Levels

The meta-analysis of SOD levels (see Figure 5) revealed a non-significant overall effect, with an SMD of  $2.51$  (95% CI:  $-0.72, 5.75$ ),  $z = 1.52$ ,  $p = 0.1274$ , suggesting that EV treatment does not significantly increase SOD levels compared to the control. The confidence interval includes zero, indicating that the effect size is uncertain and could range from negative to strongly positive. Individual studies show high variability in effect sizes, with Qu et al. (2022) [20] reporting a strong positive effect (SMD =  $7.57$ , 95% CI:  $0.51, 14.63$ ), while Zhang et al. (2022) [22] and Khamis et al. (2023) [25] report more minor, non-significant effects. The heterogeneity was moderate to high ( $I^2 = 65.0\%$ ,  $p = 0.0573$ ), with  $\tau^2 = 5.07$ , indicating considerable variation between studies. The prediction interval ( $-9.49, 14.52$ ) suggests that future studies could observe a wide range of possible effects, from a substantial decrease to a strong increase in SOD levels. Overall, while some evidence suggests a potential increase

in SOD with EV treatment, the high heterogeneity and lack of statistical significance limit the interpretability of the results.

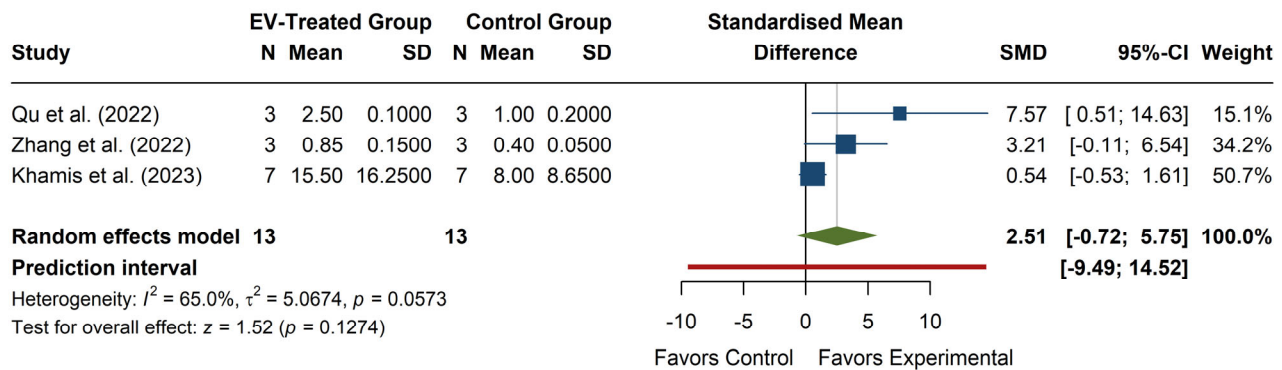


**Figure 5.** Forest plot of the effect of EVs on SOD levels. Color legend: Standardized Mean Difference of each article (blue), Average Standardized Mean Difference of all articles (green), Prediction Interval (red).

### 3.5. Effect of EV Treatment on SOD Activity

The meta-analysis of SOD activity (see Figure 6) revealed a significant overall effect, with an SMD of 7.27 (95% CI: 1.44, 13.10),  $z = 2.44$ ,  $p = 0.0146$ , indicating that EV treatment significantly increases SOD activity compared to the control. Individual studies show positive effects, with Hassan et al. (2024) [29] reporting the largest SMD (14.93, 95% CI: 7.65, 22.22), while El-Derany et al. (2021) [15] and Xu et al. (2023) [27] reported more moderate increases. The heterogeneity was high ( $I^2 = 77.6\%$ ,  $p = 0.0116$ ), with  $\tau^2 = 21.82$ , suggesting substantial variability in effect sizes across studies. The prediction interval (−16.56, 31.10) indicates a wide range of potential future outcomes, including possible adverse effects, although the overall trend favors increased SOD activity with EV treatment.

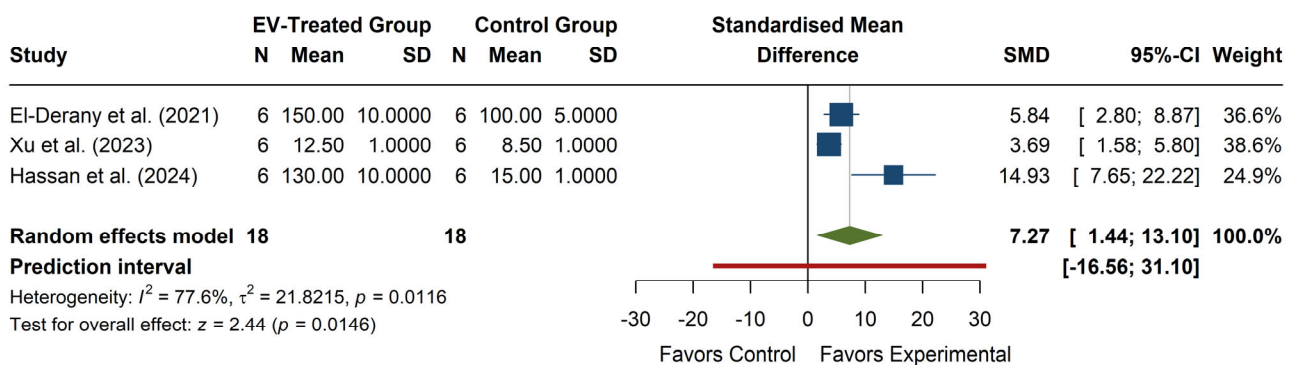


**Figure 6.** Forest plot of the effect of EVs on SOD activity. Color legend: Standardized Mean Difference of each article (blue), Average Standardized Mean Difference of all articles (green), Prediction Interval (red).

### 3.6. Effect of EV Treatment on GSH Levels

The meta-analysis of GSH levels (see Figure 7) showed a non-significant overall effect, with an SMD of 25.16 (95% CI: −18.62, 68.95),  $z = 1.13$ ,  $p = 0.2600$ , indicating that EV treatment does not lead to a consistent increase in GSH levels. Individual studies exhibited high variability, with Hassan et al. (2024) [29] reporting a considerable effect (SMD = 162.36, 95% CI: 84.12, 240.60), while Li et al. (2022) [19] showed a negative, non-significant effect (SMD = −1.58, 95% CI: −3.73, 0.57). The heterogeneity was high ( $I^2 = 91.0\%$ ,  $p < 0.0001$ ,  $\tau^2 = 2286.60$ ), reflecting substantial variation across studies. The prediction interval (−121.38, 171.70) suggests a broad range of potential outcomes in future

studies, from significant negative to extreme positive effects. The lack of statistical significance and the high heterogeneity indicate that the impact of EVs on GSH levels remains inconclusive.

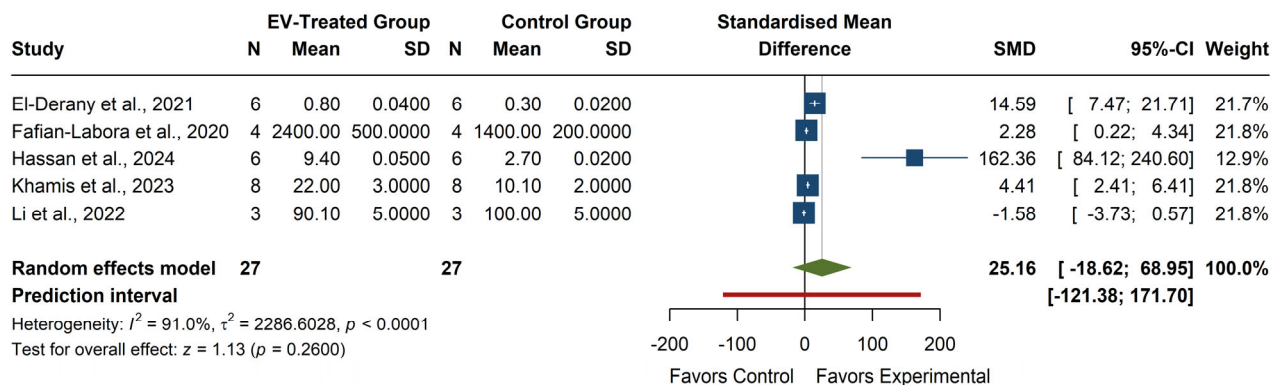


**Figure 7.** Forest plot of the effect of EVs on GSH levels. Color legend: Standardized Mean Difference of each article (blue), Average Standardized Mean Difference of all articles (green), Prediction Interval (red).

### 3.7. Effect of EV Treatment on MDA Levels

The meta-analysis of MDA levels (see Figure 8) revealed a significant overall effect, with an SMD of  $-7.51$  (95% CI:  $-14.70$ ,  $-0.32$ ),  $z = -2.05$ ,  $p = 0.0407$ , suggesting that EV treatment significantly reduces MDA levels compared to the control group. Individual studies showed high variability in effect sizes, with Xu et al. (2023) [27] reporting the most significant reduction (SMD =  $-28.95$ , 95% CI:  $-42.95$ ,  $-14.96$ ), while some studies, such as Sanz-Ros et al. (2022) [21] and Khamis et al. (2023) [25], reported small or non-significant effects. The heterogeneity was high ( $I^2 = 86.2\%$ ,  $p < 0.0001$ ,  $\tau^2 = 83.18$ ), indicating substantial differences across studies. The prediction interval ( $-31.57$ ,  $16.55$ ) suggests that future studies might observe a wide range of effects, including potential increases in MDA. Despite the significant overall effect, the considerable heterogeneity highlights the need for further investigation into factors influencing the impact of EV treatment on MDA levels.

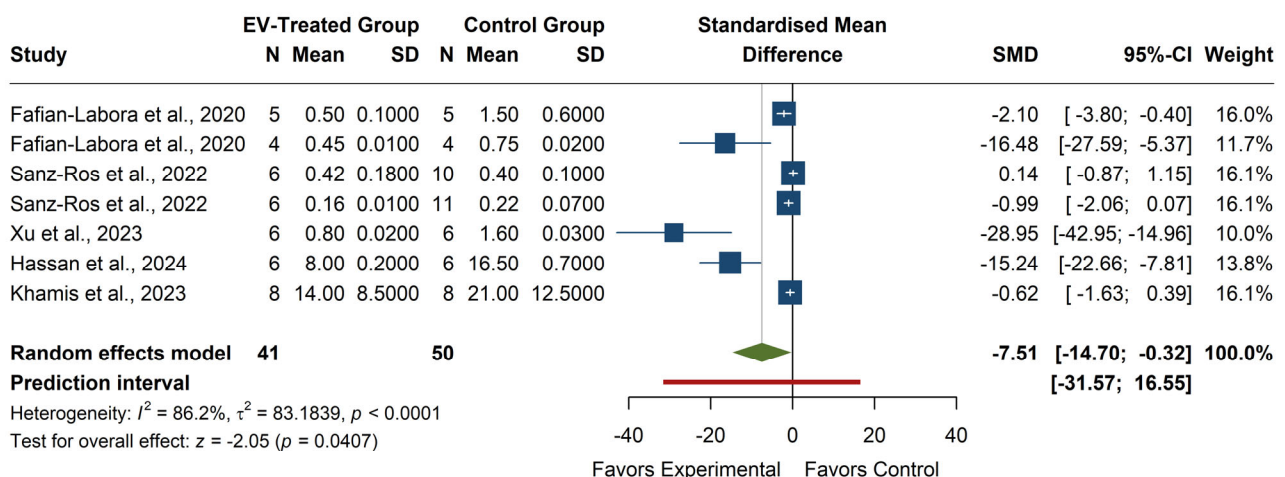


**Figure 8.** Forest plot of the effect of EVs on MDA levels. Color legend: Standardized Mean Difference of each article (blue), Average Standardized Mean Difference of all articles (green), Prediction Interval (red).

#### 4. Discussion

The findings of this systematic review with meta-analysis highlight considerable variability in EV isolation methods and characterization across different studies. The selected studies used several isolation techniques, such as ultracentrifugation (with varying forces of g and times), size exclusion chromatography, and commercial kits. Since there is no specific marker for EVs, most studies used a combination of two or more methods to characterize their EVs, such as nanoparticle tracking analysis (NTA), Western Blot (WB), Flow Cytometry (FC), Mass Spectrometry (MS), and transmission electron microscopy (TEM).

The NTA data summary illustrates that EV size and concentration can vary significantly depending on the biological source, isolation technique, and analytical method used. This heterogeneity underscores the urgent need for standardized protocols to ensure reproducibility and comparability across studies. The lack of uniformity in EV isolation and characterization remains a significant challenge in the field, limiting the translational potential of EV-based interventions. Efforts to establish consensus guidelines, such as those proposed by the International Society for Extracellular Vesicles (ISEV) [36], should be further refined and widely adopted to enhance the robustness of EV research.

Despite methodological discrepancies, our meta-analysis confirms that EV cargo transfer, particularly miRNAs, exerts beneficial effects on target cells. Specifically, EVs contribute to redox homeostasis by reducing ROS levels, enhancing SOD activity, increasing GSH levels, and lowering MDA levels. These findings suggest that EVs possess potent antioxidant properties, which could be leveraged for therapeutic applications in conditions associated with oxidative stress, such as aging, neurodegenerative disorders, and metabolic diseases [37]. The ability of EVs to modulate OS pathways aligns with previous evidence that demonstrates their role in cellular protection, tissue repair, and metabolic regulation.

One of the key mechanisms by which EVs exert their antioxidant effects is through epigenetic modifications induced by their miRNA cargo. Recipient cells can internalize EV-derived miRNAs, which regulate gene expression post-transcriptionally by targeting messenger RNAs (mRNAs) involved in OS responses. Indeed, several miRNAs carried by EVs have been implicated in the regulation of antioxidant defense systems. For example, specific miRNAs can modulate the expression of nuclear factor erythroid 2-related factor 2 (NRF2), a master regulator of the antioxidant response, by either directly targeting its inhibitors (e.g., KEAP1) or activating NRF2-responsive genes involved in detoxification and ROS scavenging [38–40].

However, evidence suggests that these miRNAs can induce more stable, long-term cellular adaptations through epigenetic modifications, including DNA methylation, histone modifications, and chromatin remodeling. EV-miRNAs have been shown to regulate histone deacetylases and DNA methyltransferases, which influence chromatin accessibility and the transcriptional activation of genes coding for antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [37,41–43].

Furthermore, EV-mediated epigenetic modifications extend beyond acute responses to OS, promoting cellular reprogramming toward a more resilient, antioxidant-enhanced phenotype. For instance, some EV-miRNAs downregulate pro-oxidant and pro-inflammatory pathways by silencing key genes in NF- $\kappa$ B and TNF- $\alpha$  signaling, leading to sustained reductions in OS and inflammatory damage [44–46]. In stem and progenitor cells, EV-mediated epigenetic modifications have also been linked to improved mitochondrial function and biogenesis, further reinforcing cellular defense mechanisms against OS [47].

These findings suggest that EVs do not merely deliver transient antioxidant benefits but can induce long-lasting changes in recipient cells through epigenetic regulation. This concept is particularly relevant for aging and age-related diseases, where the accumula-

tion of oxidative damage contributes to cellular dysfunction. By modulating epigenetic landscapes, EVs may represent a promising tool for restoring redox balance and enhancing tissue homeostasis over time.

## 5. Limitations

While our findings provide strong evidence for the beneficial effects of EVs on redox balance, several limitations should be acknowledged.

First, the heterogeneity in EV isolation and characterization methods introduces variability in the reported outcomes, which may affect the overall strength of the conclusions. Differences in isolation techniques, including ultracentrifugation, size-exclusion chromatography, and kit-based methods, can influence EV purity, yield, and cargo composition, potentially confounding the reported effects.

Second, the included studies vary regarding EV source, donor characteristics, and experimental conditions (in vivo/in vitro). Factors such as age, health status, and environmental influences may affect EV composition and bioactivity, limiting the generalizability of the findings. Additionally, most studies included in this review were conducted in preclinical models or in vitro settings, which may not fully recapitulate the complexity of clinical conditions. Further validation in clinical studies is necessary to establish the translational relevance of these findings.

Third, although our meta-analysis identified significant antioxidant effects of EVs, potential publication bias and selective reporting cannot be ruled out. Studies reporting null or negative findings may be underrepresented in the literature, which could lead to the overestimation of EV efficacy. Future systematic reviews should incorporate bias assessment tools and aim to include unpublished or negative results more comprehensively.

Finally, the variability in the methodologies used for functional assays, including differences in OS markers and measurement techniques, adds another layer of complexity. Standardized approaches for assessing EV function in OS models are needed to improve cross-study comparisons and data reproducibility.

## 6. Conclusions

In conclusion, this systematic review with meta-analysis supports the role of EVs as key modulators of OS through their miRNA cargo. Beyond their immediate effects on ROS scavenging and antioxidant enzyme activity, EVs induce long-term cellular adaptations through epigenetic regulation. This ability to modulate the expression of redox-related genes at the epigenetic level suggests that EVs may serve as powerful therapeutic tools for OS-related disorders. However, the standardization of EV isolation and characterization techniques, as well as more in-depth mechanistic studies on EV-induced epigenetic modifications, are imperative to fully harness their potential. Future research should validate these findings in clinical settings and explore strategies to optimize EV-based interventions for enhancing cellular resilience against oxidative damage.

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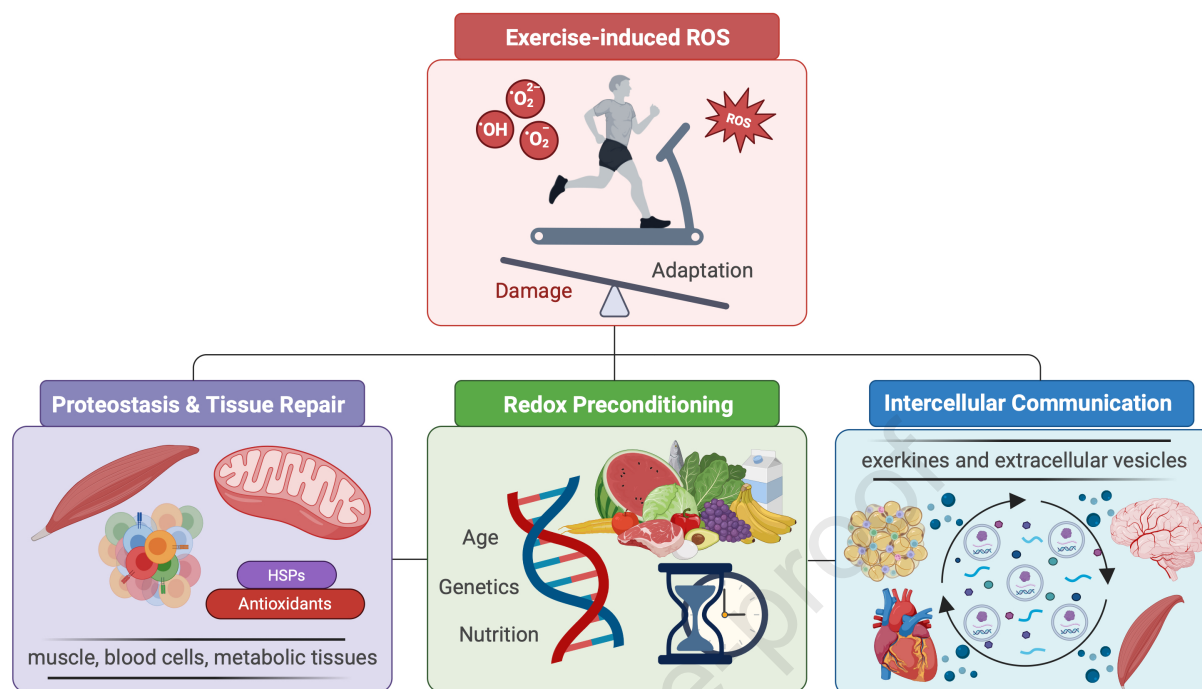
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# Reactive oxygen species in exercise biology: from adaptive stress response to cell signaling and beyond

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## Highlights

- Exercise-induced ROS act as dual regulators of stress and adaptation.
- Exercise-induced ROS modulate HSPs and antioxidant components, linking redox fluctuations to proteostasis, cytoprotection, and tissue repair in muscle, blood cells, and metabolic tissues.
- Regular physical training reprograms redox homeostasis and establishes a redox “preconditioned” phenotype influenced by age, genetics, and nutritional factors.
- Exercise drives multi-organ communication through redox-modulated exerkines and extracellular vesicles (EVs), enabling cross-talk among muscle, cardiovascular, metabolic, and neural tissues.

## Introduction

Reactive oxygen species (ROS) are continuously generated as natural by-products of cellular metabolism, especially during mitochondrial electron transport, as well as through enzymatic reactions involved in immune defense and cell signaling. When produced in excess, can disrupt redox balance, leading to oxidative modification of cellular macromolecules and structural damage. Chronic oxidative stress has been implicated in aging and in the pathogenesis of several major diseases, including cancer, cardiovascular disease, diabetes mellitus, hypertension, and neurodegenerative disorders [1–3]. Paradoxically, while exercise acutely increases ROS formation, reflected by elevated oxidative biomarkers in blood and skeletal muscle [4], regular physical activity is among the most potent interventions for reducing all-cause mortality and preventing chronic disease [5,6]. This apparent contradiction

has driven decades of research into the dual nature of exercise-induced ROS in physiology and pathology. The concept of “oxidative stress,” introduced by Sies in 1985 as an imbalance favoring oxidants over antioxidants, originally framed ROS as deleterious by-products of metabolism [7]. Early studies demonstrated that prolonged exercise induced lipid peroxidation [8], and subsequent landmark findings established that contracting skeletal muscle itself is a major source of free radicals [9,10]. While initially viewed as purely harmful, a paradigm shift in the 1990s revealed that moderate ROS production plays a critical signaling role in promoting muscle adaptation and cellular resilience [11,12]. This beneficial form of redox signaling, or “oxidative eustress” [13] regulates key adaptive processes such as mitochondrial biogenesis, antioxidant enzyme expression, heat shock protein response and enhanced contractile function [14–17].

Beyond skeletal muscle, exercise-induced ROS are likely to elicit systemic responses that extend to multiple organs through redox-sensitive signaling pathways in different tissues and cells [18–20], and circulating factors collectively termed “exerkines” [21]. These include cytokines, peptides, nucleic acids, and metabolites secreted by muscle, liver, adipose tissue, and brain, often transported via extracellular vesicles (EVs), which coordinate inter-organ communication and systemic adaptations to exercise [22,23]. Importantly, the biological effects of ROS depend on their concentration, reactivity, and subcellular localization, reflecting the highly compartmentalized redox environments within and between tissues [24,25]. Thus, understanding the fine balance between ROS-mediated damage and signaling across skeletal muscle and peripheral tissues is essential to elucidate the molecular basis of exercise-induced health benefits and the prevention of oxidative stress-related diseases. This review, inspired by the evolution of the exercise redox perspective developed by the Exercise Biology Research Group at Foro Italico University in Rome since its establishment in 2006, delineates the complex and multifaceted role of exercise-induced reactive oxygen species as both mediators of tissue damage and essential signaling molecules orchestrating adaptive responses at the cellular, tissue, and systemic levels. In doing so, it provides an integrated and comprehensive perspective on the mechanisms underpinning oxidative stress and physiological adaptation.

## **Exercise-induced ROS production: an overview**

ROS comprise both oxygen-centered radicals and non-radical derivatives formed through the partial reduction of molecular oxygen. The superoxide anion ( $O_2^{\cdot-}$ ) is the primary ROS, giving

71 rise to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) through spontaneous or superoxide dismutase-catalyzed  
 72 dismutation [26].  $\text{H}_2\text{O}_2$  is relatively stable and membrane-permeable, enabling it to act as a key  
 73 redox signaling molecule, though excessive accumulation can be cytotoxic. Further reactions  
 74 involving  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\cdot-}$  generate hydroxyl radicals ( $\cdot\text{OH}$ ), highly reactive species that cause  
 75 localized oxidative damage within cells [27].  
 76 Direct and indirect evidence from animal models and human studies shows that exercise  
 77 promotes the production of ROS in various tissues, including skeletal muscle, heart, liver,  
 78 brain and blood, in an intensity- and duration-dependent manner that contributes to both  
 79 oxidative stress and physiological adaptation [28,29].  
 80 In skeletal muscle, ROS arise from several compartmentalized sources whose relative  
 81 contributions vary with contractile state and metabolic demand. During acute contractions,  
 82 NADPH oxidases—particularly NOX2 at the sarcolemma/T-tubules and NOX4 associated  
 83 with intracellular membranes—are prominent generators that shape redox signaling underlying  
 84 glucose transport and training adaptations [30],[31] Although mitochondria continuously  
 85 produce ROS via electron leak (notably at complex III) [32], multiple lines of in situ evidence  
 86 indicate they are not the dominant source during contraction, with their contribution increasing  
 87 under specific conditions (e.g., high intensity, hypoxia, or pathology) [30]. Additional  
 88 contributors include xanthine oxidase in the myofiber microenvironment and phospholipase  
 89  $\text{A}_2$ -linked pathways that can amplify oxidant production and downstream signaling [33].  
 90 Collectively, these sources create a spatially and temporally tuned ROS landscape that  
 91 mediates both stress and adaptation in working muscle.  
 92 Similar mechanisms act in cardiac muscle, where ROS are primarily generated by  
 93 mitochondria, NADPH oxidases (NOX2 and NOX4), xanthine oxidase, and uncoupled nitric  
 94 oxide synthase (eNOS) [34,35]. During increased workload, electron leak from mitochondrial  
 95 complexes I and III produces superoxide, which is converted to hydrogen peroxide by  
 96 superoxide dismutase [35]. Moderate ROS levels regulate excitation-contraction coupling and  
 97 redox signaling [36], while excessive production—such as during ischemia-reperfusion or  
 98 chronic stress—induces oxidative damage and contributes to cardiac dysfunction [37,38].  
 99 During exercise, vascular endothelial and smooth muscle cells experience elevations in shear  
 100 stress, cyclic stretch, and metabolic activity that stimulate the production of reactive oxygen  
 101 species. The main enzymatic sources of ROS in these cells include NADPH oxidases  
 102 (particularly NOX2 and NOX4), xanthine oxidase, uncoupled eNOS, and mitochondria  
 103 [39,40]. Increased shear stress activates NOX enzymes, leading to the generation of

104 superoxide, which can be converted to hydrogen peroxide. This controlled production of  $H_2O_2$   
105 functions as a signaling molecule that regulates vascular tone, endothelial nitric oxide (NO)  
106 bioavailability, and vascular remodeling [41,42]. Mitochondrial ROS formation may also rise  
107 with increased oxygen flux during exercise, contributing to redox-sensitive pathways that  
108 enhance angiogenesis and antioxidant defenses [43]. Under physiological conditions, these  
109 mechanisms promote vascular adaptation; however, excessive or dysregulated ROS production  
110 can impair NO bioavailability and promote endothelial dysfunction [44].

111 During and following exercise, immune cells such as neutrophils and macrophages produce  
112 reactive oxygen species primarily through NADPH oxidase (NOX2) activation as part of the  
113 inflammatory and adaptive response [45]. These ROS support pathogen defense, debris  
114 clearance, and redox signaling involved in tissue repair, but excessive production can intensify  
115 inflammation and muscle damage [46]. Regular moderate exercise improves the regulation of  
116 immune-related ROS generation, enhancing antioxidant capacity and promoting systemic  
117 adaptation [47].

118 Reactive oxygen species are continuously produced in neurons and the brain as natural  
119 byproducts of cellular metabolism, particularly through mitochondrial oxidative  
120 phosphorylation. In neurons, which have high energy demands and abundant mitochondria, the  
121 electron transport chain is a major source of ROS, primarily  $O_2^{\bullet-}$  and  $H_2O_2$ . Additional ROS  
122 generation occurs through enzymatic systems such as NADPH oxidases, monoamine oxidases,  
123 and cyclooxygenases [48]. Studies from rodent models provide the direct evidence that  
124 exercise increases mitochondrial ROS production in neurons and glia, triggering redox-  
125 sensitive pathways (e.g., Nrf2, PGC-1 $\alpha$ ) that enhance antioxidant defense and neurotrophic  
126 signaling [49–51], also supported by correlational biochemical studies and neuroimaging in  
127 humans [52].

128 In summary, the mechanisms of ROS production in response to exercise are multifaceted and  
129 highly tissue-specific, reflecting the distinct metabolic and signaling demands of different  
130 tissues [Figure 1]. Readers interested in a more comprehensive understanding of these  
131 mechanisms are referred to recent in-depth reviews on exercise-induced ROS formation and  
132 redox regulation [53,54], which provide detailed insights into the molecular pathways and  
133 adaptive responses underlying this dynamic process.

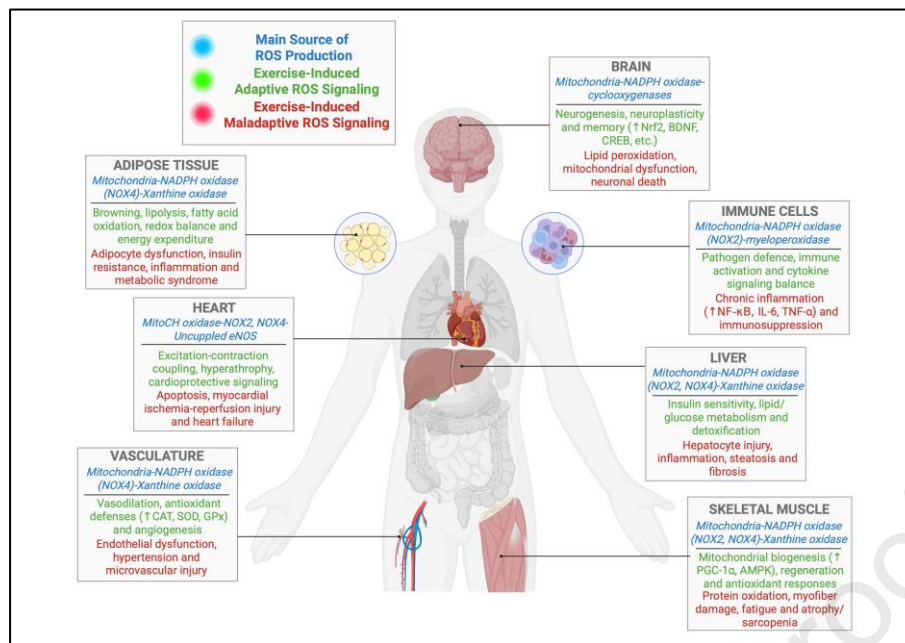


**Figure 1 - Tissue-specific sources and effects of exercise-induced ROS.** Overview of major ROS sources (blue), adaptive exercise-induced redox signaling (green), and maladaptive oxidative stress responses (red) across key organs. In skeletal muscle, heart, vasculature, adipose tissue, liver, brain, and immune cells, mitochondrial and enzymatic ROS support physiological functions such as mitochondrial biogenesis, antioxidant defense, neuroplasticity, metabolic regulation, and vascular homeostasis. When excessive or poorly regulated, ROS contribute to cellular damage, inflammation, metabolic dysfunction, and tissue injury. The figure highlights the dual role of ROS as essential mediators of exercise adaptation and potential drivers of pathology.

### Exercise and oxidative stress response: balancing benefits and risks

Physical activity (PA) is widely recognized for its health-promoting effects, including improved cardiovascular function, enhanced metabolic health, and reduced risk of chronic diseases [19,55–57]. However, PA is closely associated to oxidative stress [12]. Indeed, research advancements in both exercise and free radical biology have substantially improved our understanding of the mechanisms of exercise and oxidative stress [12,58]. Although initial investigations reported the negative effects of reactive oxygen species, several studies have shown that exercise-induced ROS can upregulate several enzymatic and non-enzymatic antioxidants in the biological system [59,60]. Exercise could be an optimizer of ROS in negating oxidative damage in the cells, while ROS can regulate signaling or act as a signaling molecule for muscular adaptation [61] [Figure 2]. This supports the concept of exercise-

induced hormesis. The term hormesis is used to define the biphasic dose-response curve in a biological system, where a smaller amount of stress can facilitate adaptation, whereas chronic and/or high doses of stressors can increase damage to the cells [53,62] [Figure 3].

The amount, source, and physiological impact of ROS depend on exercise characteristics such as intensity, duration, and type, as well as age and training status [58,63–66]. Understanding these relationships is key to optimizing exercise benefits while minimizing oxidative damage. Finally, it is important to consider that the accurate detection of ROS and associated oxidative damage following exercise is influenced by various methodological and biological factors.

Beyond the physiological occurrence of oxidative stress, the ability to observe and quantify it depends on the type of tissue sampled, timing of sample collection, and analytical characteristics of the biomarkers employed, including their sensitivity and specificity [67].

Variability in study outcomes may often be attributed to these constraints. For instance, commonly used biomarkers, such as thiobarbituric acid reactive substances (TBARS), have been questioned for their limited specificity, which may result in inconclusive or misleading findings [68]. Moreover, protocols that rely on single or infrequent sampling points or cover only a narrow post-exercise time window risk missing transient oxidative responses that may occur outside the chosen timeframe. The selection of biological tissues is another critical factor. Although blood and urine are frequently used because of their accessibility, they may not adequately reflect the oxidative processes occurring in more metabolically active or stress-sensitive tissues, such as skeletal muscle, myocardium, liver, and brain [69]. Consequently, oxidative stress may go undetected if it occurs in compartments not included in the sampling strategy. Furthermore, oxidative stress may manifest before or after the sampling period or in tissues not analyzed, which could explain the absence of detectable markers in some studies despite physiological evidence suggesting an oxidative imbalance. These considerations highlight the importance of temporal resolution, tissue specificity, and biomarker reliability in the design and interpretation of studies investigating exercise-induced oxidative stress [70].

In this chapter, we explore the effects of acute and chronic exercise-induced ROS on various human tissues, highlighting the role of stress response proteins, such as heat shock proteins (HSPs) and antioxidants, in oxidative damage and in adaptive processes. We focused on human experimental results in which the link between redox homeostasis and the expression of stress response proteins by exercise has been addressed.

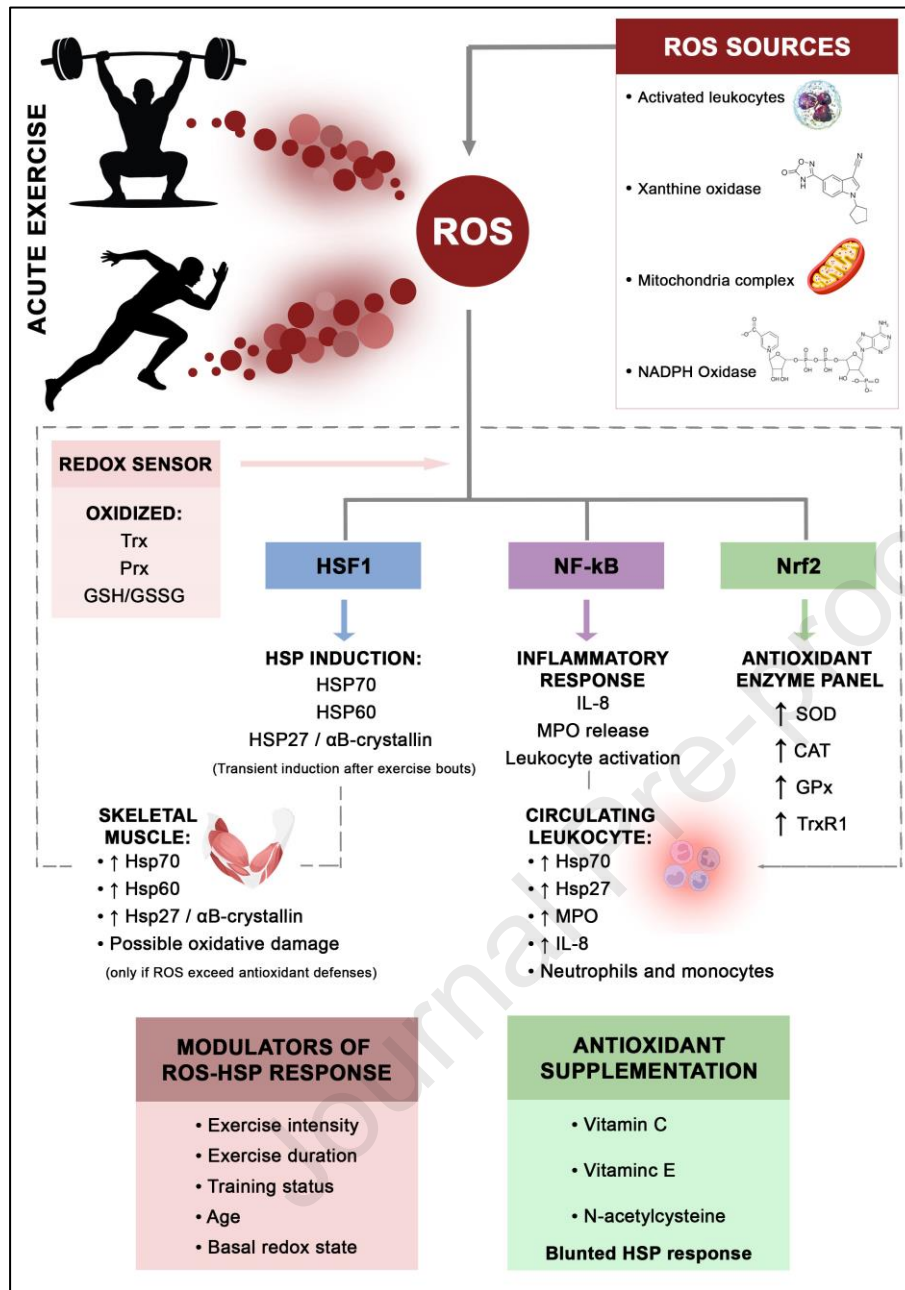


**Figure 2.** Molecular pathways activated by acute exercise-induced ROS. Acute exercise enhances ROS formation from mitochondria, xanthine oxidase, NADPH oxidase and activated leukocytes. Redox-sensitive systems (thioredoxin, peroxiredoxin, GSH/GSSG) detect these changes and activate key transcriptional pathways: (i) HSF1, initiating Hsp70, Hsp60 and small HSP expression; (ii) NF-κB, promoting pro-inflammatory mediators (MPO, IL-8) and leukocyte activation; (iii) Nrf2, upregulating the antioxidant enzyme network (SOD, CAT, GPx, TrxR1). The magnitude of these signals is modulated by exercise intensity/duration, age, training status and basal redox tone. Antioxidant supplementation (vitamin C, vitamin E, NAC)

attenuates ROS-dependent HSP responses, highlighting the role of ROS as essential signaling molecules for exercise adaptation.

### Acute Exercise-Induced ROS: The Roles of Heat Shock Proteins and Antioxidants

Acute endurance [9,71] and strength [72–74] exercise can induce an increase in free radical production, which may or may not result in acute oxidative stress, leading to oxidative damage in various tissues. It is known that, in order for oxidative stress to occur, the ROS produced during exercise must exceed the antioxidant defense system, resulting in oxidative damage to specific biomolecules [75]. Different exercise protocols may induce different levels of ROS production, as oxidative damage has been shown to be dependent on both intensity [76,77] and duration [78]. During low-intensity and short-duration exercise, antioxidant defenses appear sufficient to meet ROS production, but as the intensity and/or duration of exercise increases, these defenses are no longer adequate, potentially resulting in oxidative damage to surrounding tissues [79] [Figure 2 and 3].

HSPs are a family of molecular chaperones that are upregulated in response to cellular stress, including oxidative stress [80–82]. They contribute to protein quality control by assisting in folding, preventing aggregation, and facilitating the repair or degradation of damaged proteins [83]. Experimental evidence indicates that HSP expression is closely associated with intracellular ROS availability, supporting their role as upstream signals in HSP activation rather than solely as mediators of oxidative damage [80–82,84] (Figure 2). In parallel, the antioxidant defense system is activated to limit ROS accumulation. This response includes enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), as well as non-enzymatic antioxidants, including vitamins C and E [85]. The interaction between ROS production, HSP induction, and antioxidant activity reflects a regulated balance in which ROS initiate stress signaling, while HSPs and antioxidants act to preserve cellular homeostasis and support recovery [80].

Within this framework, numerous studies since the early 2000s have examined the relationship between ROS generation and HSP induction following acute exercise in humans. Most investigations have focused on Hsp70 and Hsp60, which are redox-sensitive and consistently upregulated in response to acute exercise stimuli [86–88]. Evidence for ROS-dependent regulation of these proteins has largely been derived from antioxidant supplementation studies. Supplementation with vitamin C [88],  $\gamma$ -tocopherol/ascorbic acid mixtures [86], or N-acetylcysteine (NAC) [89] increased basal HSP expression while preventing post-exercise

231 increases in Hsp60 and Hsp70 in the *vastus lateralis* muscle. These findings indicate that  
 232 reducing ROS availability interferes with the signaling processes required for exercise-induced  
 233 HSP upregulation.  
 234 Attempts to associate HSP induction with changes in antioxidant enzymes or conventional  
 235 oxidative stress biomarkers have produced inconsistent results. Khassaf et al. reported  
 236 simultaneous increases in Hsp60 and Hsp70 expression together with transient elevations in  
 237 SOD activity following non-damaging exercise [87], whereas a subsequent study found no  
 238 significant changes in SOD or CAT activity [88]. Similarly, increases in Hsp70, Hsc70, and  
 239 Hsp60 observed 48 h after moderate running in recreationally active individuals were not  
 240 accompanied by changes in mitochondrial MnSOD protein content or SOD and CAT activities  
 241 [90]. These observations suggest that HSP induction may be driven by transient or localized  
 242 redox perturbations that are not readily detected by conventional assays. Consistent with this  
 243 interpretation, glutathione-dependent redox systems appear more sensitive to subtle  
 244 fluctuations in ROS than thioredoxin-based systems or enzymes such as SOD and CAT [91].  
 245 Exercise-induced HSP responses have also been documented in circulating blood cells,  
 246 providing insight into systemic oxidative stress. In contrast to skeletal muscle, leukocyte  
 247 Hsp70 induction appears closely associated with substantial oxidative stress generated during  
 248 prolonged or strenuous endurance exercise [92–94]. Exhaustive treadmill running and half-  
 249 marathon races markedly increased Hsp70 mRNA and protein levels in multiple leukocyte  
 250 subpopulations for up to 24 h postexercise [92,93,95]. These responses coincided with  
 251 activation of monocytes and granulocytes, which generate considerable ROS under physical  
 252 stress [95], as well as with increased plasma myeloperoxidase (MPO) and IL-8 concentrations  
 253 [92,93,95–97]. Elevated TBARS in lymphocytes from older adults after maximal exercise  
 254 further suggest oxidative damage primarily localized to cell membranes [94]. Antioxidant  
 255 supplementation with RRR- $\alpha$ -tocopherol reduced Hsp70 transcription in granulocytes at 3 h  
 256 postexercise but not at 48 h, indicating a prominent role for ROS during the early phase of  
 257 HSP induction [95].  
 258 Age and training status further influence the relationship between oxidative stress and HSP  
 259 expression. Physically active elderly individuals exhibit higher antioxidant capacity (TEAC),  
 260 lower oxidative damage (TBARS, AOPP), and reduced leukocyte Hsp72 expression compared  
 261 with sedentary peers [98]. Similarly, professional athletes show three- to four-fold higher basal  
 262 circulating HSP70 levels and elevated anti-HSP70 antibody concentrations relative to  
 263 sedentary controls, yet demonstrate more rapid normalization of HSP70 following submaximal

exercise [99]. These findings indicate that long-term adaptations in redox homeostasis modulate both the magnitude and duration of acute HSP responses. Baseline HSP expression also appears to influence responsiveness, as a single exhaustive exercise bout increased Hsp70 and Hsp27 expression only in trained older adults with lower resting HSP levels and favorable redox balance [100]. The redox sensitivity of HSPs has also been examined under controlled oxidative conditions. High-intensity interval exercise performed under normoxia increased serum HSP27 and oxidative stress indices, whereas performing the same protocol under hyperoxia substantially attenuated both responses [101]. The positive correlation between HSP27 and oxidative markers supports its redox sensitivity and suggests that reducing hypoxia-induced ROS limits stress-driven HSP induction. In contrast, supplementation with an antioxidant-rich herbal formula increased HSP70 mRNA expression in recreational female athletes despite modest effects on conventional oxidative stress markers [102], consistent with redox hormesis.

Although modulation of small HSPs, such as Hsp27 and  $\alpha$ B-crystallin, has been widely reported following various exercise modalities [103–107], direct evidence for ROS involvement in their regulation remains limited. Non-damaging exercise protocols do not appear to alter muscle Hsp27 or  $\alpha$ B-crystallin levels or oxidative stress markers [108]. Given their high basal expression in skeletal muscle, these proteins may already provide sufficient protection against mild oxidative stress. Clear evidence linking ROS to small HSP induction has been reported primarily under conditions of pronounced oxidative stress, such as exhaustive endurance exercise, where increased leukocyte Hsp27 expression coincided with elevations in myeloperoxidase and IL-8 [92,109]. Supporting this view, no changes in 4-HNE–protein adducts were observed despite modulation of Hsp27 and  $\alpha$ B-crystallin in leukocytes following maximal but non-damaging exercise in healthy adults [110].

### Molecular Mechanisms of Acute Exercise-Induced Stress

At the molecular level, acute exercise is a brief and powerful stressor that activates a coordinated cellular defense response by activating redox-sensitive signaling pathways [111,112]. The interaction between ROS and HSPs expression, particularly Hsp70, Hsp60, and smaller chaperones, such as Hsp27 and  $\alpha$ B-crystallin, becomes a key regulatory mechanism in preserving cellular integrity and proteostasis [83,113–115]. HSP gene expression is boosted by ROS-driven activation of transcription factors like HSF1 and NF- $\kappa$ B, while the Nrf2-ARE axis

296 simultaneously increases the transcription of antioxidant enzymes, such as SOD, CAT, and  
297 GPx [16,116,117].

298 These interactions draw attention to a delicate redox balance: antioxidant systems restore redox  
299 homeostasis, HSPs stabilize unfolded or damaged proteins, and ROS trigger stress responses.  
300 Exercise duration, intensity, and the person's baseline redox state all affect how much of this  
301 response occurs. According to data from human studies, trained individuals show reduced HSP  
302 induction and quicker recovery, suggesting that stress responsiveness is modulated by prior  
303 adaptations [118,119].

304 However, the mechanistic hierarchy linking ROS sources (mitochondrial, NADPH oxidase,  
305 and xanthine oxidase) to specific HSP isoforms remains largely unresolved. Furthermore, it is  
306 still unclear which redox sensors, thioredoxin, peroxiredoxin, or glutathione-dependent  
307 systems, mediate the early signaling events leading to HSF1 activation in skeletal muscle and  
308 immune cells.

309 Future research should focus on dissecting these upstream redox–protein interactions using  
310 high-resolution proteomic and redoxomic approaches. The crosstalk between ROS signaling,  
311 HSP phosphorylation dynamics, and mitochondrial biogenesis or repair pathways (e.g., via  
312 PGC-1 $\alpha$  and sirtuins) warrants further investigation. Understanding these processes will help  
313 clarify how transient oxidative stress can shift from a potentially damaging event to a  
314 molecular trigger of cellular protection and metabolic optimization.

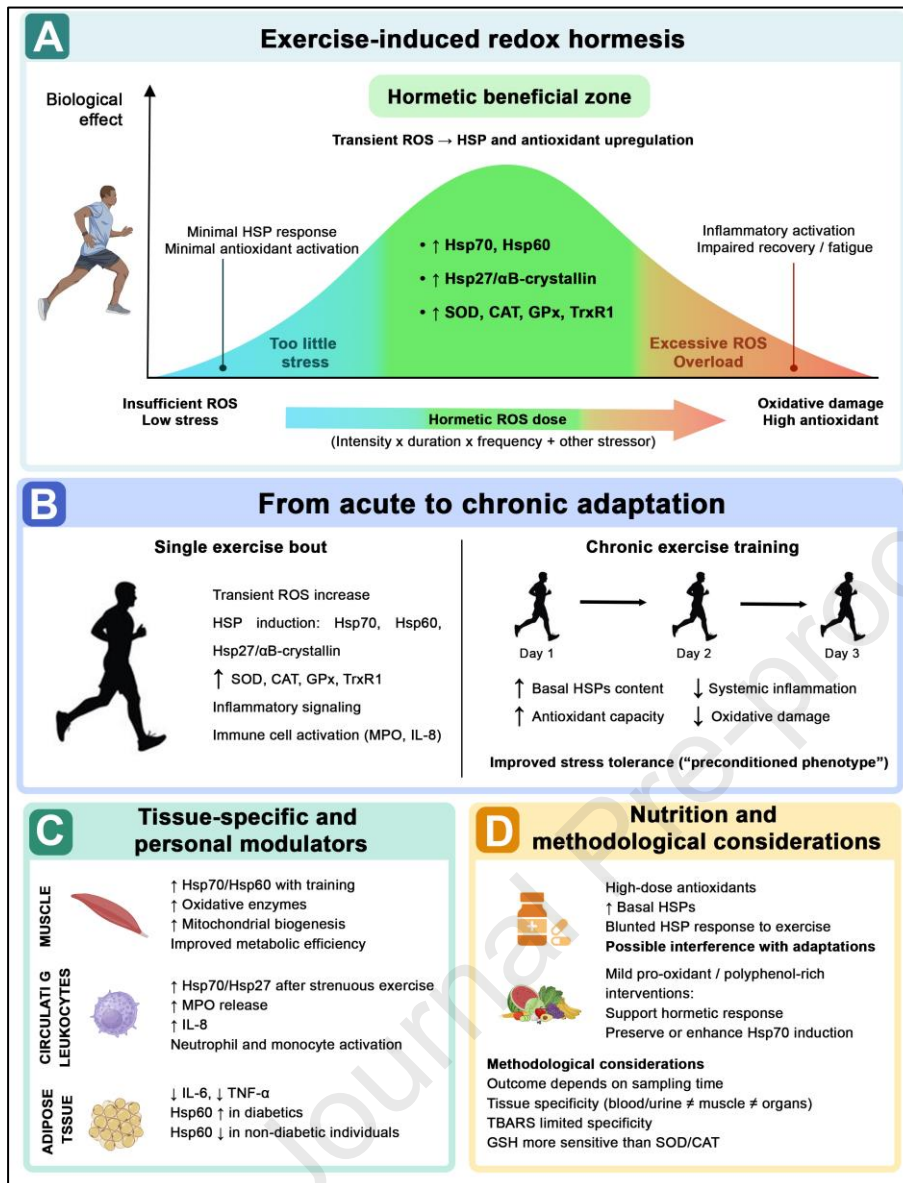


**Figure 3.** Schematic representation of exercise-induced redox hormesis and modulators of ROS–HSP responses. (A) Transient ROS generated during exercise activate a hormetic cascade promoting HSP induction (Hsp70, Hsp60, Hsp27/αB-crystallin) and antioxidant enzymes (SOD, CAT, GPx, TrxR1). Sub-threshold stress fails to activate adaptation, whereas excessive ROS elicit inflammation and oxidative damage. (B) Acute bouts trigger rapid ROS fluctuations, HSP expression, antioxidant activation and immune cell signaling, while repeated exercise leads to increased basal antioxidant capacity, lower systemic inflammation, and enhanced stress tolerance (“preconditioned phenotype”). (C) Tissue-specific and individual factors modulate these responses, including muscle-specific mitochondrial adaptations, leukocyte activation, adipose-tissue inflammatory status, training level, age, and baseline redox state. (D) Nutritional and methodological variables influence interpretation of redox-related outcomes: high-dose antioxidants can blunt HSP induction, while polyphenol-rich mild pro-oxidants may

sustain hormetic signaling. Biomarker sensitivity, tissue sampling, and timing critically affect data interpretation.

### Regular Exercise-Induced ROS: The Roles of Heat Shock Proteins (HSPs) and Antioxidants

During the last decade, several studies have suggested that ROS induction by chronic exercise could promote an adaptation process that modulates the expression of different HSPs in humans. Depending on the training status and the type and intensity of the training protocol, a close correlation has been observed between oxidative enzymes and stress protein expression in specific tissues (i.e., skeletal muscle) [120–122] and/or at the systemic level within the bloodstream (i.e., leukocytes and serum) [96,123,124]. To date, all published data seem to agree that exercise training capable of enhancing antioxidant defenses is also able to elevate the levels of specific HSPs [96,120–124] (Figure 3).

Recent findings further support this concept, showing that redox balance plays a central role in the long-term adaptations involving HSP expression. In elderly sedentary men, a 14-week concurrent (aerobic and resistance) training program significantly reduced oxidative damage markers (MDA and protein carbonyls) while increasing total antioxidant capacity (TAC) and circulating HSP70 levels, indicating that regular exercise can restore a more favorable redox status and normalize stress protein response by enhancing systemic antioxidant defense. Similarly, short-term repeated exercise stimuli trigger early redox adaptation. Dimauro et al. [125] reported that a 5-day exercise protocol induced a transient increase in PBMC expression of SOD1, HSP70, and HSP27, followed by a decline in plasma oxidative stress markers and normalization of antioxidant enzyme levels (SOD2, GPx1, TrxR1). These results suggest that even brief exercise regimens can promote biphasic redox–HSP adaptation, characterized by an early stress response followed by enhanced antioxidant efficiency and redox homeostasis.

Exercise-induced HSP modulation has also been observed in metabolic tissues. In the subcutaneous adipose tissue of obese and diabetic subjects, Khadir et al. [126] demonstrated that three months of moderate exercise decreased local inflammation (IL-6 and TNF- $\alpha$ ) and modulated HSP60 expression in a metabolic status–dependent manner, upregulated in diabetic individuals and downregulated in non-diabetic individuals, suggesting that chronic training exerts tissue-specific and redox-sensitive regulation of mitochondrial stress proteins. Further evidence has been obtained from nutritional interventions. Daneghian et al. [102] showed that a 4-week training program combined with a natural antioxidant-rich formula (ginger, cinnamon, almond, rosemary, and honey) significantly increased HSP70 mRNA

expression in lymphocytes of recreational female athletes. Despite the variable effects on oxidative stress markers such as MDA and TAC, the antioxidant formula enhanced the stress-induced chaperone response, suggesting a hormetic effect of the bioactive compounds. Kotowska et al. [127] investigated the influence of IL-6-174G/C and Hsp70 + 190G/C gene polymorphisms on redox and inflammatory responses to a 12-week swimming training in healthy men. Individuals with the GG genotype of IL-6 and Hsp70 showed lower baseline levels of TNF- $\alpha$  and HSP70, a more pronounced post-training reduction in these markers, along with decreased glutathione peroxidase (GPx) activity and lipid hydroperoxides. These findings suggest that genetic background may influence the magnitude of redox adaptation and HSP modulation, reinforcing the concept of personalized exercise prescription. Finally, nutritional antioxidant interventions may influence the magnitude of HSP induction during exercise. Harahap et al. [128] showed that acute ingestion of red dragon fruit (rich in polyphenols and betalains) before submaximal exercise attenuated post-exercise HSP70 increase compared with controls, indicating that exogenous antioxidant intake can dampen redox signaling and partially suppress the stress-induced chaperone response. These findings further emphasize the dual role of ROS as both damaging agents and essential messengers in exercise adaptation.

In conclusion, the collective evidence indicates that modulation of redox homeostasis elicited by repeated exercise stressors during training translates into physiological adaptations involving both antioxidant defense and HSP networks. Appropriate exercise protocols promote a more favorable redox balance characterized by reduced oxidative damage and controlled transient induction of HSPs, whereas chronic antioxidant supplementation or altered metabolic conditions may attenuate or reshape these adaptive responses. Genetic predisposition and targeted nutritional strategies may further enhance the efficacy of these adaptations.

### Molecular Adaptations to Chronic Exercise-Induced Stress

Chronic exercise training induces long-term reprogramming of redox-sensitive pathways, transforming acute stress responses into stable molecular adaptations [129–131] (Figure 3). Repeated bouts of ROS exposure during training promote the upregulation of endogenous antioxidant defenses (SOD, GPx, and TrxR1) and fine-tuning of HSP expression, resulting in improved redox homeostasis and enhanced cellular resilience [132,133]. This hormetic

mechanism reflects an adaptive shift toward increased antioxidant efficiency, protein quality control, and anti-inflammatory capacity (Figure 3).

At the systemic level, enhanced basal HSP70 and HSP60 expression, together with reduced oxidative damage markers, indicates the establishment of a “preconditioned” state capable of counteracting future stressors [134].

Despite consistent evidence of improved antioxidant and HSP profiles with regular training, the precise molecular intermediates linking repeated ROS signaling to stable transcriptional and epigenetic changes remain undefined. It remains unclear how chronic exercise modulates HSF1 activity over time or how mitochondrial adaptations, chaperone-assisted autophagy, and redox-sensitive kinases (e.g., AMPK, p38 MAPK, ERK1/2) integrate into a unified adaptive network. Moreover, tissue-specific responses, such as the differential regulation of HSP60 in adipose tissue or skeletal muscle, suggest complex metabolic crosstalk that depends on cellular energy status and redox tone.

Emerging data also highlight the modulatory role of genetic polymorphisms (e.g., HSPA1A and IL-6) and nutritional interventions (polyphenols and antioxidant-rich diets) in shaping redox–HSP interactions [135–137] (Figure 3). However, these effects remain inconsistent, highlighting the need for personalized approaches that consider the genetic background, training modality, and baseline oxidative phenotype.

Future studies should integrate systems biology, single-cell transcriptomics, and redox proteomics to map the full landscape of chronic exercise adaptations. Specifically, studies should aim to identify molecular “switches” that convert transient ROS–HSP signaling into long-lasting protective phenotypes. Elucidating these mechanisms could pave the way for targeted exercise-mimetic or redox-based interventions to optimize health, performance, and disease prevention.

### **Systemic adaptation to exercise-induced ROS through tissue cross-talk**

Adaptation to physical training arises from long-term compensatory mechanisms that restore homeostasis disrupted by exercise stimuli. These processes involve nearly all organ systems, underscoring the integrative and multi-level nature of physiological adaptation [138,139] [Figure 4]. Beyond localized skeletal muscle effects, exercise acts as a major regulator of systemic redox homeostasis by balancing ROS production with antioxidant defenses [18,140]. Physical activity enhances endothelial function through reduced oxidative stress and greater NO bioavailability [141], thereby improving vascular redox balance and reducing

risks of atherosclerosis and hypertension [139]. Exercise also attenuates systemic inflammation, a key contributor to redox imbalance, by downregulating pro-inflammatory cytokines (e.g., TNF- $\alpha$ , IL-6) and upregulating anti-inflammatory markers [142]. In metabolic tissues, such as the liver and adipose tissue of cachectic tumor-bearing animals, exercise mitigates lipid peroxidation and enhances insulin sensitivity through improved antioxidant capacity [143]. Moreover, exercise-induced ROS contribute to muscle hypertrophy and repair by regulating satellite cell activation and growth-related gene expression [144,145].

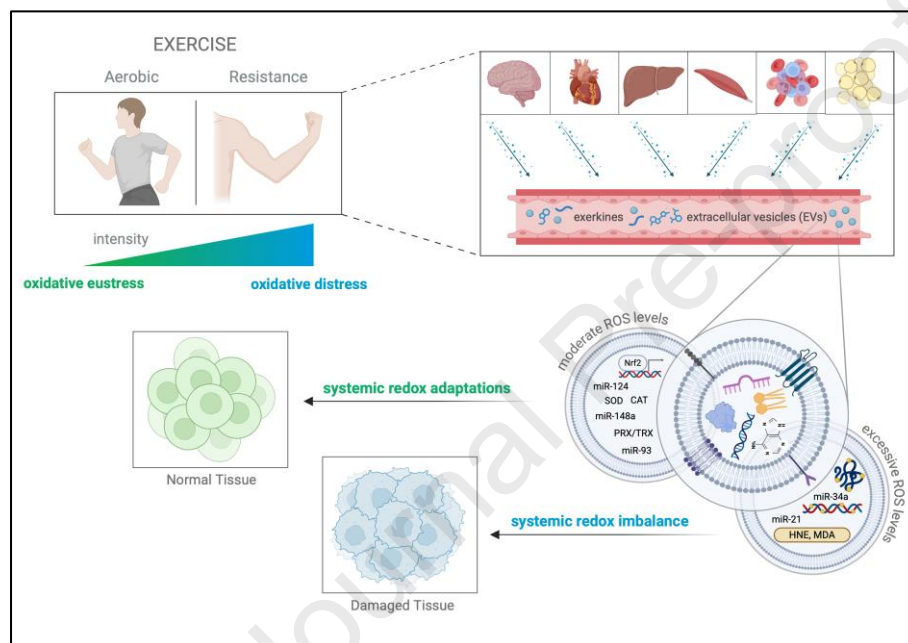


**Figure 4 - Exercise intensity-dependent ROS production and systemic redox regulation mediated by tissue cross-talk.** The figure illustrates how aerobic and resistance exercise generate varying degrees of ROS, ranging from beneficial oxidative eustress at moderate intensities to oxidative distress when ROS levels become excessive. Several tissues, including brain, heart, liver, skeletal muscle, immune cells, and adipose tissue, release exerkines and EVs into circulation. Under moderate ROS exposure, EVs carry antioxidant enzymes (SOD, CAT), stress response proteins (HSPs), redox-regulatory proteins (PRX/TRX), and protective microRNAs (miR-124, miR-148a, miR-93), contributing to systemic redox adaptations and maintenance of tissue homeostasis. In contrast, excessive ROS production leads to EVs enriched in pro-oxidant signals such as oxidized lipids (HNE, MDA) and maladaptive microRNAs (miR-21, miR-34a), promoting systemic redox imbalance and tissue dysfunction.

#### *Exercise- induced redox modulation in exerkine release*

Exercise-induced systemic adaptations collectively improve metabolic health, reduce inflammation, and enhance resistance to chronic disease. These adaptations are mediated not only by local muscle responses but also by endocrine and paracrine signaling that coordinate cross-talk among organs such as the heart, brain, liver, and adipose tissue [146]. Increasing evidence identifies exerkines and EVs as pivotal mediators of this interorgan communication, with redox biology central to their regulation and function [147,148].

Exerkines comprise metabolites, hormones, and cytokines secreted during or after exercise that act as endocrine, autocrine, and paracrine signaling molecules. They include myokines (e.g., IL-6, irisin), hepatokines (e.g., FGF21), adipokines (e.g., adiponectin), and others released from various organs [149]. Broadly, exerkines can be divided into two categories: (1) “metabolic exerkines, such as lactate, which are by-products of macronutrient metabolism and energy turnover [150], and (2) “physiologic exerkines”, including hormones, growth factors, and cytokines that act on specific tissues, cells, or organelles [151].

The term “myokine” was first introduced by Pedersen et al. (2003) [152] when IL-6 was identified as an exercise-induced cytokine. IL-6 rises acutely during exercise, enhancing glucose uptake and lipid oxidation [153]. Alongside lactate and adiponectin, IL-6 supports brain mitochondrial function, complementing the role of Brain-Derived Neurotrophic Factor (BDNF) derived from immune cells [154–156]. Another key myokine, irisin—cleaved from FNDC5—promotes mitochondrial biogenesis, dynamics, and mitophagy in adipocytes, and is associated with increased ITGA5 receptor expression in human adipose tissue, suggesting a compensatory adaptation to enhance mitochondrial efficiency and fat browning [157].

Although its detectability and precise role in humans remain under debate, accumulating evidence highlights irisin’s importance in metabolic regulation and neuroprotection, positioning it as a potential therapeutic target for metabolic and neurodegenerative disorders [158,159]. Collectively, metabolic and physiologic exerkines integrate to coordinate energy utilization, mitochondrial function, and overall systemic adaptation to physical activity.

Many exerkines exhibit redox sensitivity. IL-6 expression in skeletal muscle, for instance, is stimulated by ROS through NF- $\kappa$ B and redox-dependent p38 MAPK pathways [146,160,161]. Both NOX2 inhibition [17] and antioxidant supplementation suppress IL-6 release, confirming redox signaling as central to its regulation [162]. IL-6 subsequently acts systemically to mobilize energy substrates and modulate inflammation, linking muscle

oxidative stress with whole-body homeostasis [153]. ROS also enhance BDNF expression via CREB-dependent signaling [163–165]; thus, exercise-induced oxidative eustress in the hippocampus and peripheral tissues may elevate BDNF levels, supporting cognitive and mood improvements [110]. Irisin expression, meanwhile, is promoted by PGC-1 $\alpha$ —a redox-sensitive coactivator upregulated through mitochondrial ROS signaling—while FGF21 expression is modulated by AMPK and SIRT1 activity [166]. These mechanisms position irisin as a redox-dependent exerkine connecting muscle oxidative state with systemic energy metabolism. For more detail, see Félix-Soriano and Stanford [167].

Aging attenuates the exerkine response to exercise [151,168]. Moreover, downstream signaling via pathways such as Nrf2—a principal regulator of antioxidant gene expression—is often compromised in aged tissues, limiting redox adaptability [169]. Nonetheless, regular physical activity in older adults can improve mitochondrial efficiency, stimulate residual exerkine release, and enhance antioxidant defenses, although to a lesser extent than in younger individuals [170,171].

In conclusion, exerkines serve as pivotal mediators linking exercise-induced redox dynamics with systemic health. By supporting mitochondrial integrity, energy balance, and cellular resilience, they form the molecular bridge between muscle activity and multi-organ adaptation. Although aging diminishes exerkine responsiveness, consistent exercise can partially restore redox balance and mitigate functional decline, highlighting exerkines as promising targets for promoting healthy aging and disease prevention.

### **From exerkines to extracellular vesicles: a multi-organ network of exercise-induced communication**

Traditionally, exerkines have been described as soluble mediators released into the circulation in response to exercise [21]. However, increasing evidence indicates that a substantial fraction of these factors is packaged and secreted within extracellular vesicles [172], which protect them from enzymatic degradation, allow the co-delivery of multiple molecular species, and facilitate their selective uptake by recipient cells [173,174].

Importantly, beyond serving as carriers of exerkines, EVs represent an autonomous system of intercellular communication that shapes cellular responses by transporting a wide range of bioactive molecules (proteins, nucleic acids, lipids, and metabolites) across different tissues and organs [148,175]. EVs are small, lipid bilayer-enclosed particles released into the extracellular space by all cell types. Due to their heterogeneous nature, they are commonly

categorized into three main subtypes that differ in size, origin, and molecular composition [176]. Exosomes (30–150 nm) originate primarily from the endolysosomal system through a tightly regulated biogenetic pathway, during which the inward budding of the endosomal membrane generates multivesicular bodies (MVBs) that subsequently fuse with the plasma membrane to release their intraluminal vesicles (ILVs) [177]. Microvesicles (100–1000 nm) are shed from the plasma membrane by outward budding and fission, a process primarily driven by cytoskeletal remodeling and alterations in lipid membrane asymmetry [178]. Finally, apoptotic bodies (1000–5000 nm) arise during the late stages of apoptosis, where cell shrinkage and chromatin condensation culminate in plasma membrane blebbing and nuclear fragmentation, producing membrane-bound vesicles enriched in cytoplasmic portions, organelles, and nuclear fragments from dying cells [179]. Although this classification is widely adopted, EV subtypes partially overlap in size and composition, and current isolation techniques often fail to clearly discriminate between them and their origin; as a consequence, circulating EVs analyzed in the context of exercise usually represent mixed populations in which exosomes and microvesicles are generally regarded as the most relevant, both originating from multiple cellular and tissue sources [180]. Among them, skeletal muscle, the primary effector organ of exercise, contributes a small but functionally relevant fraction (approximately 1–5%) of circulating EVs, characterized by  $\alpha$ -sarcoglycan<sup>+</sup>/CD81<sup>+</sup> vesicles immediately released after exercise and carrying molecular mediators involved in metabolic, inflammatory, and redox regulation [181]. Brown adipose tissue (BAT) increases EV secretion with training, promoting lipid metabolism and anti-inflammatory signaling, while white adipose tissue (WAT) remains less responsive [182]. Endothelial and immune cells release vesicles that contribute to vascular adaptation, tissue repair, and immune regulation in response to exercise-induced shear stress and cell activation [183]. The liver and brain also participate, producing EVs enriched in metabolic and neurotrophic factors that support mitochondrial function, neuroplasticity, and systemic energy balance [184,185]. In addition, mesenchymal stem cells, cardiomyocytes, platelets, and antigen-presenting cells represent further contributors, emphasizing the coordinated, multi-organ nature of the EV response to exercise [186,187]. Physical activity is now widely recognized as a powerful physiological stimulus for EV secretion into the circulation, with both acute and chronic exercise regimens influencing EV biology and function. A single bout of acute exercise typically induces a rapid increase in circulating EVs, peaking during or immediately after exercise and returning to baseline within a few hours [188]. This transient effect has been consistently observed

across different aerobic modalities (cycling, running, swimming) and in both human and animal models, although the extent of EV response varies with exercise intensity and duration [189]. Fitness level and individual characteristics further modulate this phenomenon: untrained or lean individuals generally exhibit a greater EV response than trained, obese [190], or metabolically impaired subjects [191], and sex-related differences have also been reported [192]. In contrast, repeated bouts of exercise sustained over time induce more subtle and long-lasting remodeling of EV abundance and composition [193,194]. Elevated baseline levels of circulating EVs [195] carrying a more specialized cargo enriched in mediators of metabolic and immune modulation, antioxidant defense, and regenerative signalling indicate that chronic exercise promotes adaptive patterns of intercellular communication that support cellular resilience and the maintenance of tissue homeostasis [196].

#### *Redox modulation of extracellular vesicle biogenesis and cargo remodelling during exercise*

Increasing evidence indicates that the cellular redox environment critically influences EV secretion rate and molecular identity [197]. ROS/RNS and other redox-active molecules affect multiple stages of the vesiculation process, from endosomal trafficking to selective cargo loading [198]. Although exercise is a well-known modulator of redox homeostasis [199], no study to date has specifically investigated how the resulting oxidative fluctuations impact EV dynamics. Nevertheless, the molecular pathways activated during muscle contraction and those known to govern EV formation share common ROS- and calcium-dependent mechanisms [200,201]. This overlap suggests that exercise-induced redox changes act as a trigger for EV-mediated communication, dictating how cells translate oxidative signals into coordinated extracellular messages that ultimately promote systemic redox adaptations[202]. Supporting this hypothesis, a wide array of antioxidant enzymes has been identified in the EV cargo following acute or chronic exercise. Among the most consistently enriched molecules, members of the superoxide dismutase family, particularly SOD1 and SOD2, together with catalase represent core components of the EV antioxidant response. EV-mediated delivery of SOD1 during endurance exercise contributes to ROS regulation, nutrient sensing, and tissue regeneration [202], whereas SOD2 and CAT levels and activity in plasma EVs rise transiently after acute exercise and normalize with training, reflecting adaptive redox remodelling [203,204]. Similarly, endurance training elevates peroxiredoxin 1 (PRDX1) levels in circulating EVs from obese participants and in vesicles released by stimulated myotubes [205], while also increasing TRX abundance in post-exercise EVs from endurance-trained females [206]. Exercise further enhances EV content and activity of

glutathione-related enzymes, such as glutathione reductase (GR), and glutathione biosynthetic proteins, such as CNDP2, GCLM, and MGST2, which sustain the glutathione pool and overall antioxidant capacity [204]. In addition to classical antioxidants, EVs released after exercise also carry eNAMPT, a redox-regulatory enzyme crucial for NAD<sup>+</sup> biosynthesis that enhances NAD<sup>+</sup>/SIRT1 signaling in recipient tissues, promoting metabolic efficiency and redox resilience [207]. In parallel, vesicular enrichment of mitogen-activated protein kinase kinase 1 (MAP2K1) and HSP27 further highlights the role of EVs as carriers of stress-response proteins that coordinate antioxidant defense and proteostasis during exercise [23]. Beyond their protein cargo, EVs released in response to exercise also carry a repertoire of miRNAs, suggesting an additional layer of post-transcriptional regulation in systemic adaptation to oxidative stress [147]. Among them, miR-124 has emerged as a key modulator of the pro-oxidant enzyme NOX2. Its levels are altered in EVs from both endurance-trained individuals performing anaerobic-threshold exercise and from young women subjected to high-intensity interval training, supporting a role in the post-transcriptional repression of NOX2 and the downstream control of ROS production, inflammation, and neuronal integrity [208]. Similarly, miR-148a, another NOX2-targeting miRNA, is upregulated in circulating EVs from endurance-trained elderly men compared with sedentary counterparts, suggesting that enhanced expression of both miR-124 and miR-148a across ages contributes to exercise-mediated NOX2 inhibition and redox protection [209]. Exercise also increases miR-146a abundance in EVs released after both endurance and high-intensity interval training, whose elevation likely dampens NOX4-dependent ROS production and inflammation in endothelial cells, aligning with the vascular antioxidant adaptations observed with training [210]. Additional miRNAs influence the Nrf2/ARE axis, a central regulator of cellular redox homeostasis. In circulating EVs from exercised Wistar rats, the levels of miR-93 and miR-142-5p, both repressors of Nrf2 translation, are reduced, consistent with increased Nrf2 protein expression and enhanced antioxidant defense in target tissues. Likewise, miR-128, which inhibits MAFG, a transcriptional co-factor of Nrf2, is downregulated in EVs following exercise, relieving repression of the Nrf2–MAFG complex and facilitating ARE activation [22]. Interestingly, miR-128 levels rise with aging, potentially limiting this adaptive antioxidant response in older individuals [211]. Taken together, these findings indicate that exercise reshapes EV-mediated signalling toward enhanced antioxidant capacity and stress resilience, orchestrating adaptive responses across tissues.

*Redox-dependent shifts in extracellular vesicles profiles and their functional implications*

The nature of oxidative stimulus is a key determinant of the vesicular response to exercise, ultimately defining the functional phenotype of circulating EVs. Under resting physiological conditions, circulating EVs mirror the steady-state redox balance of their tissues of origin, with moderate exercise inducing controlled ROS production that fine-tunes their molecular content toward a cytoprotective phenotype [15]. *Lisi et al.* showed that, while untrained individuals display a more oxidized EV cargo reflecting a heightened pro-oxidant environment, short-term aerobic training restores a more antioxidant signature, providing clear evidence that PBMC-derived EV profiles dynamically adapt to the systemic redox environment to counteract oxidative imbalance and promote redox homeostasis [203]. Functionally, by both delivering active antioxidant enzymes and enhancing endogenous defense pathways, exercise-derived EVs exert potent protective effects in multiple target tissues. In human cardiomyocytes, treatment with post-exercise plasma EVs increased the activity of glutathione peroxidase and glucose-6-phosphate dehydrogenase (G6PD), activated the Nrf2–ARE pathway, and upregulated SOD1 and HMOX1 expression, while promoting HSP27 phosphorylation, collectively reinforcing intrinsic antioxidant defenses under both basal and stress conditions [204]. In cerebral microvascular endothelial cells, EVs isolated from chronically trained mice (8-week treadmill protocol) decreased Nox2 expression and attenuated angiotensin II- and hypoxia-induced oxidative stress, thereby limiting ROS overproduction and enhanced cellular protection against oxidative stress [212]. However, when oxidative stress exceeds the adaptive threshold, circulating EVs acquire a pro-oxidant profile, reflecting cellular distress rather than adaptation. Excessive ROS accumulation promotes the release of EVs enriched in oxidized lipids [213], carbonylated proteins [214], and stress-associated miRNAs such as miR-21 [215,216] and miR-34a [217], which further amplify oxidative damage propagation, inflammation, senescence and mitochondrial dysfunction in target tissues [218–220]. Although exhaustive exercise and overtraining are well known to elicit pronounced oxidative stress [221], the characterization of circulating EVs under these conditions remains limited. Recently, *Slusher et al.* reported that acute maximal exercise induces oxidative stress–dependent alterations in circulating EVs, particularly in individuals with obesity. In these participants, lipid peroxidation within exosome-like vesicles markedly increased, whereas glutathione levels declined, indicating a shift toward redox imbalance. Such oxidatively modified EVs were proposed to activate Toll-like receptor 4 signalling and promote NF- $\kappa$ B–dependent inflammatory responses, linking

excessive exercise-induced ROS generation to maladaptive vesicular signalling [222]. Collectively, these observations highlight the hormetic nature of EV-mediated redox signaling, whereby mild and transient ROS act as beneficial signals, while chronic or excessive oxidative stress converts EVs into vectors of cellular injury. Understanding how exercise intensity, redox load, and training status shape this vesicular phenotype is crucial to harness EV-mediated signaling for both health and performance [53]. In this context, the molecular signatures carried by circulating EVs may represent a powerful and minimally invasive tool to monitor systemic redox balance, detect early transitions to oxidative stress-related dysfunctions and guide individualized exercise strategies [223].

## Conclusion and Future Perspectives

The role of reactive oxygen species in exercise biology has shifted from a simplistic view of oxidative damage toward a nuanced understanding of redox signaling as a fundamental determinant of adaptation. Exercise-induced ROS, once considered mere by-products of metabolism, are now recognized as central regulators of cellular and systemic remodeling. The extent, localization, and temporal pattern of ROS generation critically determine whether their effects are detrimental or beneficial. Within this redox-sensitive continuum, the dynamic interplay between ROS, antioxidant systems, and stress response proteins such as heat shock proteins (HSPs) emerges as a key mechanism preserving proteostasis, mitochondrial integrity, and metabolic flexibility. Regular exercise fine-tunes this network, fostering a resilient redox phenotype characterized by enhanced antioxidant capacity, reduced basal oxidative stress, and improved stress tolerance.

The impact of ROS extends beyond the contracting muscle, encompassing systemic effects mediated through circulating factors such as exerkines and extracellular vesicles. These redox-modulated mediators enable inter-organ communication, linking skeletal muscle activity with cardiovascular, metabolic, immune, and neural adaptations. The integration of redox biology within this multi-organ signaling network provides a mechanistic basis for the broad health benefits of physical activity, including improved vascular function, metabolic homeostasis, and neuroprotection. Nonetheless, the influence of aging, genetic polymorphisms, and nutritional factors on the redox-HSP-exerkine axis remains incompletely defined, underscoring the need for individualized approaches to exercise and antioxidant modulation.

Future investigations should aim to elucidate the molecular mechanisms by which transient ROS signals are translated into long-term adaptive responses. Advanced methodologies integrating redox proteomics, metabolomics, and single-cell transcriptomics will be essential to delineate the spatiotemporal landscape of redox regulation in response to exercise. Particular attention should be directed toward the redox-dependent remodeling of EV cargo, the role of mitochondrial-derived ROS in epigenetic reprogramming, and the identification of redox-sensitive molecular switches governing stress resilience. Understanding how exercise variables—intensity, duration, and recovery—shape these pathways across physiological and pathological contexts will be crucial for optimizing the balance between oxidative stress and adaptive signaling.

In conclusion, the evolving redox perspective in exercise biology establishes ROS not as mere agents of damage but as essential modulators of health and adaptation.

Recognizing their dual nature provides a conceptual framework to explain how regular physical activity maintains redox homeostasis, promotes systemic resilience, and contributes to the prevention of chronic diseases. Continued interdisciplinary research integrating molecular, physiological, and translational approaches will be pivotal to harness the full therapeutic potential of exercise as a redox-based intervention for human health and longevity.

#### **Credit authorship contribution statement**

**Luara Sireno:** Writing – original draft, Investigation, Visualization. **Ivan Dimauro:** Writing – review & editing, Writing – original draft, Investigation, Visualization, Conceptualization. **Daniela Caporossi:** Writing – review & editing, Writing – original draft, Investigation, Visualization, Conceptualization.

#### **Conflicts of interest**

The authors have no conflicting interests to disclose.

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716 List of abbreviations

|     |                  |                                                            |
|-----|------------------|------------------------------------------------------------|
| 717 | 4-HNE:           | 4-Hydroxynonenal,                                          |
| 718 | AMPK             | AMP-activated protein kinase                               |
| 719 | AOPP             | Advanced Oxidation Protein Products                        |
| 720 | ARE              | Antioxidant Response Element                               |
| 721 | BAT              | Brown Adipose Tissue                                       |
| 722 | BDNF             | Brain-Derived Neurotrophic Factor                          |
| 723 | CAT              | Catalase                                                   |
| 724 | CNDP2            | Carnosine Dipeptidase 2                                    |
| 725 | CREB             | cAMP Response Element-Binding Protein                      |
| 726 | eNAMPT           | Extracellular Nicotinamide Phosphoribosyltransferase       |
| 727 | eNOS             | Endothelial Nitric Oxide Synthase                          |
| 728 | ERK1/2           | Extracellular Signal-Regulated Kinases 1 and 2             |
| 729 | EVs              | Extracellular vesicles                                     |
| 730 | FGF21            | Fibroblast Growth Factor 21                                |
| 731 | FNDC5            | Fibronectin Type III Domain Containing 5                   |
| 732 | GCLM             | Glutamate-Cysteine Ligase Modifier subunit                 |
| 733 | GPx              | Glutathione Peroxidase                                     |
| 734 | GR               | Glutathione Reductase                                      |
| 735 | GSH              | Glutathione                                                |
| 736 | GSSG             | Oxidized Glutathione                                       |
| 737 | HMOX1            | Heme Oxygenase 1                                           |
| 738 | Hsc70            | Heat Shock Cognate protein 70, gene                        |
| 739 | HSF1             | Heat Shock Transcription Factor 1                          |
| 740 | Hsp60            | Heat Shock Protein 60, gene                                |
| 741 | Hsp70            | Heat Shock Protein 70, gene                                |
| 742 | Hsp72            | Heat Shock Protein 72, gene                                |
| 743 | HSP27            | Heat Shock Protein 27                                      |
| 744 | HSP60            | Heat Shock Protein 60                                      |
| 745 | HSP70            | Heat Shock Protein 70                                      |
| 746 | HSPs             | Heat Shock Proteins                                        |
| 747 | IL-8             | Interleukine-8                                             |
| 748 | IL-6             | Interleukine-6                                             |
| 749 | ILVs             | Intraluminal Vesicles                                      |
| 750 | ITGA5            | Integrin Subunit Alpha 5                                   |
| 751 | MAFG             | MAF BZIP Transcription Factor G                            |
| 752 | MAP2K1           | Mitogen-Activated Protein Kinase kinase 1                  |
| 753 | MDA              | Malondialdehyde                                            |
| 754 | MGST2            | Microsomal Glutathione S-transferase 2                     |
| 755 | MnSOD            | Manganese Superoxide Dismutase                             |
| 756 | MPO              | Myeloperoxidase                                            |
| 757 | MVBs             | Multivesicular Bodies                                      |
| 758 | NAC              | N-acetyl cysteine                                          |
| 759 | NAD <sup>+</sup> | Nicotinamide Adenine Dinucleotide                          |
| 760 | NADPH            | Nicotinamide Adenine Dinucleotide Phosphate (reduced form) |

|     |                |                                                                      |
|-----|----------------|----------------------------------------------------------------------|
| 761 | NF- $\kappa$ B | Nuclear factor kappa-light-chain-enhancer of activated B cells       |
| 762 | NO             | Nitric Oxide                                                         |
| 763 | NOX2           | NADPH oxidase 2                                                      |
| 764 | NOX4           | NADPH oxidase 4                                                      |
| 765 | Nrf2           | Nuclear Factor Erythroid 2-Related Factor 2                          |
| 766 | p38 MAPK       | p38 Mitogen Activated Protein Kinase                                 |
| 767 | PA             | Physical Activity                                                    |
| 768 | PGC-1 $\alpha$ | Peroxisome proliferator-activated receptor gamma coactivator 1-alpha |
| 769 | PRDX1          | Peroxiredoxin 1                                                      |
| 770 | PRX            | Peroxiredoxin protein family                                         |
| 771 | ROS            | Reactive Oxygen Species                                              |
| 772 | SIRT1          | Sirtuin 1                                                            |
| 773 | SOD            | Superoxide Dismutase                                                 |
| 774 | TAC            | Total Antioxidant Capacity                                           |
| 775 | TBARS          | Thiobarbituric Acid Reactive Substances                              |
| 776 | TEAC           | Trolox Equivalent Antioxidant Capacity                               |
| 777 | TNF- $\alpha$  | Tumor Necrosis Factor alpha                                          |
| 778 | TRX            | Thioredoxin protein family                                           |
| 779 | TrxR1          | Thioredoxin Reductase 1                                              |
| 780 | WAT            | White Adipose Tissue                                                 |

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## UNIVERSITÀ DEGLI STUDI DI ROMA "FORO ITALICO"

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To whom it might concerns

I hereby confirm no potential competing financial or non-financial interests on behalf of all authors of the manuscript.

A handwritten signature in black ink, appearing to read 'Caporossi', followed by a horizontal line.

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**Small extracellular vesicles biogenesis and release in a murine model of proliferating and differentiated skeletal muscle cells: role of redox modulation.**

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**Abstract**

Physical exercise is among the most potent physiological stimuli promoting skeletal muscle adaptation and systemic health, largely through the activation of signalling pathways that preserve redox homeostasis. In addition to its contractile and metabolic functions, skeletal muscle operates as a secretory organ, communicating with distant tissues via the release of soluble factors and extracellular vesicle (EV)–associated cargo. Accumulating evidence indicates that exercise-induced reactive oxygen species (ROS) in muscle tissue act as essential signalling molecules that orchestrate redox-dependent adaptations; however, whether these redox cues directly regulate EV biogenesis and secretion remains unclear. In this study, we investigated whether oxidative eustress modulates EV production in skeletal muscle cells, with particular emphasis on small extracellular vesicles (sEVs, exosome-like) biogenesis and release.

Murine C2C12 myoblasts and myotubes were employed as in vitro models of proliferative and differentiated muscle cells and exposed to low, non-cytotoxic concentrations of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to mimic the transient ROS increase that occurs during exercise. Gene expression analyses revealed that H<sub>2</sub>O<sub>2</sub> induces a coordinated transcriptional reprogramming of multiple components of the endosomal sorting complex required for transport (ESCRT) machinery and the tetraspanin family, indicating redox-sensitive regulation of sEV biogenesis pathways. Consistent with these

findings, nanoparticle tracking analysis (NTA) demonstrated that H<sub>2</sub>O<sub>2</sub> modulates sEV release in a cell state-dependent manner without affecting vesicle size distribution. Western blot analysis confirmed the enrichment of canonical EV markers (ALIX, TSG101, CD81) in isolated vesicles, supporting their endosome-derived origin.

Collectively, these results demonstrate that physiological redox signals regulate exosome-like vesicle production in skeletal muscle cells and identify H<sub>2</sub>O<sub>2</sub> as a key modulator of vesicular trafficking. By linking cellular redox status to intercellular communication, this mechanism may contribute to the systemic adaptations induced by physical exercise.

**Keywords:** skeletal muscle, redox signalling, reactive oxygen species, extracellular vesicles, exosome biogenesis.

## Introduction

Skeletal muscle (SkM) is the largest organ in the human body, accounting for approximately 40% of body weight in lean individuals, and has traditionally been regarded as an organ primarily responsible for posture maintenance, locomotion, force generation, and the regulation of whole-body energy balance [1]. Over the past two decades, however, growing evidence has revealed that skeletal muscle also functions as a secretory organ capable of producing and releasing hundreds of bioactive molecules that act in autocrine, paracrine, or endocrine manners [2]. These products include soluble factors such as cytokines and peptides—collectively termed myokines [3], or exerkines when released in response to exercise from different tissues [4]—as well as extracellular vesicles (EVs) [5].

EVs are small, lipid membrane-bound vesicles released by virtually all cell types into the extracellular space [6]. Due to their heterogeneous nature, they have traditionally been classified into three main subtypes—exosomes, microvesicles, and apoptotic bodies—distinguished by differences in size, biogenesis, and composition [7]. Exosomes (30–150 nm) originate primarily from the endolysosomal system through a tightly regulated pathway in which intraluminal vesicles (ILVs) form within multivesicular bodies (MVBs) that subsequently fuse with the plasma membrane [8]. Microvesicles (100–1000 nm) are generated by direct budding from the plasma membrane [9], whereas apoptotic bodies (1000–5000 nm) arise from membrane blebbing during the terminal stages of apoptosis [10]. Given the heterogeneity of EVs and the complexity of defining their biogenesis, the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines recommend classifying EVs using operational criteria based on (1) size, distinguishing small EVs (sEVs, <200 nm) from large EVs (lEVs, >200 nm), (2) biochemical composition, and (3) cell of

69 origin [7]. EVs facilitate the transfer of a wide range of bioactive molecules—including proteins,  
70 nucleic acids (DNA, RNA, and microRNA), lipids, and metabolites—from donor cells to both  
71 proximal and distal recipient cells [11], thereby playing a pivotal role in maintaining local and  
72 systemic homeostasis. When selectively packaged within EVs, this cargo reflects the physiological  
73 or pathological state of the cell of origin and influences target cell behaviour by modulating gene  
74 expression, signal transduction, and regulatory responses [12].

75 Although skeletal muscle contributes only a small proportion (approximately 1–5%) of circulating  
76 EVs [13], its involvement is multifaceted and derives from a diverse array of cellular sources  
77 [14,15]. Myogenic cells at different stages of differentiation, including satellite cells [16], myoblasts  
78 and myotubes [17], as well as distinct muscle fiber types (I, IIa, and IIx) [18], collectively shape the  
79 SkM-derived EV landscape, giving rise to vesicles with distinct molecular profiles and biological  
80 activities [19]. Importantly, both physiological and pathological states of skeletal muscle markedly  
81 influence EV production, cargo composition, and function, highlighting their potential dual role as  
82 mediators of adaptation and biomarkers for disease detection [20]. In this context, physical exercise  
83 represents one of the most potent physiological stimuli modulating EV secretion rates and  
84 composition [21]. Several studies have demonstrated that both acute and chronic exercise induce the  
85 release of EVs enriched with bioactive molecules that promote tissue regeneration [22,23], improve  
86 redox homeostasis, and enhance energy metabolism [23], thereby contributing to overall health.

87 Despite substantial advances in EV biology, the regulatory mechanisms controlling EV production  
88 and secretion in skeletal muscle cells in response to exercise remain incompletely understood.

89 During aerobic exercise, contracting muscle fibers experience moderate and transient increases in  
90 ROS levels [24]. These molecules, primarily generated by NADPH oxidases (NOXs),  
91 mitochondria, xanthine oxidase (XO), and other oxidant-producing systems, function as critical  
92 second messengers rather than mere by-products of metabolism, orchestrating adaptive responses  
93 through redox-sensitive signalling pathways [25]. Physiological levels of ROS, particularly  
94 hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), activate kinases and transcriptional regulators such as AMPK, p38  
95 MAPK, ERK1/2, Nrf2, and PGC-1 $\alpha$ , which coordinate gene expression programs involved in  
96 mitochondrial biogenesis, angiogenesis, myogenesis, antioxidant defence, and metabolic  
97 remodelling [26]. Notably, recent evidence has shown that pharmacological activation of the  
98 mitochondrial electron transport chain (ETC) enhances EV release via a ROS-dependent  
99 mechanism, whereas antioxidant treatment or inhibition of Complex I abolishes this effect [27].

100 These findings identify ROS as potential regulators of vesicular trafficking and establish a  
101 mechanistic link between cellular redox status and EV secretion, suggesting that exercise-induced  
102 redox changes in muscle tissue may act upstream of the EV biogenesis machinery.

103 In this study, we investigated whether oxidative eustress in skeletal muscle cells regulates the  
104 expression of genes involved in exosome biogenesis and modulates the release of small EVs, which  
105 likely represent the predominant EV subtype secreted by skeletal muscle cells [19], [28]. To this  
106 end, we employed C2C12 myoblasts and myotubes, a well-established in vitro model of  
107 proliferative and differentiated muscle cells, whose redox modulation recapitulates key features of  
108 exercise-induced oxidative eustress in striated muscle tissues [29,30]. Cells were exposed to low,  
109 supra-physiological but non-cytotoxic concentrations of H<sub>2</sub>O<sub>2</sub> to mimic transient redox  
110 perturbations elicited by a single bout of exercise. Analyses were performed at multiple time points  
111 following H<sub>2</sub>O<sub>2</sub> exposure to assess markers of pro-oxidant environments, the expression of genes  
112 associated with the endosomal sorting complex required for transport (ESCRT) machinery and the  
113 tetraspanin family, as well as sEV release from both myoblasts and myotubes into the culture  
114 medium at baseline and 24 hours post-exposure. Collectively, the findings provide new insights into  
115 the impact of H<sub>2</sub>O<sub>2</sub>-induced oxidative eustress on EV dynamics in skeletal muscle cells and support  
116 a potential role for SkM-derived EVs in sensing and propagating adaptive signals that contribute to  
117 exercise-induced systemic redox homeostasis.

118

## 119 **Materials and Methods**

### 120 *Cell culture and differentiation*

121 C2C12 myoblasts (ATCC® CRL-1772™) were maintained in high-glucose Dulbecco's Modified  
122 Eagle's Medium (DMEM; 4.5 g/L glucose; Sial) supplemented with 10% fetal bovine serum (FBS;  
123 Capricorn Scientific), 1% penicillin–streptomycin, and 2 mM L-glutamine. Cells were cultured in a  
124 humidified incubator at 37 °C with 5% CO<sub>2</sub> and routinely passaged at approximately 70–80%  
125 confluence. Myogenic differentiation was induced by replacing the growth medium with  
126 differentiation medium consisting of DMEM supplemented with 2% horse serum (Capricorn  
127 Scientific), as previously described [29]. Medium was renewed every 48 hours and multinucleated  
128 myotube formation was monitored by phase-contrast microscopy. Fully differentiated myotubes were  
129 obtained after 6 days, as confirmed by morphological and molecular analyses (Figure 1A–C).  
130 Differentiation was marked by reduced expression of Myogenic Factor 5 (Myf5) gene and increased  
131 levels of myoblast determination protein 1 (MYOD1) and myogenin (MYOG) genes, reflecting  
132 progression from early myogenesis to terminal differentiation. These transcriptional changes were  
133 supported by elevated protein levels of myogenin (MyoG), desmin, and myosin heavy chain (MyHC),  
134 confirming the establishment of a mature myotube phenotype.

135 For extracellular vesicle (EV) isolation and EV-related analyses, FBS and horse serum were depleted  
136 of serum-derived EVs prior to use to avoid confounding effects [31]. Serum was pre-cleared by

centrifugation at  $2,000 \times g$  for 20 min to remove cellular debris and large particles, followed by ultracentrifugation at  $110,000 \times g$  for 18 h at 4 °C using an SW41 Ti rotor (Beckman Coulter, Brea, CA, USA) to eliminate small EVs. The resulting supernatant was filtered through a 0.2 µm membrane and stored at 4 °C until use. Myoblasts and myotubes were subsequently adapted for 24 h in media containing EV-depleted serum prior to experimental treatments.

#### *H<sub>2</sub>O<sub>2</sub> treatments and cell viability assays*

Cells were exposed to increasing concentrations of H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich, St. Louis, MO, USA) for 1 h to reproduce the transient pro-oxidant environment elicited by an acute bout of exercise in mice [29,30]. Fresh working solutions were prepared immediately before use from a 30% stock solution diluted in sterile phosphate-buffered saline (PBS; Sial) and subsequently added to the culture medium to achieve the desired final concentrations. Myoblasts were treated with 50, 100, 300, and 500 µM H<sub>2</sub>O<sub>2</sub>, whereas myotubes were exposed to 100, 300, 500, and 1000 µM H<sub>2</sub>O<sub>2</sub>; control cells were maintained in DMEM containing vehicle alone. Following treatment, the culture medium was replaced, and cells were allowed to recover for the indicated time points prior to sample collection. Cell viability following H<sub>2</sub>O<sub>2</sub> exposure was evaluated using both Trypan Blue exclusion and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. For Trypan Blue analysis, cells were detached with 0.05% trypsin–EDTA, resuspended in PBS, and mixed 1:1 with a 0.4% Trypan Blue solution (Gibco). Viability was expressed as the percentage of viable cells relative to the total cell number. Cellular metabolic activity was assessed using the (MTT) assay (Promega, Madison, WI, USA), according to the manufacturer’s instructions. Absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Results were normalized to vehicle-treated controls and expressed as relative viability values.

#### *Protein extraction and Western Blot*

Total proteins from C2C12 cells were lysed in ice-cold RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS; pH 7.4) supplemented with a complete protease and phosphatase inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined by a colorimetric assay using the Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific), according to the manufacturer’s instructions. Equal amounts of protein (20–25 µg per lane) were resolved on 10–12% SDS–polyacrylamide gels (Bio-Rad) and transferred onto nitrocellulose membranes (Amersham).

Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and incubated overnight at 4 °C with the appropriate primary antibodies diluted in

171 blocking buffer. After three washes in TBS-T (10 min each), membranes were incubated with  
172 horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature.  
173 Immunoreactive bands were visualized using an enhanced chemiluminescence substrate (Santa Cruz)  
174 and acquired with a LAS imaging system. Densitometric analysis was performed using ImageJ  
175 software (NIH, Bethesda, MD, USA), and results were expressed as arbitrary densitometric units  
176 relative to control samples. A complete list of primary and secondary antibodies, including source,  
177 catalogue number, host species, and working dilution, is provided in Table 1.

178

#### 179 *RNA extraction and RT-qPCR*

180 Total RNA was extracted from C2C12 myoblasts and myotubes using TRIzol™ reagent (Invitrogen,  
181 Carlsbad, CA, USA) in accordance with the manufacturer's instructions. RNA concentration and  
182 purity were determined spectrophotometrically by measuring absorbance at 260 and 280 nm using a  
183 NanoDrop 2000 system (Thermo Fisher Scientific). Gene expression was quantified by one-step  
184 reverse transcription quantitative PCR (RT-qPCR) using the Luna® Universal One-Step RT-qPCR  
185 Kit (New England Biolabs, Ipswich, MA, USA). Each reaction was performed in quadruplicate in a  
186 final volume of 20 µL containing 5 ng of total RNA, 0.4 µM of each primer, and Luna Universal  
187 One-Step Reaction Mix.

188 Real-time RT-qPCR was carried out on a 7500 Real-Time PCR System (Applied Biosystems, Life  
189 Technologies) under the following cycling conditions: reverse transcription at 55 °C for 10 min,  
190 initial denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 10 s and  
191 annealing/extension at 60 °C for 30 s. Primers were designed using the Primer-BLAST tool (NCBI)  
192 and synthesized by Sial. Primer specificity was confirmed by melt-curve analysis of the amplified  
193 products. Gene expression levels were normalized to GAPDH. Relative expression was calculated  
194 using the  $2^{-\Delta\Delta C_t}$  method and expressed as fold change relative to control samples. Primer  
195 sequences used for amplification are listed in Table 2.

196

#### 197 *Protein carbonylation (OxyBlot assay)*

198 Protein carbonylation was assessed using the OxyBlot Protein Oxidation Detection Kit (Merck  
199 Millipore, Darmstadt, Germany) in accordance with the manufacturer's instructions. Briefly,  
200 derivatized proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes,  
201 which were subsequently probed with the anti-dinitrophenyl (DNP) antibody provided in the kit.  
202 Immunoreactive bands were detected by enhanced chemiluminescence using a LAS imaging system  
203 and quantified by densitometric analysis with ImageJ software. Signals were normalized to total  
204 protein loading, assessed by Ponceau S staining, and expressed as arbitrary units relative to control

205 samples.

206

#### 207 *Isolation, purification and characterization of extracellular vesicles from culture medium*

208 Small extracellular vesicles (sEVs) were isolated from conditioned media collected from equivalent  
209 numbers of producer cells (average  $3.0 \times 10^7$  cells per condition), all maintained in identical  
210 volumes of collection medium for 24 h. The isolation protocol was performed in accordance with  
211 the guidelines of the International Society for Extracellular Vesicles (ISEV) [7]. Briefly, conditioned  
212 media were centrifuged at  $290 \times g$  for 7 min to remove intact cells, followed by a second  
213 centrifugation at  $2,000 \times g$  for 20 min to eliminate residual cellular debris. The resulting  
214 supernatants were then subjected to differential ultracentrifugation. Medium and large EVs  
215 (m/lEVs) were pelleted at  $15,000 \times g$  for 20 min at 4 °C, after which the supernatant was further  
216 ultracentrifuged at  $100,000 \times g$  for 2 h 30 min to isolate small EVs.

217 The resulting pellets were kept on ice for 15 min, gently resuspended in sterile phosphate-buffered  
218 saline (PBS), and washed by an additional ultracentrifugation step at  $110,000 \times g$  for 2 h 30 min at 4  
219 °C. Purified sEVs were finally resuspended in 50–150 µL of sterile PBS and stored at 4 °C for  
220 short-term use (up to one week) or lysed and stored at –80 °C for long-term preservation and  
221 downstream analyses.

222 The particle concentration and size distribution of isolated sEVs were determined by nanoparticle  
223 tracking analysis (NTA) using a NanoSight NS300 instrument (Malvern Panalytical,  
224 Worcestershire, UK) equipped with a 532 nm laser module. Samples derived from three  
225 independent isolations were analysed under identical acquisition settings. Measurements were  
226 performed at a camera level of 15, with five 60 s videos recorded per sample. Instrument calibration  
227 parameters and detection thresholds were maintained constant throughout all acquisitions to ensure  
228 data comparability. Prior to analysis, samples were diluted 1:50–1:500 in sterile, 0.22 µm-filtered  
229 phosphate-buffered saline (PBS) to achieve particle concentrations within the optimal range for  
230 accurate tracking. Data were processed using NanoSight NTA software (version X.X), and results  
231 were expressed as particle size distribution and particle concentration (particles/mL).

232 Total sEV protein content was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher  
233 Scientific, Waltham, MA, USA) according to the manufacturer's instructions. All ultracentrifugation  
234 steps were performed at 4 °C using an SW41 Ti rotor (Beckman Coulter, Brea, CA, USA).

235

#### 236 *Statistical Analysis*

237 All data are presented as mean ± standard deviation (SD) from at least three independent biological  
238 replicates. Statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad

239 Software, San Diego, CA, USA). Differences between two groups were assessed using an unpaired  
240 two-tailed Student's *t*-test, whereas comparisons among multiple groups were analyzed by one-way  
241 or two-way analysis of variance (ANOVA), followed by Tukey's or Dunnett's post hoc tests, as  
242 appropriate. A *p* value < 0.05 was considered statistically significant.

243

## 244 **Results**

245

### 246 *Characterization of C2C12 responses to H<sub>2</sub>O<sub>2</sub>-induced eustress*

247 Based on consolidated data from our laboratory [32,33], distinct concentration ranges were applied  
248 for 1 h to the two cell types (0–50–100–300–500  $\mu$ M for proliferating myoblasts and 0–100–300–  
249 500–1000  $\mu$ M for differentiated myotubes) to identify the range of H<sub>2</sub>O<sub>2</sub> concentrations reflecting  
250 supraphysiological conditions (eustress range) (Figures 2 and 3).

251 In C2C12 myoblasts, a 1 h exposure to exogenous H<sub>2</sub>O<sub>2</sub> induced a concentration- and time-  
252 dependent reduction in cell viability and metabolic activity, assessed after 12 and 24 h of recovery,  
253 with significant decreases observed at 300 and 500  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Figures 2A–B). Notably, cells  
254 exposed to 300  $\mu$ M partially recovered viability and metabolic activity after 24 h, whereas those  
255 treated with 500  $\mu$ M remained markedly impaired. Western blot analysis showed a rapid, dose-  
256 dependent increase in p38 MAPK phosphorylation immediately after treatment (Figure 2C).  
257 Markers of oxidative damage indicated a selective vulnerability of lipid components, as levels of 4-  
258 HNE-modified proteins increased significantly at 24 h following exposure to 500  $\mu$ M H<sub>2</sub>O<sub>2</sub>, while  
259 protein carbonylation remained largely unchanged across most conditions (Figures 2D–E).

260 In myotubes, metabolic activity assessed by the MTT assay after 12 h of recovery showed a modest  
261 increase at 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, followed by a significant decline at 500  $\mu$ M and 1 mM. In contrast, after  
262 24 h of recovery, metabolic activity remained substantially reduced only at 1 mM (Figure 3A).

263 Western blot analysis revealed a dose-dependent increase in ERK1/2 phosphorylation at all H<sub>2</sub>O<sub>2</sub>  
264 concentrations tested, with the strongest activation observed at 500  $\mu$ M and 1 mM (Figure 3B).

265 Consistent with the findings in myoblasts, oxidative damage in myotubes predominantly affected  
266 lipids: accumulation of 4-HNE-modified proteins was evident after 24 h at 500 and 1000  $\mu$ M H<sub>2</sub>O<sub>2</sub>,  
267 whereas protein carbonylation levels remained unchanged or slightly decreased at the highest  
268 concentration tested (Figures 3C–D).

269 Collectively, these data indicate that a 1 h exposure of C2C12 myoblasts to 50–100  $\mu$ M H<sub>2</sub>O<sub>2</sub> and of  
270 C2C12 myotubes to 300–500  $\mu$ M H<sub>2</sub>O<sub>2</sub> effectively triggered redox-responsive signalling pathways  
271 with minimal induction of lipid and protein oxidative modifications, respectively, and without  
272 substantial impairment of cell viability or metabolic activity. These H<sub>2</sub>O<sub>2</sub> concentrations were

273 therefore selected as optimal for inducing measurable oxidative responses without overt  
274 cytotoxicity.

275

276 *Comparative transcriptional profiling of exosome-like biogenesis pathway in C2C12 myoblasts*  
277 *challenged with H<sub>2</sub>O<sub>2</sub> eustress.*

278 To determine whether physiological concentrations of H<sub>2</sub>O<sub>2</sub> modulate small extracellular vesicle  
279 (sEV) exosome-like biogenesis, we analysed the expression of genes involved in distinct stages of  
280 the vesiculation process—multivesicular body (MVB) formation (TSG101, CD9, CD63, CD81),  
281 intracellular trafficking (RAB7, RAB11, RAB35), and docking at the plasma membrane (VAMP3,  
282 VAMP5, VAMP7, SNAP23)—in myoblasts (Figure 4) and myotubes (Figure 5) at 6, 12, and 24 h  
283 following a 1 h exposure to H<sub>2</sub>O<sub>2</sub>.

284 Overall, acute H<sub>2</sub>O<sub>2</sub> exposure in myoblasts induced a transient modulation of most genes,  
285 predominantly activation, which was mainly evident at 12 h with the higher pro-oxidant  
286 concentration and returned to basal levels by 24 h after treatment.

287 Among genes involved in MVB formation, FLOT1, CD9, CD63, and CD81 expression increased at  
288 12 h following exposure to 100 µM H<sub>2</sub>O<sub>2</sub>. In contrast, TSG101 was not upregulated at any  
289 concentration or time point but was transiently downregulated at 6 and 12 h (Figure 4A).

290 A comparable pattern was observed for genes involved in MVB trafficking (RAB7, RAB11, and  
291 RAB35). Notably, RAB35 exhibited a biphasic response, with increased expression at 6 h following  
292 treatment with 50 µM H<sub>2</sub>O<sub>2</sub>, decreased expression at the same time point with 100 µM, and a  
293 subsequent increase at 12 h under 100 µM exposure; expression levels returned to control values by  
294 24 h (Figure 4B).

295 Genes associated with MVB docking displayed heterogeneous regulation. VAMP3 and VAMP5  
296 were upregulated at 12 h and/or 6 h, respectively, but were reduced below control levels at 24 h.  
297 VAMP7 expression increased significantly only at 6 h following treatment with 50 µM H<sub>2</sub>O<sub>2</sub> and  
298 returned to basal levels thereafter, whereas SNAP23 was upregulated at both 6 and 12 h in response  
299 to 50 and 100 µM H<sub>2</sub>O<sub>2</sub>, respectively, and normalized by 24 h (Figure 4C).

300 Thus, acute physiological H<sub>2</sub>O<sub>2</sub> exposure transiently activates key genes involved in sEV biogenesis  
301 in myoblasts without sustained transcriptional reprogramming.

302

303 *Comparative transcriptional profiling of exosome-like biogenesis pathway in C2C12 myotubes*  
304 *challenged with H<sub>2</sub>O<sub>2</sub> eustress.*

305 In myotubes, genes involved in MVB formation exhibited both shared and gene-specific expression  
306 patterns in response to H<sub>2</sub>O<sub>2</sub> treatment. A first group, comprising CD81 and FLOT1, showed early to

intermediate downregulation followed by partial or concentration-dependent recovery. Both genes were significantly reduced at 12 h following H<sub>2</sub>O<sub>2</sub> exposure; expression returned to baseline at 24 h with 300  $\mu$ M H<sub>2</sub>O<sub>2</sub>, whereas suppression persisted under the 500  $\mu$ M condition. A second response pattern was observed for TSG101 and CD9, which displayed transient repression followed by late recovery or overcompensation. TSG101 expression was unchanged at 6 h, significantly reduced at 12 h with 300  $\mu$ M H<sub>2</sub>O<sub>2</sub>, and restored by 24 h; notably, treatment with 500  $\mu$ M resulted in a significant upregulation at 24 h. Similarly, CD9 expression decreased at early time points, remained reduced at 12 h with both concentrations, but normalized at 24 h with 300  $\mu$ M and increased significantly with 500  $\mu$ M H<sub>2</sub>O<sub>2</sub>. In contrast, CD63 exhibited a distinct regulatory profile characterized by delayed induction followed by suppression: its expression was unchanged at 6 h, significantly upregulated at 12 h in response to 500  $\mu$ M H<sub>2</sub>O<sub>2</sub>, and markedly reduced at 24 h. Overall, these data indicate that MVB formation genes share a general tendency toward early or intermediate downregulation following H<sub>2</sub>O<sub>2</sub> exposure, while diverging at later time points in a concentration-dependent manner, with some genes recovering baseline expression and others exhibiting sustained repression or late upregulation (Figure 5A).

Among MVB trafficking genes, RAB7, RAB11, and RAB35 also showed an overall tendency toward early downregulation, albeit with distinct recovery dynamics. RAB7 exhibited sustained suppression, with reduced expression at 6 h under both concentrations, persistence of downregulation at 12 h with 300  $\mu$ M H<sub>2</sub>O<sub>2</sub>, and significant reduction again at 24 h under both treatments, indicating heightened sensitivity to H<sub>2</sub>O<sub>2</sub>. In contrast, RAB11 and RAB35 displayed transient responses, with significant decreases at 6 and 12 h following treatment with 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> and complete normalization by 24 h (Figure 5B).

A similar transient regulatory pattern was observed among MVB docking genes, although with greater heterogeneity. VAMP3 and VAMP5 shared an early downregulation at 6 h under both H<sub>2</sub>O<sub>2</sub> concentrations; VAMP3 remained suppressed at 12 h with 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> but recovered by 24 h, whereas VAMP5 showed a more pronounced decrease at 12 h with 300  $\mu$ M before returning to baseline at 24 h under both conditions. VAMP7 displayed a delayed response, remaining unchanged at 6 h, decreasing at 12 h with 300  $\mu$ M H<sub>2</sub>O<sub>2</sub>, and normalizing by 24 h. SNAP23 showed an early reduction at 6 h with both concentrations but recovered more rapidly, returning to control levels at 12 h under the 500  $\mu$ M condition and at 24 h with 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Figure 5C).

Taken together, these findings indicate that in C2C12 myotubes, exposure to physiological concentrations of H<sub>2</sub>O<sub>2</sub> predominantly induces a transient downregulation of genes involved in extracellular vesicle biogenesis, followed by recovery or modest upregulation at later time points.

341 *Cell state-dependent redox regulation of sEV exosome-like release in C2C12 myoblasts and*  
342 *myotubes*

343 To determine whether the H<sub>2</sub>O<sub>2</sub>-induced transcriptional modulation of exosome biogenesis genes is  
344 associated with changes in vesicle release, we analysed the amount and biophysical characteristics  
345 of sEV secreted by C2C12 myoblasts and myotubes into the culture media 24 h after a 1 h  
346 exposure to the pro-oxidant stimulus (Figures 6 and 7).

347 In C2C12 myoblasts, sEV size distribution remained within the expected exosomal range (150–180  
348 nm) under all experimental conditions (0  $\mu$ M H<sub>2</sub>O<sub>2</sub>: 170,15  $\pm$  5,83 nm; 50  $\mu$ M H<sub>2</sub>O<sub>2</sub>: 172,87  $\pm$  6.82  
349 nm; 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>: 165,8  $\pm$  6,46 nm), and particle concentration was not significantly affected by  
350 the acute H<sub>2</sub>O<sub>2</sub> treatments (0  $\mu$ M H<sub>2</sub>O<sub>2</sub>: 1,68  $\times$  10<sup>10</sup>  $\pm$  4,22  $\times$  10<sup>9</sup> particles/ml; 50  $\mu$ M H<sub>2</sub>O<sub>2</sub>: 1,14  $\times$   
351 10<sup>10</sup>  $\pm$  1,98  $\times$  10<sup>9</sup> particles/ml; 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>: 1,69  $\times$  10<sup>11</sup>  $\pm$  5,22  $\times$  10<sup>10</sup> particles/ml) (Figure 3A).

352 Western blot analysis confirmed the exosomal identity of the isolated vesicles through the detection  
353 of canonical markers—ALIX, TSG101, and CD81—in exosome lysates. These proteins were  
354 largely absent or only faintly detectable in the microvesicle fraction, whereas TSG101 was also  
355 present in total cell lysates, consistent with its known intracellular functions (Figure 6B).

356 In C2C12 myotubes, exosomes displayed a size distribution comparable to that observed in  
357 myoblast-derived vesicles, remaining within the same exosomal range (0  $\mu$ M H<sub>2</sub>O<sub>2</sub>: 167  $\pm$  0,44 nm;  
358 300  $\mu$ M H<sub>2</sub>O<sub>2</sub>: 168,67  $\pm$  8,20 nm; 500  $\mu$ M H<sub>2</sub>O<sub>2</sub>: 167,30  $\pm$  8,0 nm). In contrast to myoblasts,  
359 particle concentration increased in a dose-dependent manner following acute H<sub>2</sub>O<sub>2</sub> exposure (0  $\mu$ M  
360 H<sub>2</sub>O<sub>2</sub>: 5,87  $\times$  10<sup>10</sup>  $\pm$  1,77  $\times$  10<sup>10</sup> particles/ml; 300  $\mu$ M H<sub>2</sub>O<sub>2</sub>: 7,26  $\times$  10<sup>10</sup>  $\pm$  1,17  $\times$  10<sup>10</sup> particles/ml;  
361 500  $\mu$ M H<sub>2</sub>O<sub>2</sub>: 1,07  $\times$  10<sup>11</sup>  $\pm$  3,65  $\times$  10<sup>10</sup> particles/ml) (Figure 7A). The presence of exosomal  
362 markers (ALIX, TSG101, and CD81) was confirmed in exosome lysates and, to a lesser extent, in  
363 total cell lysates, while their absence in the microvesicle fraction further supported the purity of the  
364 exosome preparations (Figure 7B).

365 Collectively, these findings indicate that under physiological pro-oxidant conditions, the biophysical  
366 properties of extracellular vesicles are preserved, whereas vesicle release is regulated in a cell state-  
367 dependent manner. Specifically, differentiated myotubes exhibit an enhanced vesiculation response  
368 compared with proliferating myoblasts, consistent with their distinct functional state and biological  
369 role.

370

371 **Discussion**

372 Skeletal muscle function extends well beyond contraction and energy metabolism, encompassing a  
373 pronounced secretory activity that supports tissue crosstalk and both local and systemic  
374 homeostasis. This function is particularly relevant during exercise, when contracting fibers generate

transient ROS bursts that act as physiological messengers, activating redox-sensitive signaling cascades that drive metabolic and structural adaptations [27]. Among the diverse molecules released by muscle cells, small extracellular vesicles (sEVs) have emerged as key mediators of intercellular communication. Their biogenesis, release, and molecular composition reflect the functional state of the cells of origin and, in turn, contribute to a broad spectrum of adaptive responses [34]. However, whether exercise-associated oxidative perturbations directly influence EV dynamics in muscle cells has remained unclear.

In this study, by comparing proliferating and differentiated muscle cells, we show that physiological, non-cytotoxic levels of H<sub>2</sub>O<sub>2</sub> differentially modulate sEV exosome-related pathways, supporting the concept that redox signaling acts as a regulatory cue in skeletal muscle vesiculation. Adaptation to exercise or stress requires coordinated communication between the contractile apparatus and the regenerative niche [35], a process in which exosome-mediated signaling from both compartments plays a critical role [36,37]. Mature fibers and satellite-like cells—represented in vitro by myotubes and myoblasts, respectively—do not respond uniformly to redox signals [38], as they differ in antioxidant capacity [32], dependence on oxidative metabolism [39,40], and transcriptional programs [41]. Consequently, vesicle production and release, particularly in response to redox changes, cannot be assumed to be homogeneous across the myogenic lineage. This notion is supported by previous work in C2C12 cells showing that quiescent myoblasts secrete increased levels of exosomes with distinct uptake properties, cargo, and signaling capacity compared with proliferating myoblasts and differentiated myotubes [32].

These differences are especially relevant in the context of physiological H<sub>2</sub>O<sub>2</sub>, a central redox mediator generated during muscle contraction [26] and employed here to mimic exercise-induced oxidative fluctuations [42]. By selectively oxidizing cysteine residues in target proteins [43], H<sub>2</sub>O<sub>2</sub> functions as a molecular switch that translates transient redox changes into coordinated biochemical and transcriptional responses, including Nrf2- [44], NF-κB- [45], AMPK-, and PGC-1α-dependent programs [46], as well as pathways involved in vesicular trafficking [47]. In our experimental model, physiological concentrations of H<sub>2</sub>O<sub>2</sub> activated distinct signaling cascades in proliferating versus differentiated muscle cells. Myoblasts exhibited a rapid and robust activation of p38 MAPK, consistent with their progenitor-like identity [48], lower antioxidant capacity, and heightened sensitivity to oxidative fluctuations. As a canonical stress-responsive kinase, p38 promotes growth arrest, DNA damage responses, and apoptosis when oxidative stress exceeds the buffering capacity of proliferating cells [49], reflecting a protective strategy that prioritizes damage containment over secretory activation.

408 In contrast, myotubes preferentially activated ERK1/2 [50], a pathway closely associated with  
409 metabolic regulation, cytoskeletal organization, and membrane trafficking [51]. This response  
410 aligns with the functional priorities of mature fibers, which rely on robust mitochondrial activity  
411 and enhanced antioxidant defenses to interpret physiological oxidative signals as cues for adaptive  
412 remodeling rather than cellular stress [52,53]. The differential activation of redox-sensitive kinases  
413 in myoblasts and myotubes thus provides a mechanistic link between oxidative stimuli and the  
414 regulation of exosome biogenesis and release.

415 Consistent with this framework, physiological H<sub>2</sub>O<sub>2</sub> exposure in myoblasts induced a transient  
416 upregulation of several components of the vesiculation machinery—including ESCRT proteins,  
417 tetraspanins, RAB GTPases, and SNARE/VAMP proteins [54]—most prominently at 12 h, with  
418 expression returning to baseline by 24 h. This short-lived transcriptional response is characteristic  
419 of p38-driven stress adaptation in proliferating progenitors, which prioritize membrane integrity and  
420 genomic stability over secretory activity [55]. Importantly, myoblasts did not exhibit increased sEV  
421 exosome-like release despite these transcriptional changes, indicating that low-level oxidative  
422 stimuli are effectively buffered to preserve progenitor homeostasis. This observation is consistent  
423 with evidence showing that myoblast-derived EVs contain pro-differentiative cargo [59] and that  
424 their untimely release in the absence of differentiation or damage signals could disrupt the balance  
425 of the satellite cell-like pool.

426 Myotubes, by contrast, displayed a distinct regulatory pattern. Physiological H<sub>2</sub>O<sub>2</sub> initially induced  
427 a downregulation of multiple biogenesis-related transcripts at 6–12 h, followed by partial recovery  
428 or modest upregulation at 24 h. Despite this transient transcriptional suppression, myotubes showed  
429 a clear increase in exosome release, while vesicle size and the expression of canonical exosomal  
430 markers remained unchanged. These findings are in agreement with recent work by Obi et al. [56],  
431 demonstrating that acute and chronic electrically induced contractile activity in C2C12 myotubes  
432 leads to a progressive increase in sEV concentration without altering vesicle size.

433 The dissociation between transcriptional changes and functional outcomes suggests a substantial  
434 contribution of post-transcriptional mechanisms in the regulation of exosome biogenesis in  
435 differentiated fibers [57]. This interpretation is supported by accumulating evidence indicating that  
436 redox stimuli directly modulate the endolysosomal system in mammalian cells: ROS can influence  
437 RAB GTPase activity, regulate MVB maturation and motility, modulate lysosome–MVB fusion,  
438 and alter SNARE complex assembly [58]. The ability of differentiated myotubes to couple  
439 physiological oxidative signals to enhanced exosome release has important implications for skeletal  
440 muscle biology. Muscle-derived EVs are known to increase in circulation following both acute and  
441 chronic exercise [59], and their cargo is enriched in factors that promote mitochondrial biogenesis

442 [60], angiogenesis [61], immune modulation [62], and satellite cell activation [63]. Our findings  
443 suggest that physiological H<sub>2</sub>O<sub>2</sub> directly contributes to this process by promoting exosome  
444 secretion, thereby coordinating vesicle-mediated communication during exercise-induced muscle  
445 remodeling.

446 Conversely, the absence of a secretory response in proliferating myoblasts appears to preserve  
447 intracellular homeostasis and prevent premature activation of differentiation or intercellular  
448 signaling pathways. This restraint ensures that vesicle-mediated communication is engaged  
449 selectively in response to appropriate regenerative cues, such as fiber damage, inflammation, or  
450 substantial metabolic stress, rather than transient physiological fluctuations.

451

### 452 **Limitations of the study**

453 Despite providing new insights into the redox-dependent regulation of exosome biogenesis and  
454 release, this study has several limitations that should be considered when interpreting the results.  
455 First, the experiments were conducted using C2C12 cells, a well-established but simplified in vitro  
456 model that does not fully capture the structural, mechanical, and functional complexity of skeletal  
457 muscle in vivo, where contraction-induced calcium fluxes and mechanical forces critically influence  
458 both redox signaling and vesicle trafficking. Second, although H<sub>2</sub>O<sub>2</sub> is a key redox mediator, its  
459 exogenous application cannot fully reproduce the spatially restricted and temporally regulated ROS  
460 production generated by mitochondria and NADPH oxidases during muscle contraction. Third,  
461 while this study assessed transcriptional regulation and exosome abundance, it did not examine the  
462 molecular cargo of the released vesicles—including proteins, lipids, and RNA species—which is  
463 essential for determining the functional consequences of the redox-dependent modulation observed.

464

### 465 **Conclusions and future perspectives**

466 In summary, this study shows a connection between redox signaling and exosome-mediated  
467 communication in skeletal muscle. Our results suggest that physiological oxidative signals can  
468 influence the vesiculation machinery, supporting the idea that changes in redox state help shape  
469 how muscle cells communicate during physical activity. Understanding the molecular effectors and  
470 functional consequences of this redox-sensitive regulation, together with its validation in primary  
471 muscle fibers and in vivo exercise models, will be essential for clarifying how skeletal muscle  
472 adapts and contributes to systemic redox homeostasis during exercise.

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### 474 **Declaration of competing interest**

475 The authors declare no conflict of interest.

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**Author contribution**

Conceptualization, LS; DC; methodology, VL; FG; DA; CF; formal analysis, LS; DC; investigation, LS; FG; DA; data curation, LS; FG; DA; DC; writing-original draft preparation, LS; DC; writing—review and editing, LS, FG, EA; DA, CF, DC; supervision, FG, DA, CF, DC; project administration and grant collection, DC.

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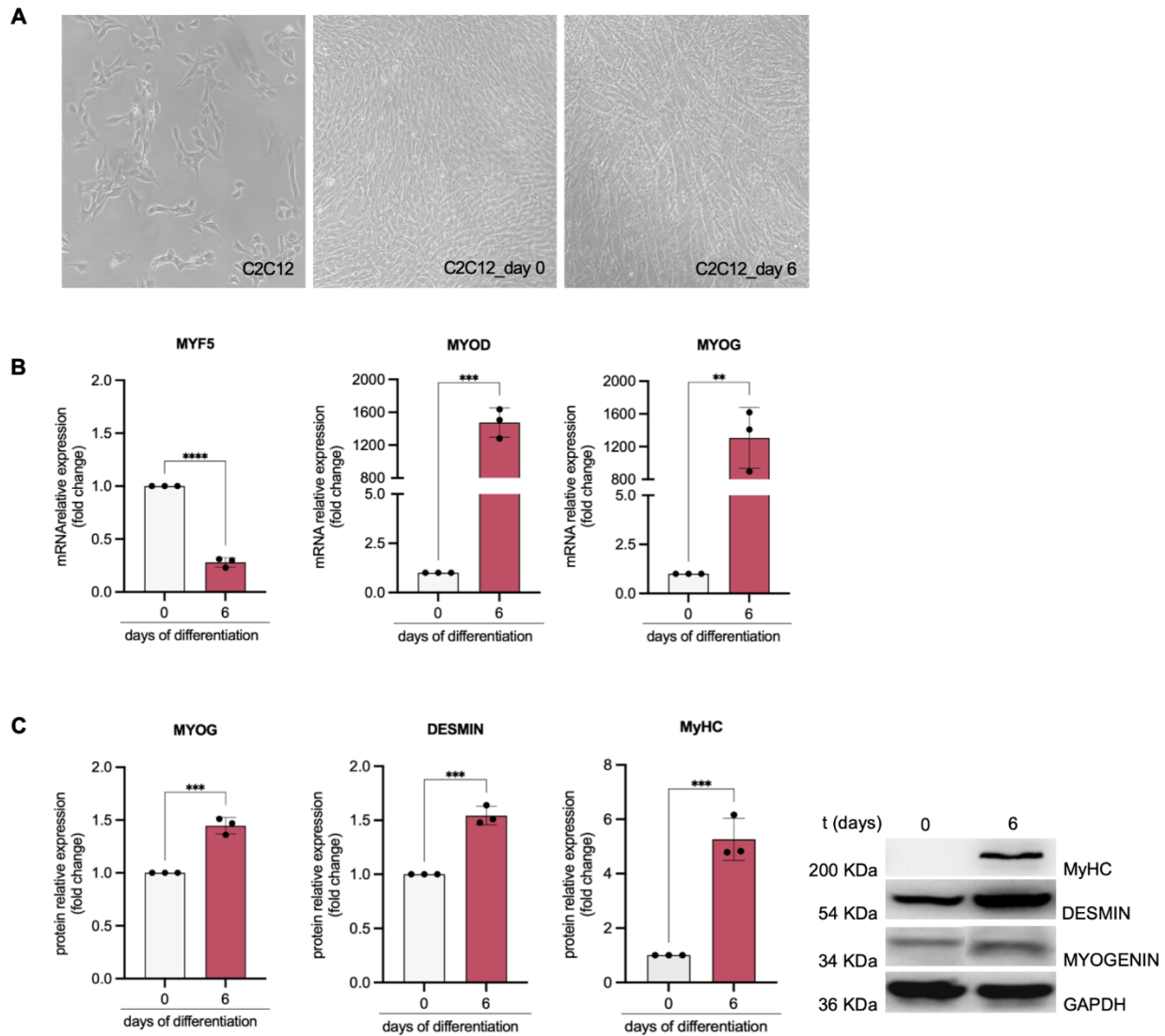


**Figure 1. C2C12 differentiation.** (A) Phase-contrast images of C2C12 cells at different stages of differentiation: proliferating myoblasts (*left panel*), myoblasts at day 0 following induction of differentiation (*middle panel*), and multinucleated myotubes at day 6 (*right panel*). (B) Relative mRNA expression levels of key myogenic markers, *Myf5*, *Myogenin*, and *MyoD* at day 0 and day 6 of differentiation, quantified by qRT-PCR. (C) Protein expression levels of MyHC, MYOGENIN and DESMIN at day 0 and day 6 of differentiation, assessed by Western blotting. GAPDH is used as both housekeeping gene and loading control. Data are expressed as mean  $\pm$  SEM of  $n = 3$  independent experiments. Statistical analysis was performed using unpaired two-tailed  $t$ -test;  $p < 0.05$  was considered significant (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

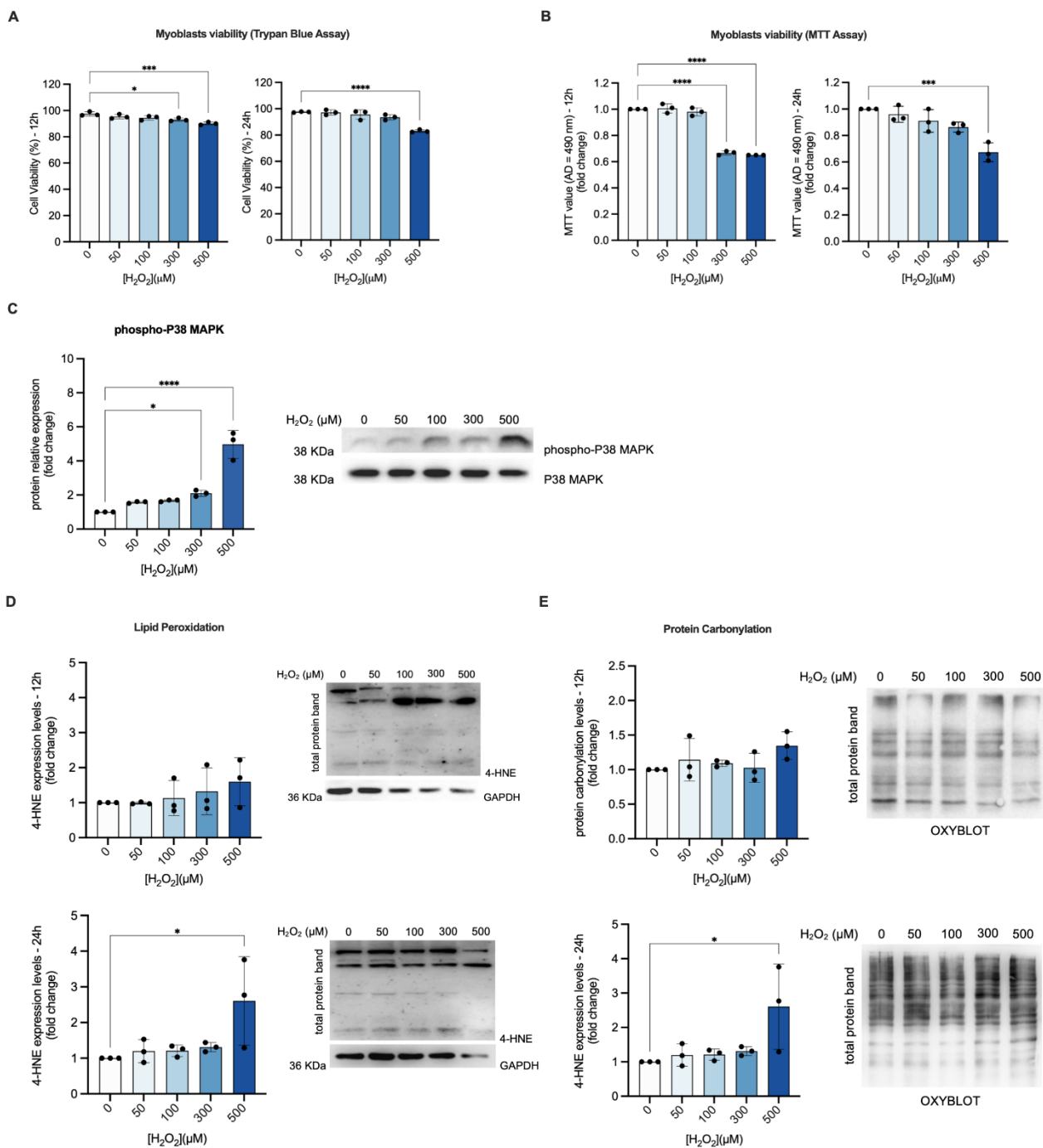
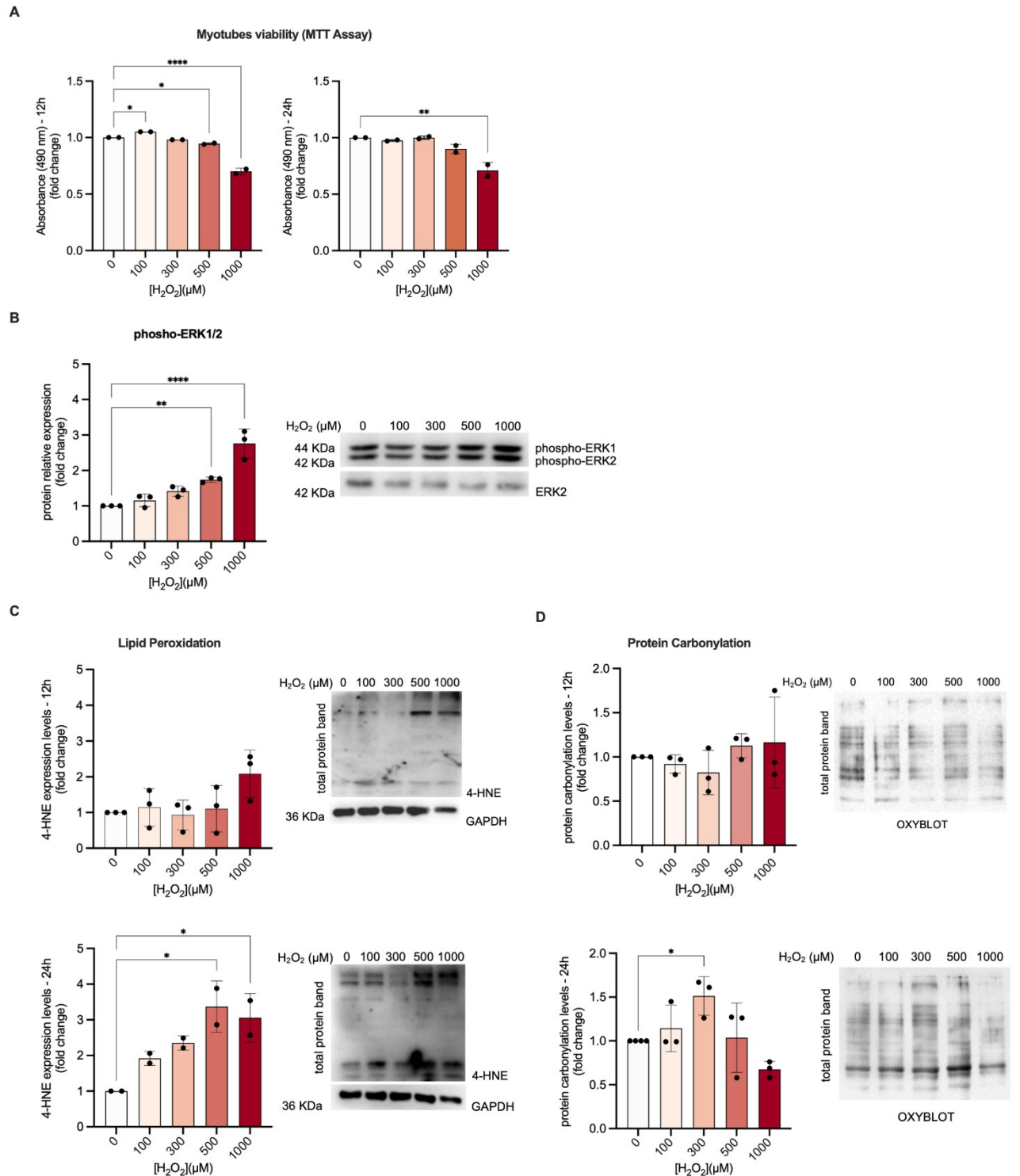


**Figure 2. Response of C2C12 myoblasts to acute H<sub>2</sub>O<sub>2</sub> concentrations.** (A) Myoblast viability and (B) metabolic activity measured at 12 and 24 h following a 1 h treatment with increasing concentrations of H<sub>2</sub>O<sub>2</sub> (0, 50, 100, 300, and 500 μM), assessed using the Trypan Blue exclusion assay and the MTT assay, respectively. (C) Protein expression of phosphorylated p38 MAPK immediately after a 1 h treatment with increasing concentrations of H<sub>2</sub>O<sub>2</sub>, assessed by Western blotting. Total p38 MAPK levels were used as loading controls. (D) Levels of 4-HNE–modified proteins at 12 and 24 h following a 1 h treatment with increasing concentrations of H<sub>2</sub>O<sub>2</sub>, assessed by Western blotting. Ponceau S staining was used as a loading control. (E) Protein carbonylation levels at 12 and 24 h following a 1 h treatment with increasing concentrations of H<sub>2</sub>O<sub>2</sub>,

747 assessed by OxyBlot analysis. Ponceau S staining was used as a loading control. Data are presented as mean  
 748  $\pm$  SD from three independent experiments and are expressed as fold changes relative to control conditions.  
 749 Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test; \* $p < 0.05$  was  
 750 considered statistically significant (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

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755 **Figure 3. Response of C2C12 myotubes to acute H<sub>2</sub>O<sub>2</sub> concentrations.** (A) Myotube metabolic activity  
756 measured at 12 and 24 h following a 1 h treatment with increasing concentrations of H<sub>2</sub>O<sub>2</sub> (0, 100, 300, 500,  
757 and 1000  $\mu$ M), assessed using the MTT assay. (B) Protein expression of phosphorylated ERK1/2 immediately  
758 after a 1 h treatment with increasing concentrations of H<sub>2</sub>O<sub>2</sub>, assessed by Western blotting. Total ERK1/2 levels  
759 were used as loading controls. (C) Levels of 4-HNE–modified proteins at 12 and 24 h following a 1 h treatment  
760 with increasing concentrations of H<sub>2</sub>O<sub>2</sub>, assessed by Western blotting. Ponceau S staining was used as a loading  
761 control. (D) Protein carbonylation levels at 12 and 24 h following a 1 h treatment with increasing  
762 concentrations of H<sub>2</sub>O<sub>2</sub>, assessed by OxyBlot analysis. Ponceau S staining was used as a loading control. Data  
763 are presented as mean  $\pm$  SD from three independent experiments and are expressed as fold changes relative to  
764 control conditions. Statistical analysis was performed using one-way ANOVA followed by Tukey’s post hoc  
765 test; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

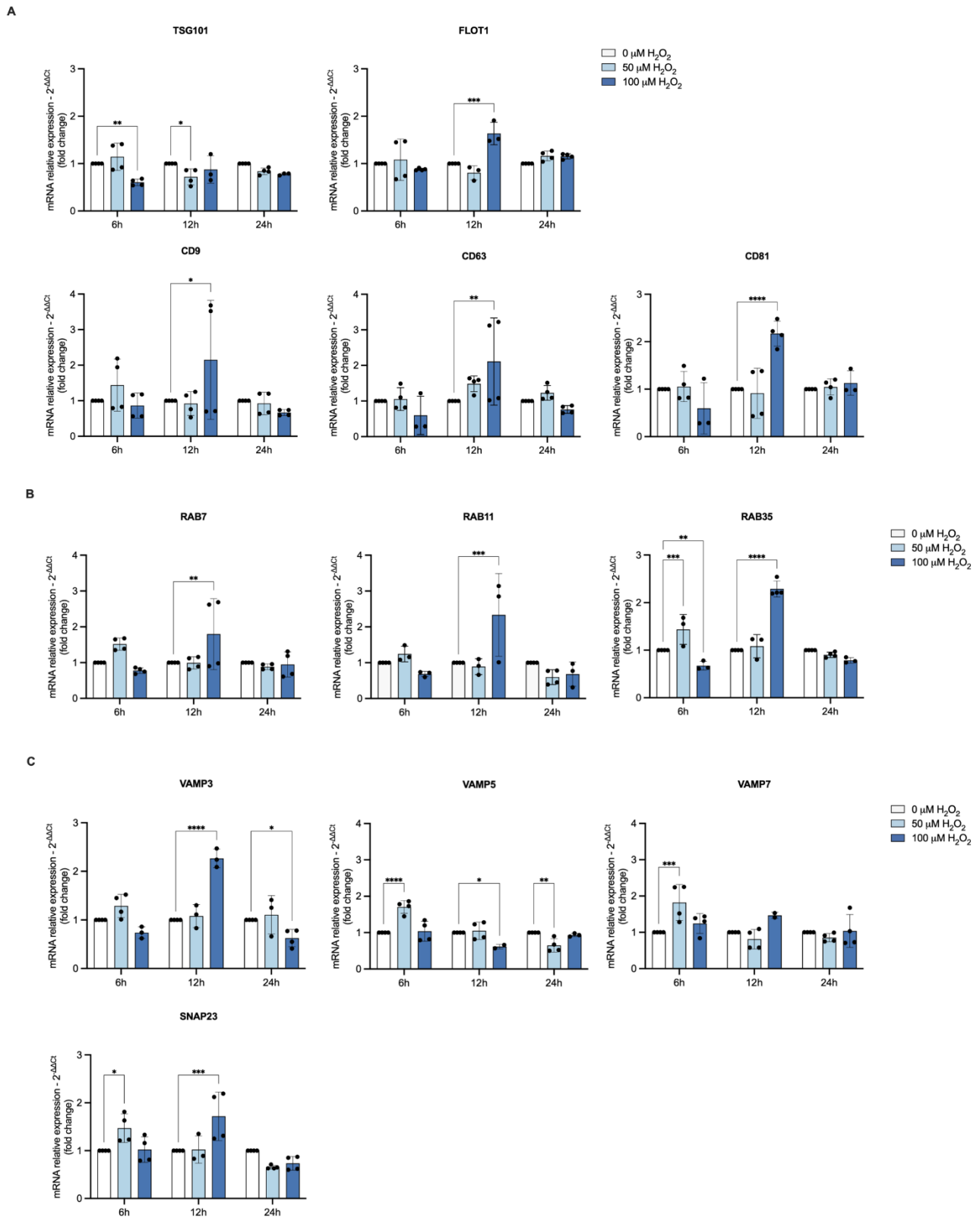


**Figure 4. Expression of genes involved in sEV exosome-like biogenesis in C2C12 myoblasts under standard and pro-oxidant conditions. (A–C) Relative mRNA expression of genes involved in (A) multivesicular body (MVB) formation (TSG101, CD9, CD63, CD81), (B) MVB trafficking (RAB7, RAB11, RAB35), and (C) MVB docking to the plasma membrane (VAMP3, VAMP5, VAMP7, SNAP23) in C2C12**

772 myoblasts at 6, 12, and 24 h following a 1 h treatment with physiological concentrations of H<sub>2</sub>O<sub>2</sub>. Gene  
773 expression was quantified by RT-qPCR using GAPDH as the housekeeping gene. Data are presented as mean  
774  $\pm$  SD from four independent experiments and are expressed as fold changes relative to untreated controls.  
775 Statistical analysis was performed using two-way ANOVA followed by Dunnett's multiple-comparison test  
776 (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001).

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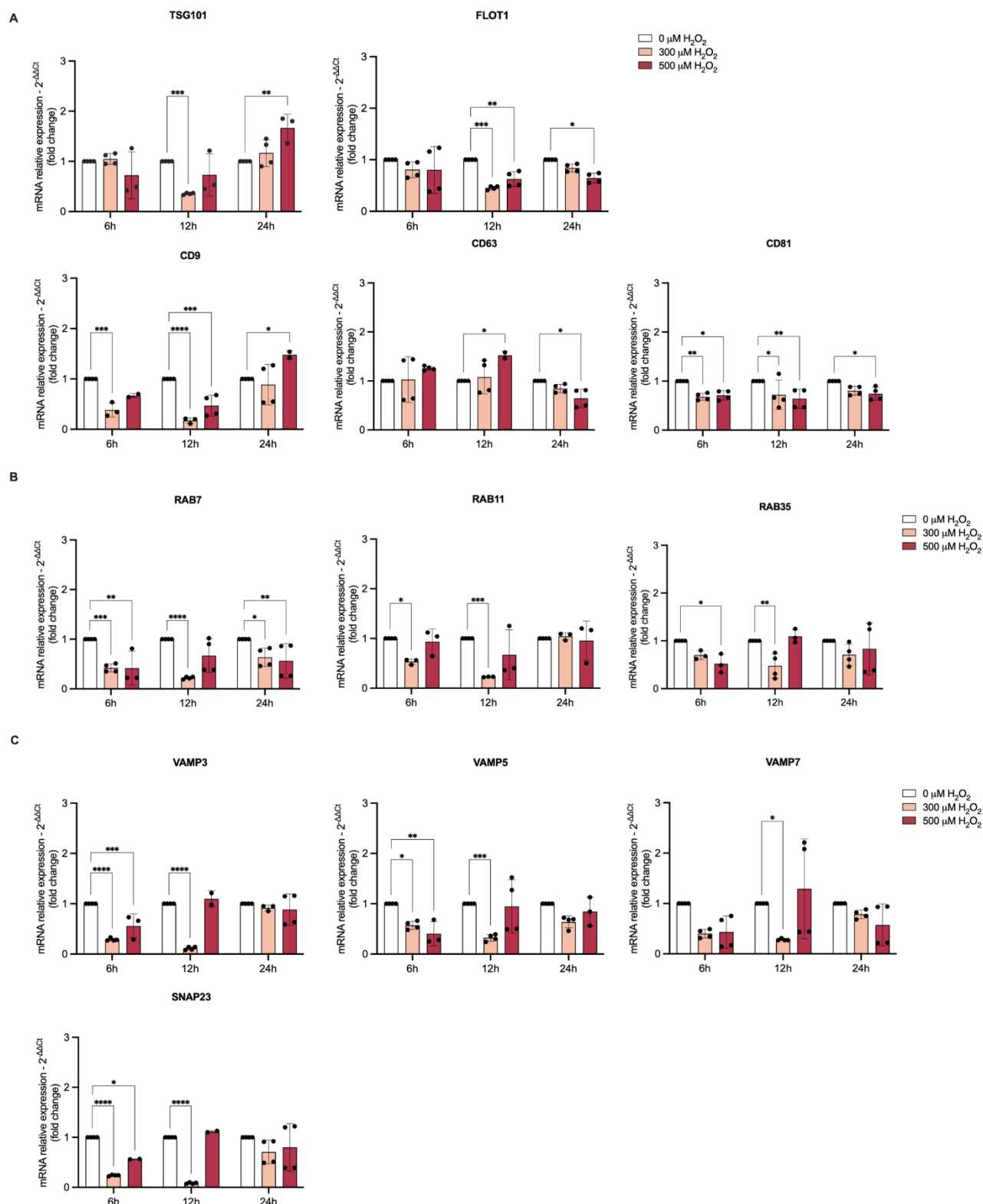


**Figure 5. Expression of genes involved in sEV exosome-like biogenesis in C2C12 myotubes under standard and pro-oxidant conditions. (A–C) Relative mRNA expression of genes involved in (A) multivesicular body (MVB) formation (TSG101, CD9, CD63, CD81), (B) MVB trafficking (RAB7, RAB11, RAB35), and (C) MVB docking to the plasma membrane (VAMP3, VAMP5, VAMP7, SNAP23) in C2C12 myotubes at 6, 12, and 24 h following a 1 h treatment with physiological concentrations of  $\text{H}_2\text{O}_2$ . Gene**

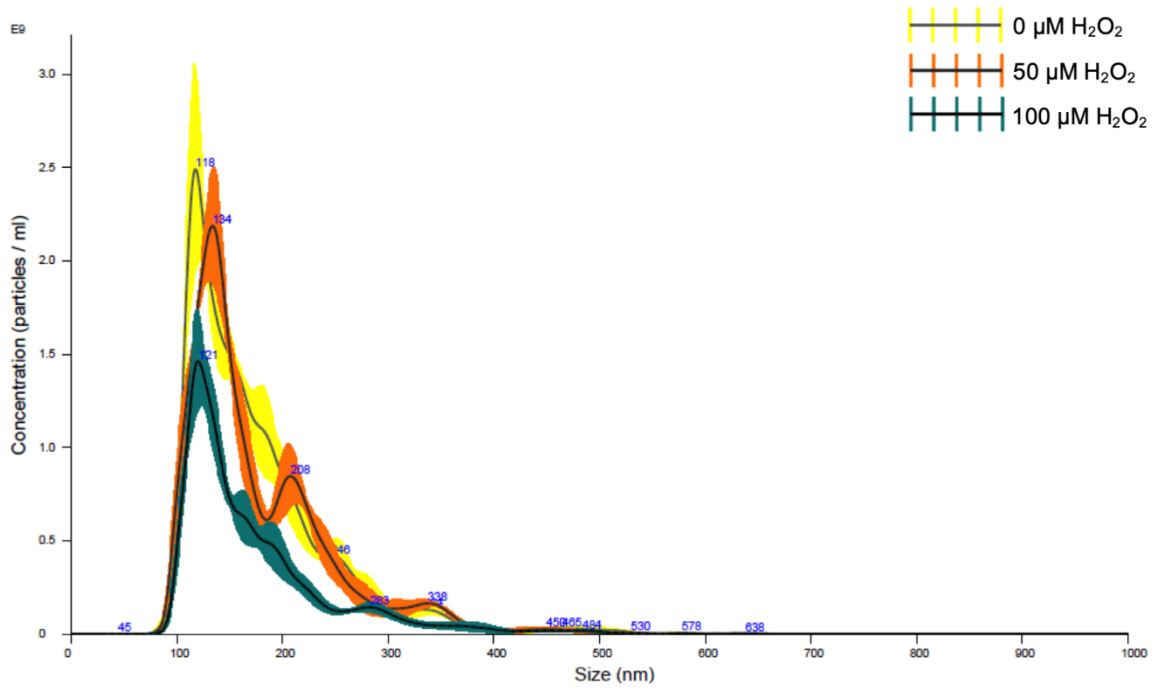
787 expression was quantified by RT-qPCR using GAPDH as the housekeeping gene. Data are presented as mean  
788  $\pm$  SD from four independent experiments and are expressed as fold changes relative to untreated controls.  
789 Statistical analysis was performed using two-way ANOVA followed by Dunnett's multiple-comparison test  
790 (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

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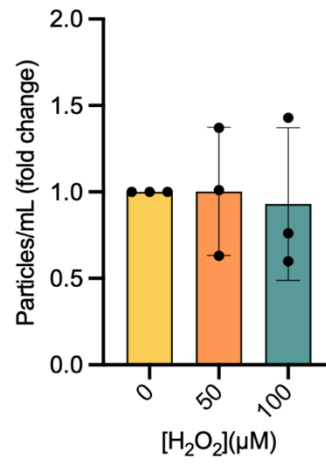
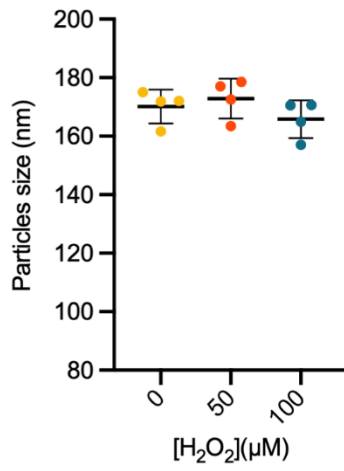
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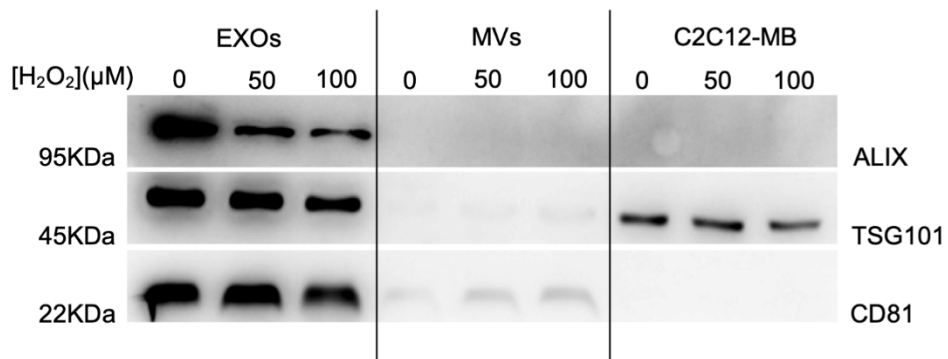


**Exosome size**

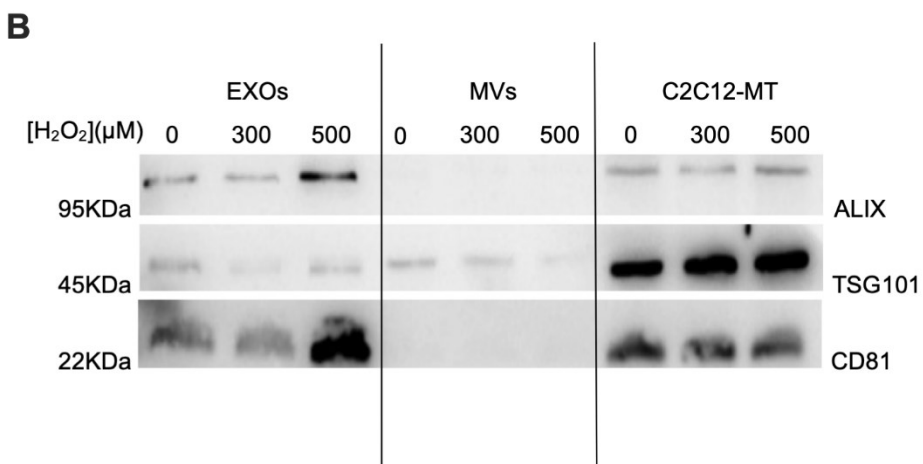
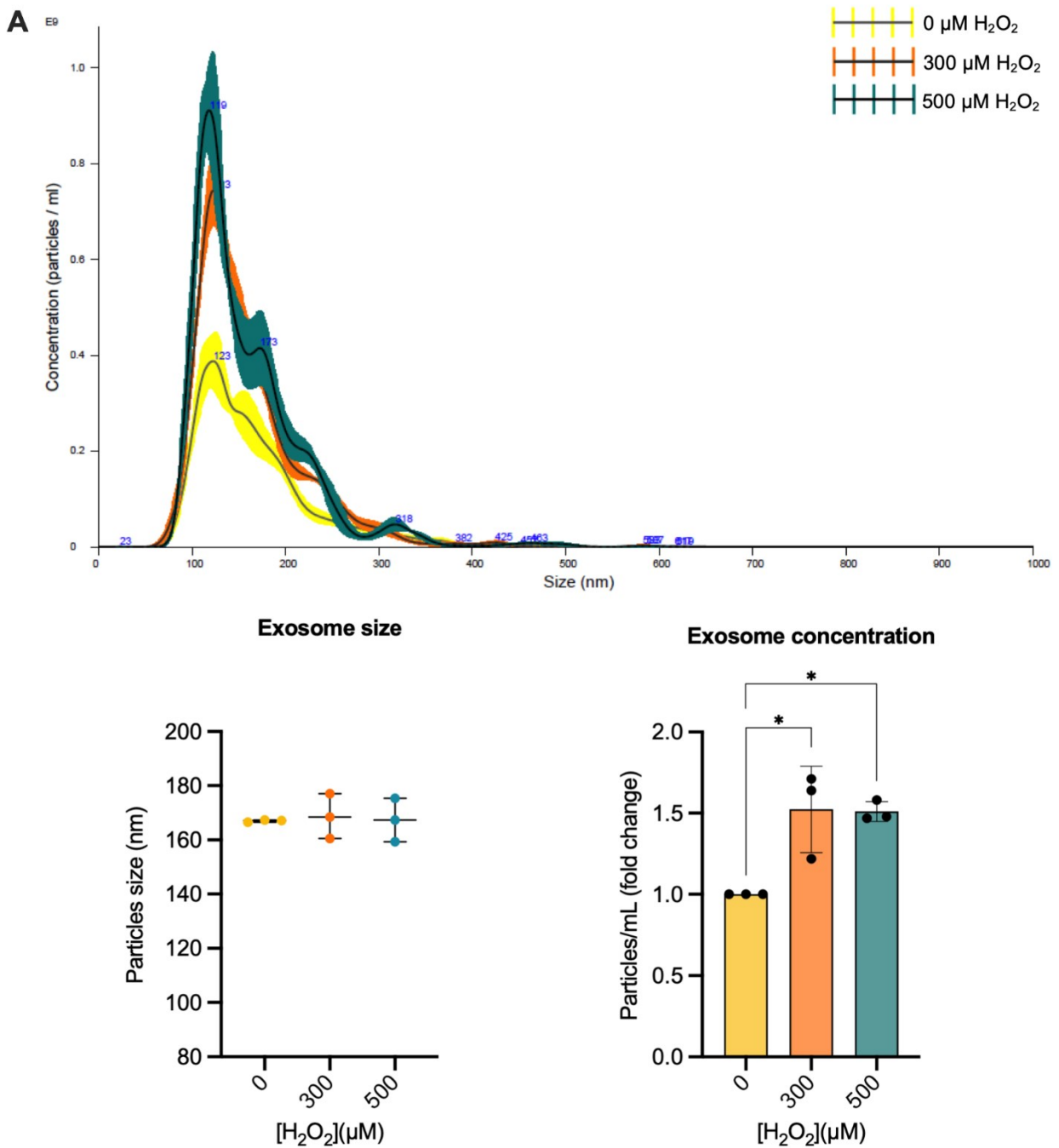
**Exosome concentration**



**B**



795 **Figure 6. sEV exosome-like release and characterization in C2C12 myoblasts exposed to acute pro-**  
796 **oxidant conditions.** (A) Size distribution and particle concentration of extracellular vesicles (EVs) released  
797 by C2C12 myoblasts 24 h after a 1 h treatment with physiological concentrations of H<sub>2</sub>O<sub>2</sub>, quantified by  
798 nanoparticle tracking analysis (NTA). (B) Protein expression of exosomal markers ALIX, TSG101, CD81, and  
799 HSC70 assessed by Western blotting in exosome, microvesicle, and total cell lysates. Data are presented as  
800 mean ± SD from four independent experiments and are expressed as fold changes relative to untreated controls.  
801 Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test (\*p < 0.05, \*\*p  
802 < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001).  
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807 **Figure 7. sEV exosome-like release and characterization in C2C12 myotubes exposed to acute pro-**  
808 **oxidant conditions.** (A) Size distribution and particle concentration of extracellular vesicles (EVs) released  
809 by C2C12 myotubes 24 h after a 1 h treatment with physiological concentrations of H<sub>2</sub>O<sub>2</sub>, quantified by  
810 nanoparticle tracking analysis (NTA). (B) Protein expression of exosomal markers ALIX, TSG101, CD81,  
811 and HSC70 assessed by Western blotting in exosome, microvesicle, and total cell lysates. Data are presented  
812 as mean  $\pm$  SD from four independent experiments and are expressed as fold changes relative to untreated  
813 controls. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test (\*p <  
814 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001).



## Article

# The Effect of *Moringa oleifera* Leaf Extract on C2C12 Myoblast Proliferation and Redox Status Under Oxidative Insult

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**Abstract:** Skeletal muscle tissue can regenerate after damage through the action of satellite cells, which proliferate as myoblasts when activated. Oxidative stress, marked by high rates of reactive oxygen species (e.g., hydrogen peroxide, H<sub>2</sub>O<sub>2</sub>), impairs this process by increasing myoblast cell death. *Moringa oleifera* leaf extract (MOLE), known for its antioxidant properties, was tested for its protective effects on C2C12 myoblasts under oxidative stress. We assessed MOLE's impact on total antioxidant capacity (TAC), glutathione homeostasis (GSH/GSSG), cell viability, and wound recovery. The metabolomic analysis of MOLE using an LC-MSMS ZenoTOF 7600 mass spectrometry system identified key compounds, including peculiar glucosinolates (42.1%) and flavonoids (18.8%), as well as phenolic acids (4.5%) and other significant metabolites (34.6%; among them, amino acids, vitamins, and fatty acids). H<sub>2</sub>O<sub>2</sub> disrupted myoblast redox balance and caused cell death, but MOLE treatment restored the GSH/GSSG ratio, improved TAC, and increased cell viability. Additionally, MOLE promoted faster wound closure in myoblasts exposed to H<sub>2</sub>O<sub>2</sub>. These findings suggest that MOLE can protect C2C12 myoblasts by restoring redox balance and enhancing recovery under oxidative stress.

**Keywords:** *Moringa oleifera* leaf extract (MOLE); C2C12 skeletal muscle cells; myoblast proliferation; glutathione; redox homeostasis; oxidative stress; LC-MSMS ZenoTOF 7600 system metabolomics analysis



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## 1. Introduction

Skeletal muscle constitutes up to 50% of total body mass and plays an important role in maintaining optimal energy expenditure across the entire organism [1]. Rapid changes in fuel selection are a hallmark of metabolically healthy muscle. Consequently, preserving healthy skeletal muscle mass is essential throughout life in preventing or mitigating possible metabolic diseases.

Skeletal muscle can regenerate through the activation of stem cells (satellite cells) by environmental signals [2]. Once activated, satellite cells exit cell-cycle arrest, proliferate as myoblasts, and form multinucleated cells with a complex process of cell alignment and fusion that is able to repair damaged sections of the myofibers and maintain muscle mass [3,4]. This is a complex process involving many cellular changes, and defects in the

activation or proliferation of myoblasts can profoundly impair muscle regeneration [5–7]. In fact, a reduction in the regenerative potential of the fibers characterizes several physiological conditions. Muscle mass loss (atrophy) and ALS (amyotrophic lateral sclerosis) are some examples of pathological conditions that involve the failure to activate myoblasts and severely affect human ability and health [8–11].

Among the molecules that profoundly influence muscle regeneration, reactive oxygen species (ROS), such as hydrogen peroxide ( $H_2O_2$ ), play a critical regulatory role [12–14]. Low-to-moderate ROS levels are essential for muscle adaptation, cell signaling, the regulation of gene expression, and muscle growth [13,15]. However, when ROS are produced at high levels, they lead to the oxidation of macromolecules, which results in impaired myoblast function, increased cell death, and hindered muscle repair. Elevated ROS levels occur under physiological conditions such as aging, or are associated with metabolic diseases like diabetes, as well as conditions of muscle mass loss, such as atrophy and sarcopenia [16].

The metabolic activity of muscles, which occurs in many types of physical exercise, especially intense or unaccustomed, leads to an increase in reactive oxygen species, and consequently, in oxidative stress, as indicated by elevated biomarkers of molecule oxidation in both skeletal muscle and blood tissues [15,17,18]. Additionally, prolonged periods of muscle disuse may induce processes that promote ROS production in skeletal muscle fibers [19].

On the other hand, an increase in oxidative stress can compromise muscle functionality and the ability to regenerate after damage, which can result in vigorous contraction. The ability of satellite cells to repair muscle damage and facilitate recovery can be severely impaired by ROS-induced molecular and cellular damage [16,20,21]. While low-to-moderate levels of ROS are essential for muscle adaptation, high levels of ROS significantly impair muscle function [22].

In this context, there is an ever-increasing demand for strategies that enhance physical performance by preventing muscle damage or aiding recovery after exertion. Among these, nutritional strategies to mitigate or reduce the damage caused by elevated ROS levels to macromolecules are the subject of current research [23,24].

In recent years, the natural products derived from *Moringa oleifera* Lam. (family Moringaceae; order Brassicaceae) have been the subject of numerous studies comprising different experimental models aiming to evaluate their antioxidant properties [25–29]. Different parts of the plant (i.e., leaves, seeds, roots) contain numerous bioactive molecules with antioxidant, antimicrobial, and/or anti-carcinogenic properties [30,31]. Extracts from *Moringa oleifera* demonstrate activity due to the presence of bioactive molecules such as isothiocyanates, tannins, saponins, flavonoids, and terpenoids, especially in the leaves [30–33]. Due to the remarkable efficacy of these bioactive molecules, and especially some unique isothiocyanates, *Moringa oleifera* is gaining global interest for a wide range of applications. Among the notable glucosinolates/isothiocyanate present, moringin, a potent isothiocyanate produced by the hydrolysis of the glucosinolate glucomoringin by the enzyme myrosinase, exhibits potent anti-inflammatory and indirect cytoprotective antioxidant activity, supporting the therapeutic use of *Moringa oleifera* extracts for various pathologies [33–35].

Moreover, flavonoids, other glycosides, and phenolic acids (e.g., chlorogenic acid, ferulic acid) are present in moderate concentrations in *Moringa oleifera* leaf extracts. These molecules have proven to be effective in counteracting the increase in reactive oxygen species [36–39], acting as primary antioxidants by either inactivating lipid free radicals or preventing the decomposition of hydroperoxides to produce free radicals [40–42].

Recently, our group demonstrated that *Moringa oleifera* leaf extract (MOLE) improved the capacity of C2C12 myotubes to respond to oxidative stress via the activation of the SIRT1-PPAR pathway [43]. Moreover, MOLE was able to induce the nuclear factor erythroid 2-related factor (Nrf2) and its target gene heme oxygenase-1 (HO-1) pathway [29], resulting in an increase in the antioxidant system of skeletal muscle cells. This restored the redox balance (assessed by total free thiols, Trx, and GSH/GSSG ratio increase levels) and increased the

antioxidant enzymatic system (catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione S-transferase (GST)), significantly reducing markers of cellular oxidative damage (thiobarbituric acid reactive substance (TBAR) and carbonylated protein (PrCAR)) [44]. Based on these findings, we hypothesize that MOLE could efficiently protect C2C12 myoblasts subjected to a pro-oxidant environment.

MOLE extract was analyzed for metabolic qualitative profiling by ultra-high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UHPLC/QTOF-MS), as previously described [45].

The aim of this research was to provide scientific evidence about the effect of *Moringa oleifera* leaf extract on restoring redox balance in myoblasts subjected to an oxidative insult mimicking (for example) the high stress condition that occurs during intense muscle contractions. To this end, proliferative C2C12 myoblasts were treated with MOLE and/or exposed to 0.3 mM H<sub>2</sub>O<sub>2</sub> in single and combined treatments for 24 h and then analyzed for (a) cell viability; (b) GSH homeostasis; (c) total antioxidant capacity (TAC); and (d) the ability of myoblasts to replicate and migrate in the culture plate under different experimental conditions through the “wound closure assay”.

## 2. Materials and Methods

The chemical reagents used in this research were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

### 2.1. MOLE: Ethanolic Extract of *Moringa oleifera* Leaves

The ethanolic extract of *Moringa oleifera* leaves was prepared as previously described [44]. Briefly, *Moringa oleifera* leaf powder (PureBodhi Nutraceuticals Ltd., London, UK) (1 g powder/10 mL ethanol 100%) was gently sonicated (Vibra-Cell CV 18 SONICS VX 11, Sonics & Materials, Newtown, CT, USA) twice for 10 min at +4 °C. After centrifugation (2000 × g for 10 min at +4 °C), the resulting supernatant was collected and stored at −20 °C (stock solution equivalent to 15 mg/mL of dried leaves).

### 2.2. MOLE Qualitative Profiling

MOLE metabolomic qualitative profiling was carried out via ultra-high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UHPLC/QTOF-MS) on a SCIEX ZenoTOF 7600 mass spectrometer (AB SCIEX GmbH, Landwehrstraße 54, Darmstadt, Germany), as previously described [43,45]. For deeper qualitative profiling, a sample was acquired using the approaches of Data-Dependent Acquisition (zeno DDA) and Data-Independent Acquisition (zenoSWATH). The obtained data were processed using SciexOS Software 3.3 (AB SCIEX GmbH, Landwehrstraße 54, Darmstadt, Germany) and the SCIEX Natural Products 2.1 Library (AB SCIEX GmbH, Landwehrstraße 54, Darmstadt, Germany) and NIST 2017 Library in order to search for compound spectra databases.

The Formula Finder and Chemspider tools in SciexOS software (SciexOS software version 3.3) were used for the analysis of molecules that had no correspondence with the library but had accurate TOF MS and TOF MS/MS spectra. ZenoSWATH allowed for more confident identification, particularly for cases of a very low abundance of secondary metabolites. This method enhances the detection of these very-low-abundance metabolites by searching for precursor ions and detecting their diagnostic fragment ions through accurate mass measurements [43,45].

### 2.3. Cell Cultures

Mouse C2C12 myoblasts (ATCC, Manassas, VA, USA) were cultured in sterile conditions at 37 °C with 5% CO<sub>2</sub> in a humidified atmosphere at a density of  $2 \times 10^3$ /cm<sup>2</sup> in Dulbecco's modified Eagle's medium (DMEM; HyClone, Oud-Beijerland, The Netherlands). The media were supplemented with Glutamax-I (4 mM l-alanyl-l-glutamine), 4.5 g/L glucose (Invitrogen, Carlsbad, CA, USA), and 10% heat-inactivated fetal bovine

serum (Hy-Clone, Oud-Beijerland, The Netherlands). Cells were split 1:6 twice weekly and fed 24 h before each experiment, as described previously [44,46].

#### 2.4. Cell Viability

C2C12 viability was assessed by the Trypan blue exclusion assay, as previously described [47]. Briefly, cells were seeded at low density (<15%) in 25 cm<sup>2</sup> culture flasks. After 24 h (≈25% confluence), they were treated with different concentration of H<sub>2</sub>O<sub>2</sub> (0.1–1 mM) (H<sub>2</sub>O<sub>2</sub> dose dependence), and various dilutions of the MOLE stock solution (1:50–1:1000 working solution) for 24 h (MOLE dose dependence). Additionally, different H<sub>2</sub>O<sub>2</sub> exposure times (6–48 h, time-dependent) were evaluated. Cells reached a maximum confluence of 75%. Accordingly, for the combined treatments, concentrations of 0.3 mM H<sub>2</sub>O<sub>2</sub> and a 1:100 dilution of MOLE were chosen to assess their effects on myoblast viability and oxidative stress responses. Under these experimental conditions, the ethanol concentration in the working solutions (0.1%, *v/v*) did not affect myoblast viability. After the treatments, cells were trypsinized and collected by centrifugation (1200× *g* for 10 min at room temperature), and then cell viability was assessed using the Trypan blue exclusion assay (cells/dye 0.05% *v/v* solution in PBS mixed in a ratio of 1:1). With this method, it is possible to count the live cells that have rejected the Trypan blue dye (alive) from the colored cells that have incorporated it (dead cells) by analyzing the sample on a hemocytometer. Experiments were performed in triplicate with different cell preparations and the results are expressed as the number of cells counted.

#### 2.5. Trolox<sup>®</sup> Equivalent Antioxidant Capacity

Cellular total antioxidant capacity was evaluated as previously described [29,48]. The Trolox<sup>®</sup> equivalent antioxidant capacity (TAC) assay evaluates the ability of samples (cell lysates) to prevent ABTS<sup>+</sup> radical formation compared to Trolox<sup>®</sup> (vitamin E analogue) standards in a pro-oxidative buffer. This assay allows the measurement of the lipo- and hydro-philic antioxidants present in a sample. Briefly, 10 mL of cell lysate or standard (0.125–2.0 mM) was incubated in ABTS-met-Myo-PBS buffer, and the absorbance at 734 nm was monitored for 2 min. Then, the pro-oxidative insult was initiated by the addition of H<sub>2</sub>O<sub>2</sub> (0.45 mM) and the variation in absorbance was recorded in the following 10 min of reaction. The variation in absorbance detected was compared to that obtained using the different Trolox<sup>®</sup> standards (standard curve). Cell lysate TAC is expressed as micromoles Trolox equivalent/mg protein tested.

#### 2.6. Wound Closure Assay

The “wound closure assay” evaluates the ability of myoblasts to replicate and migrate in a culture plate under different experimental conditions [49]. Briefly, C2C12 myoblasts (2 × 10<sup>3</sup>/cm<sup>2</sup>) were cultured in proliferative conditions in 6-well plates until approximately 50% confluence was reached. To create a discontinuity in the cell culture, a vertical wound (1 mm) down through the cell monolayer was created by using a 1000 µL pipette tip in a sterile environment, pressing firmly against the tissue culture plate and taking care not to scratch the support. Afterward, culture media and cell debris were carefully removed, and fresh media were added under sterile conditions. Then, an initial picture was taken (named time = 0), and after a recovery period of two hours, cells were treated with MOLE (1:100), H<sub>2</sub>O<sub>2</sub> (0.3 mM) and the combined treatments for the subsequent 24 h. At the end of the experimental time, the width of the wound was measured in triplicate and photographed. The distance between the sides of the wound were measured (in triplicate) using ImageJ software (version 1.51, Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA) to evaluate wound closure after different treatments.

#### 2.7. Glutathione Homeostasis

C2C12 glutathione homeostasis, including reduced (GSH) and oxidized (GSSG) glutathione levels and the GSH/GSSG ratio, was quantified by a DTNB–glutathione reduc-

tase recycling assay, as previously described [50]. Briefly, an adequate number of cells ( $>10^7$  cell collected) were suspended in (1:1) (*v/v*) mL 5% sulfosalicylic acid (SSA). The cells were then lysed (by freezing and thawing the samples three times) and then immediately centrifuged at  $10,000\times g$  for 5 min at  $+4\text{ }^{\circ}\text{C}$ . The resulting deproteinized supernatant was analyzed for total glutathione content (tGSH). Oxidized glutathione (GSSG) was selectively measured in samples in which reduced glutathione (GSH) was masked by the pretreatment with 2-vinylpyridine (2%) [43]. During the assay, 10 mL of the sample was mixed with the reaction buffer, which consisted of 700  $\mu\text{L}$  NADPH (0.3 mM), 100  $\mu\text{L}$  DTNB (6 mM), and 190  $\mu\text{L}$   $\text{H}_2\text{O}$ . The reaction was started by adding 2.66 U/mL glutathione reductase. The variation in absorbance recorded at 412 nm by the TNB stoichiometric formation was noted and compared to those obtained by using glutathione standards. The results were then normalized to protein content.

### 2.8. Statistical Analysis

The Kolmogorov–Smirnov test was used to test the normal distribution of the data. For all experiments, data are expressed as the means  $\pm$  standard deviation (SD) of three independent experiments, each performed in triplicate. To evaluate the statistically significant differences of the data among the groups for each variable tested, one-way ANOVA for repeated measures followed by Bonferroni post hoc analyses were performed. SPSS for Windows (Version 17.0; SPSS Inc., Chicago, IL, USA) was used and statistical significance was set at  $p < 0.05$ . The comparisons between the untreated controls and the control vehicles showed no statistical differences for all variables tested [29,43].

## 3. Results

### 3.1. MOLE Metabolomic Fingerprint

Table 1 shows the relative percentages of metabolite groups identified in the MOLE metabolomic fingerprint. Glucosinolates (GLs) made up the largest share at 42.1%, followed by flavonoids (18.8%), as well as phenolic acids (4.5%) and other metabolites (34.6%; among them, amino acids, vitamins, and fatty acids). The table shows the three most represented bioactive molecules for each identified category.

**Table 1.** Relative amounts of constituents of MOLE <sup>1</sup>.

|                                        |                                  |
|----------------------------------------|----------------------------------|
| <b>Glucosinolates (relative %)</b>     | <b>42.1 <math>\pm</math> 0.3</b> |
| 4-O-acetyl-rhamnopyranosyloxybenzyl-GS | 31.6 $\pm$ 0.4                   |
| Glucomoringin                          | 10.3 $\pm$ 0.2                   |
| 4-O-acetyl-glucopyranosyloxybenzyl-GS  | 0.2 $\pm$ 0.1                    |
| <b>Flavonoids (relative %)</b>         | <b>18.8 <math>\pm</math> 0.2</b> |
| Isoquercitrin                          | 6.3 $\pm$ 0.3                    |
| Astragalin                             | 4.5 $\pm$ 0.2                    |
| Rutin                                  | 2.4 $\pm$ 0.2                    |
| <b>Phenolic acids (relative %)</b>     | <b>4.5 <math>\pm</math> 0.1</b>  |
| Cryptochlorogenic acid                 | 2.2 $\pm$ 0.1                    |
| Chlorogenic acid                       | 1.8 $\pm$ 0.2                    |
| Quinic acid                            | 0.3 $\pm$ 0.1                    |
| <b>Others (relative %)</b>             | <b>34.6 <math>\pm</math> 0.4</b> |
| Alpha-linolenic acid                   | 19.2 $\pm$ 0.4                   |
| Linoleic acid                          | 9.2 $\pm$ 0.3                    |
| Pantothenic acid                       | 1.6 $\pm$ 0.2                    |

<sup>1</sup> Metabolomic analysis was conducted on the MOLE working solution. The relative percentages of the most abundant biomolecules in MOLE are categorized into different groups: glucosinolates, flavonoids, phenolic acids, and others, with their respective percentages for each category. The table shows the three most represented bioactive molecules for each identified category. The data presented are the mean  $\pm$  SD of three different extractions, each tested in triplicate.

Notably, the most intense peaks corresponded to GLs. Due to the absence of reference standards for some GLs and their associated MS/MS spectral information, an alternative identification approach was necessary. Glucosinolates are thioglucoside compounds containing a sulfated aldoxime moiety and a variable side chain derived from amino acids. This unique chemical structure produces diagnostic fragment ions in MS/MS spectra. Specifically, a sulfated glucose moiety (fragment at 259.0122  $m/z$ ) and a sulfate group (fragment at 96.9601  $m/z$ ) were readily identifiable in the spectra [33,51]. Using this method, the following glucosinolates were identified from the ion chromatogram (XIC), MS, and SWATH MS/MS spectra, with the following retention times (tR) and relative percentages: glucosoonjnain (tR: 3.8), glucomoringin (tR: 4.2), sinalbin (tR: 4.3), 4-O-acetyl-rhamnopyranosyloxybenzylGS (tR: 6.0, 6.4, 7.7), and 4-O-acetyl-glucopyranosyloxybenzylGS (tR: 5.3, 7.0).

### 3.2. Effect of Hydrogen Peroxide Treatment on C2C12 Myoblast Viability and Total Antioxidant Capacity

C2C12 myoblast viability was assessed using the Trypan blue exclusion assay, which distinguishes dead from living cells through direct cell counting under an inverted microscope. For the dose-dependent experiments, cells were treated with a range of  $H_2O_2$  concentrations (0.1–1 mM) for 24 h. We observed a dose-dependent effect of  $H_2O_2$  treatment, indicated by a decrease in the number of viable cells and a concomitant rise in dead cells (Figure 1A). A concentration of 0.3 mM of  $H_2O_2$  was then selected for all subsequent experiments.

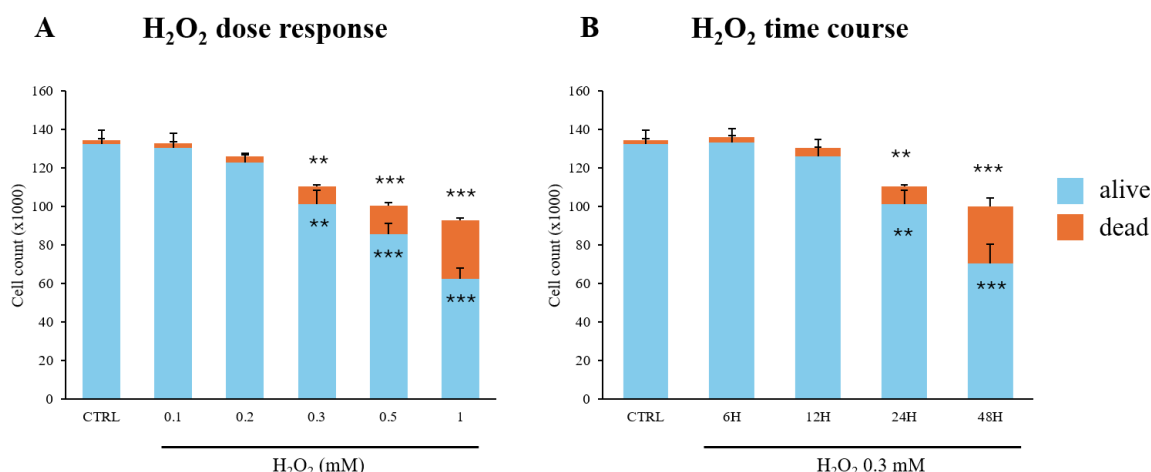


**Figure 1.** C2C12 myoblast viability under oxidative insult. (A) C2C12 myoblasts were treated with varying concentrations of  $H_2O_2$  (0.1–1 mM) for 24 h to assess dose-dependent effects; (B) C2C12 myoblasts were treated with  $H_2O_2$  0.3 mM for different durations (6–48 h) to evaluate time-dependent effects. Cell viability was assessed using the Trypan blue exclusion assay. Results are expressed as the number of cells ( $\times 1000$ ) counted. Data presented are the mean  $\pm$  SD of three different experiments, each performed in triplicate. Statistical analyses were conducted using one-way ANOVA followed by Bonferroni's multiple comparisons test. \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  vs. CTRL.

In the time-course (6–48 h) experiments, a statistically significant decrease in viable cells, along with an increase in dead cells, was noted after 24 h of treatment (Figure 1B).

To evaluate whether the  $H_2O_2$  treatment altered the antioxidant capacity in the myoblasts, we tested the time-dependent effects of 0.3 mM  $H_2O_2$  administration on cellular total antioxidant capacity (TAC) over a period of 6 to 48 h. We observed a statistically significant decrease in TAC values over time ( $p < 0.05$ ), with a reduction of up to 45% after 48 h of treatment ( $p < 0.01$ , Figure 2).

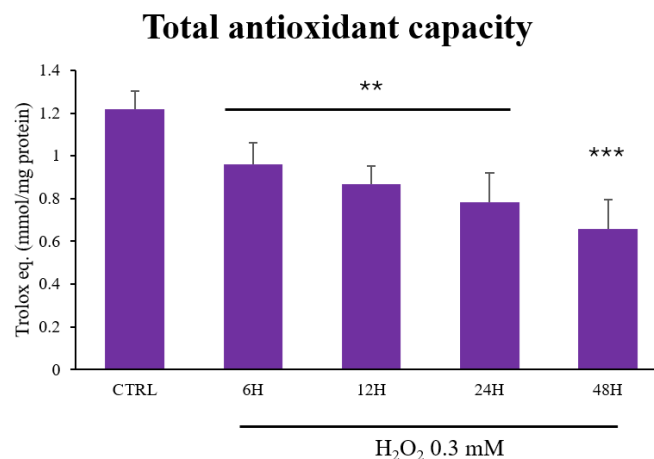


**Figure 2.**  $\text{H}_2\text{O}_2$  treatment and total antioxidant capacity. C2C12 myoblasts were treated with  $\text{H}_2\text{O}_2$  0.3 mM for different durations (6–48 h). The total antioxidant capacity (TAC) of C2C12 myoblasts is expressed as micromoles of Trolox equivalent/mg protein tested. Data presented are the mean  $\pm$  SD of three different experiments, each performed in triplicate. Statistical analysis was conducted using one-way ANOVA followed by Bonferroni's multiple comparisons test. \*\*\*  $p < 0.01$  and \*\*  $p < 0.05$  compared to control (CTRL).

### 3.3. Effect of MOLE Supplementation on C2C12 Myoblast Viability

To evaluate the effect of MOLE administration on C2C12 myoblasts, cells were treated with a range of working solutions (1:50–1:1000 stock solution dilutions) for 24 h. No statistically significant effect of MOLE administration was observed (Figure 3A).

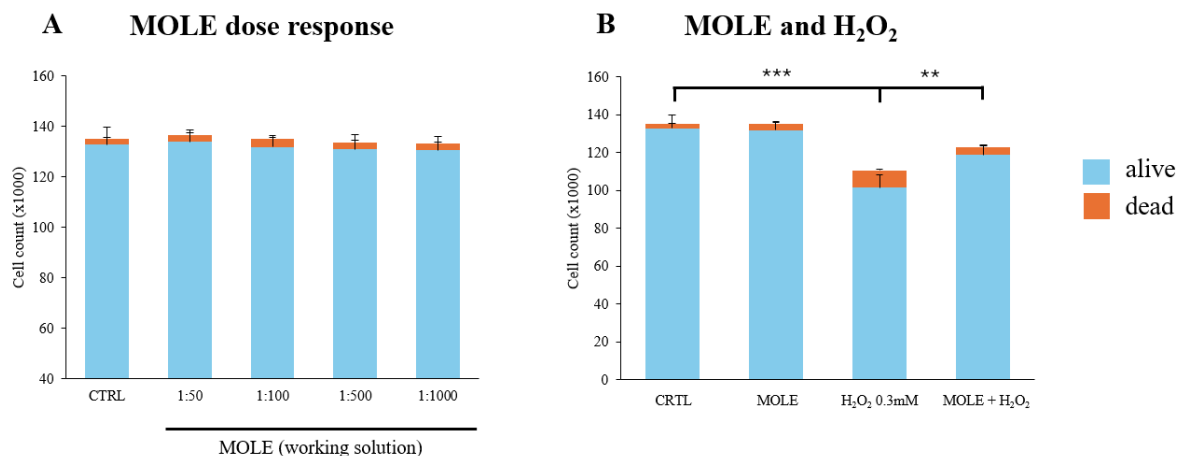


**Figure 3.** Effect of MOLE and  $\text{H}_2\text{O}_2$  in single and combined treatments on C2C12 myoblast viability. (A) C2C12 myoblasts were treated with different MOLE dilutions (1:50–1:1000 of stock solution) for 24 h; (B) C2C12 myoblasts were treated single and combined treatments of  $\text{H}_2\text{O}_2$  0.3 mM and MOLE (1:100 working solution) for 24 h. Cell viability was assessed using the Trypan blue exclusion assay. Results are expressed as the number of cells ( $\times 1000$ ) counted. Data presented are the mean  $\pm$  SD of three experiments, each performed in triplicate. One-way ANOVA was performed, followed by Bonferroni's multiple comparisons tests for panel (A) and (B), respectively. \*\*\*  $p < 0.001$  vs. CTRL; \*\*  $p < 0.01$  vs.  $\text{H}_2\text{O}_2$ .

For the subsequent experiments, we selected  $\text{H}_2\text{O}_2$  at 0.3 mM and a MOLE dilution of 1:100. Among the different working solutions of MOLE, we chose the 1:100 dilution because it has been shown to have important biological effects in in vitro experiments on muscle cells [29,43–45].

We found that hydrogen peroxide exposure induced a decrease in the total number of cells (24% reduction,  $p < 0.001$ ) and a fourfold increase in the number of dead cells ( $p < 0.001$ ) compared to control cells (Figure 3B). Notably, MOLE administration significantly mitigated the harmful effects induced by hydrogen peroxide ( $p < 0.01$ , Figure 3B).

### 3.4. Effect of MOLE on C2C12 Myoblast Migrative Ability

To investigate the effects of our treatments on the migratory ability of myoblasts, C2C12 grown under proliferative conditions (<50% confluence) was subjected to a scratch wound assay, and wound closure was measured after 24 h of recovery [49]. Briefly, in the wound closure assay, a wound of 1 mm was created in the culture plate before each treatment, and after 24 h, the width of the wound was measured in the control and treated cell samples.

After 24 h, reductions in wound width of 48% and 44% were observed for the CTRL and MOLE samples, respectively (Figure 4). After a single 24 h  $H_2O_2$  treatment, a minor reduction was observed compared to the control (20% reduction,  $p < 0.05$ ).

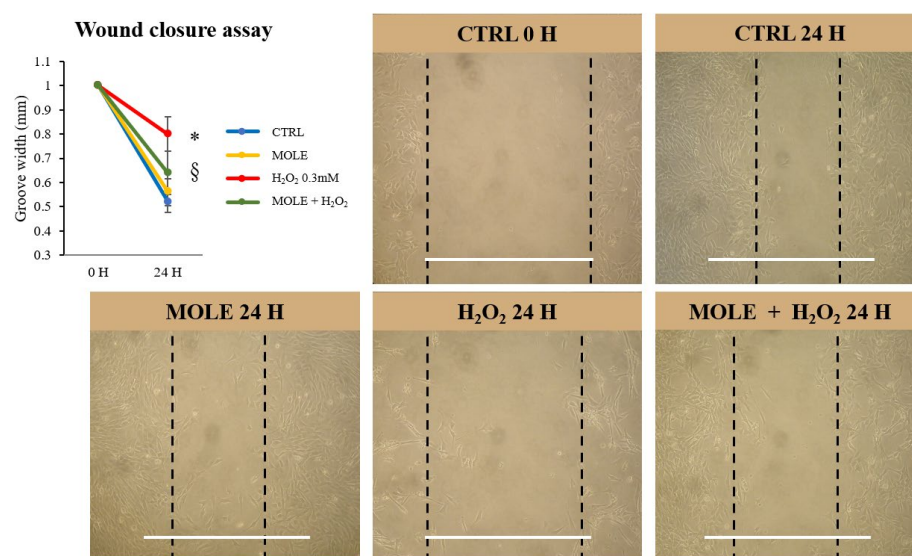


**Figure 4.** Wound closure assay. A 1 mm sulcus was created in the cell culture under sterile conditions before the experimental protocols were carried out. Cells were treated with MOLE (1:100 working solution),  $H_2O_2$  0.3 mM, or combined treatments, as well as a vehicle control, for 24 h. The sulcus width was then measured in both the control and treated cell samples. On the graph, the effects of MOLE,  $H_2O_2$ , and combined treatment on sulcus width are shown. The images shown are representative of the experiments and all treatments performed. The white lines indicate the 1 mm measurement of the groove created before the treatments. Data and representative images presented are the mean  $\pm$  SD of three experiments, each performed in triplicate. One-way ANOVA was performed followed by Bonferroni's multiple comparisons tests. \*  $p < 0.05$  vs. CTRL; §  $p < 0.05$  vs.  $H_2O_2$ .

The combined MOLE +  $H_2O_2$  treatment showed that the presence of MOLE enhanced the cells' ability to close the wound, resulting in a 36% reduction in wound width compared to  $H_2O_2$  alone ( $p < 0.05$ , Figure 4).

### 3.5. Effect of MOLE on C2C12 Myoblast Redox Status and Total Antioxidant Capacity

The hydrogen peroxide-induced perturbation of the redox state and the effect of MOLE were evaluated by analyzing glutathione homeostasis. In particular, the GSH/GSSG ratio is a well-known marker of redox status. We found statistically significant differences in the GSH/GSSG ratio between the different treatments of the C2C12 myoblasts. The  $H_2O_2$  treatment induced a decrease in total glutathione levels (tGSH, 13%  $p < 0.01$ ), an increase in GSSG levels (152%  $p < 0.05$ ), and a decrease in the GSH/GSSG ratio (51%  $p < 0.05$ ) compared

to the control (Table 2). No statistically significant differences in glutathione homeostasis were found after the MOLE treatments. Interestingly, the combination of MOLE with H<sub>2</sub>O<sub>2</sub> significantly prevented the H<sub>2</sub>O<sub>2</sub>-induced increase in oxidized glutathione ( $p < 0.01$ , Table 2) and the decrease in the GSH/GSSG ratio ( $p < 0.01$ , Table 2).

**Table 2.** Glutathione homeostasis evaluation <sup>1</sup>.

| Total glutathione (tGSH)                         |                |
|--------------------------------------------------|----------------|
| CTRL                                             | 206.5 ± 7.5    |
| MOLE                                             | 222.9 ± 7.8    |
| H <sub>2</sub> O <sub>2</sub> 0.3 mM             | 181.6 ± 3.3 ** |
| MOLE + H <sub>2</sub> O <sub>2</sub>             | 199.4 ± 13.5   |
| Oxidized glutathione (GSSG)                      |                |
| CTRL                                             | 20.5 ± 4.3     |
| MOLE                                             | 25.4 ± 0.8     |
| H <sub>2</sub> O <sub>2</sub> 0.3 mM             | 31.2 ± 1.5 **  |
| MOLE + H <sub>2</sub> O <sub>2</sub>             | 26.6 ± 0.4 §   |
| Reduced to oxidized glutathione ratio (GSH/GSSG) |                |
| CTRL                                             | 9.5 ± 1.9      |
| MOLE                                             | 7.8 ± 0.1      |
| H <sub>2</sub> O <sub>2</sub> 0.3 mM             | 4.8 ± 0.4 *    |
| MOLE + H <sub>2</sub> O <sub>2</sub>             | 6.5 ± 0.4 §    |

<sup>1</sup> Total glutathione (tGSH) nanomol/mg protein, oxidized glutathione (GSSG, nanomol/mg protein), and the reduced-to-oxidized glutathione ratio (GSH/GSSG) were evaluated in C2C12 myoblasts after 24 h in the presence of MOLE (1:100 working solution), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, 0.3 mM), and combined treatments. Data are presented as the mean ± SD of three independent experiments, each performed in triplicate. Statistical analysis was conducted using a two-way ANOVA followed by Bonferroni's multiple comparisons test. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. CTRL; §  $p < 0.01$  vs. H<sub>2</sub>O<sub>2</sub>.

The analysis of total antioxidant capacity revealed a statistically significant reduction following 24 h of H<sub>2</sub>O<sub>2</sub> treatment ( $p < 0.001$ , Figure 5). The combined treatment with MOLE significantly reversed this decrease ( $p < 0.01$ , Figure 5).

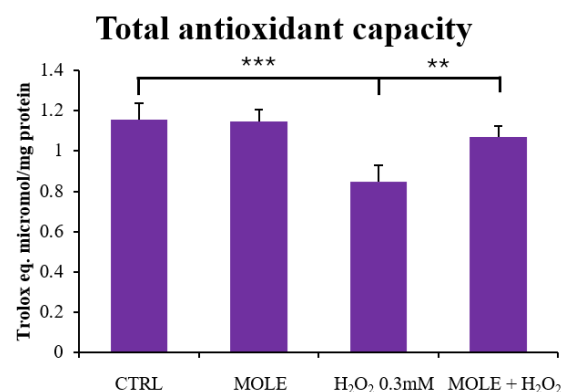


**Figure 5.** Effect of MOLE and H<sub>2</sub>O<sub>2</sub> on total antioxidant capacity in C2C12 myoblasts. C2C12 myoblasts were treated with H<sub>2</sub>O<sub>2</sub> 0.3 mM, MOLE (1:100 working solution), and combined treatments for 24 h. The total antioxidant capacity (TAC) of cell lysates was measured and is expressed as micromoles of Trolox equivalent/mg protein. Data are presented as the mean ± SD of three independent experiments, each performed in triplicate. Statistical analysis was conducted using one-way ANOVA followed by Bonferroni's multiple comparisons test. \*\*\*  $p < 0.001$  vs. CTRL; \*\*  $p < 0.01$  vs. H<sub>2</sub>O<sub>2</sub>.

#### 4. Discussion

Oxidative stress is a condition that can seriously compromise cellular functionality, primarily through the disruption of cellular antioxidant homeostasis. One potential strategy to counteract oxidative stress is the use of bioactive molecules with antioxidant properties.

This approach is particularly important in areas that focus on muscle metabolism, muscle function, and sports performance.

In this context, to meet the increasing demand for nutritional interventions targeting oxidative stress, many studies have focused on strategies aimed at enhancing physical performance, and especially reducing fatigue and increasing exercise endurance. These conditions are closely linked to elevated oxidative stress levels [24,52–54].

Skeletal muscle tissue is particularly vulnerable to the harmful effects of oxidative stress due to its high oxygen consumption. During physical exercise, especially in intense or unaccustomed activities, muscle contractions are often accompanied by high ROS production due to increased metabolic activity. This activity can lead to oxidative stress and potential myofibrillar damage, which is evidenced by elevated biomarkers of oxidation in both skeletal muscle and blood [55,56].

To preserve muscle function and protect cells from excessive ROS exposure, the use of antioxidants has become a common strategy. Along these lines, appropriate antioxidant use has been shown to effectively balance the oxidant–antioxidant ratio in most physiopathological conditions [52,57,58].

Among the natural extracts gaining recent popularity, *Moringa oleifera* extract has shown promising results in muscle cell models as a supplement capable of counteracting oxidative stress. We previously demonstrated that *Moringa oleifera* leaf extract (MOLE) shows dose-dependent total antioxidant capacity in cell-free systems, and this indicates that the efficacy of the leaf extract depends on the concentration of all the antioxidant molecules present [43,44]. Moreover, MOLE has been shown to activate the antioxidative metabolism through the SIRT1-PPAR and Nrf2 pathways (including its target gene, HO-1), both of which are regulators of cellular resistance to oxidants in C2C12 myotubes.

MOLE also enhanced cellular antioxidant capacity by improving glutathione redox homeostasis and increasing antioxidant enzyme activities in C2C12 myotubes [29,43]. These effects were significant in protecting myotubes under oxidative insult conditions induced by H<sub>2</sub>O<sub>2</sub> treatment [44].

Muscle microtrauma commonly occurs after exercise and is normally associated with elevated inflammatory processes and reactive oxygen species (ROS) production, which may lead to the secondary damage of myofibers [59]. In response to such injury, satellite cells are activated, exiting their quiescent state, entering the cell cycle, and proliferating. These cells give rise to new cells that either return to quiescence (self-renewal) or proceed to terminal differentiation. During this phase, the cells become myoblasts, which fuse with damaged myofibers to form new muscle fibers or, alternatively, assist in growing old uninjured fibers [2,60,61].

In this study, we focused on myoblasts under oxidative insult and supplementation with a mix of molecules with antioxidant action (for example, polyphenols, phenolic acids, etc.) because the efficiency of myoblasts is a key factor for optimal muscle regeneration.

Recently, studies utilizing antioxidant compounds to mitigate excess ROS production in skeletal muscle during the repair process following myotrauma have provided evidence of enhanced muscle recovery. This recovery is characterized by the upregulation of the myogenic potential, viability, maintenance, and activity of satellite cells in both in vitro and in vivo experimental models [62–64].

In this study, we employed C2C12 myoblasts, which are derived from satellite cells and serve as a robust model for investigating muscle proliferation and differentiation in vitro [65–67]. C2C12 cells are widely used to study muscle regeneration due to their capacity, under appropriate stimuli, to transition from a proliferative phase into differentiated myofibers, resembling the behavior of satellite cells.

The present study demonstrated that mild, non-cytotoxic H<sub>2</sub>O<sub>2</sub> treatment caused growth inhibition, perturbation in redox status, and a decrease in total antioxidant capacity in C2C12 myoblasts. These negative effects induced by oxidative insults can seriously compromise the metabolism of satellite cells and, consequently, the regenerative capacity of muscle fibers. Given that antioxidant supplementation is one strategy to counteract these

effects, in this work we also evaluated the effect of *Moringa oleifera* leaf extract (MOLE) on the vitality and proliferation, as well as the redox state, of myoblasts subjected to oxidative insult.

Interestingly, we found that MOLE supplementation had a significant effect in counteracting the harmful effects of hydrogen peroxide by restoring redox status and improving the vitality and proliferation of C2C12 myoblasts.

*Moringa oleifera* Lam. is commonly referred to as the “miracle tree” due to its numerous beneficial properties, which have significant implications for both therapeutic and nutritional applications. In fact, different parts of the plant, such as the leaves, seeds, and roots, are used by different populations in the world to treat various pathological conditions. For example, *Moringa oleifera* preparations are used to support cardiovascular health and regulate blood glucose levels, exhibiting antioxidant, anti-inflammatory, and potential anti-cancer properties [68–76].

In particular, the leaves of *Moringa oleifera* are highly nutritious, making it easy to incorporate them into one’s daily diet to benefit from their therapeutic and nutritional properties. Leaves can be eaten fresh, such as in salads, or dried to produce moringa powder and added to dishes. However, the powder should not be heated above about 40 °C so as not to denature the myrosinase. Many valuable bioactive molecules are present in fresh leaves and many of these molecules are retained in significant quantities even after preparation for food consumption [35,77].

First, we conducted a metabolomics analysis of the ethanolic extract of the leaves. The highly sensitive high-resolution LC-MS/MS technique [78], specifically using a ZenoTOF 7600 mass spectrometer, significantly enhanced the detection and confident identification of a higher number of bioactive molecules in the MOLE extracts by combining the Zeno-DDA and Zeno-SWATH approaches [45]. This analysis identified the presence of many bioactive molecules, including glucosinolates, flavonoids, phenolic acids, and other metabolites. Among them were amino acids, vitamins, and fatty acids with important nutritional value.

Most of these molecules show antioxidant properties, which is further supported by their effectiveness in mitigating the oxidative damage induced by H<sub>2</sub>O<sub>2</sub> in the cell culture model. The antioxidant capacity of the leaf extract is likely attributable to the collective properties of the various bioactive molecules present in Brassicaceae extracts, particularly in *Moringa oleifera*, as previously documented [79–85]. Isothiocyanates, polyphenols, flavonoids, and phenolic acids are known to exhibit antioxidant abilities, either preventing hyperperoxide decomposition or inactivating lipid free radicals [86–88]. Glucosinolates (GSLs) are typical of the order Brassicales (e.g., Brassicaceae, Capparaceae, Caricaceae, etc.), and are known for their health-promoting and antioxidative properties, which are mediated by the bioactive molecules present [89–93]. Glucosinolates are hydrolyzed by myrosinase activity into isothiocyanates, such as benzyl isothiocyanate, phenyl isothiocyanate, and sulforaphane [94]. These compounds are potent activators of the enzymatic antioxidant system, primarily by upregulating Nrf2-mediated gene induction, which includes increases in the mRNA levels of GCLC, NQO1, and HO-1, as well as elevated HO-1 protein levels. Additionally, the p38 MAPK signaling pathway [95], known for its role in regulating Nrf2 phosphorylation and nuclear translocation, is also activated by isothiocyanates [96,97].

Isothiocyanates support mitochondrial function and help maintain protein integrity under oxidative stress. It has been demonstrated that sulforaphane, one of the most studied isothiocyanates, is able to reduce muscle damage and inflammation in individuals subjected to oxidative stress induced by physical exercise [98]. Furthermore, it alleviates muscle soreness by upregulating Nrf2-target NQO1 expression [99].

Isothiocyanates also exhibit Nrf2-independent effects, including the inhibition of mitochondrial fission and the modulation of the mTOR pathway [100].

Moreover, glucosinolates, in addition to regulating oxidative stress levels, influence inflammation, cell proliferation, cell-cycle arrest, apoptosis, angiogenesis, and invasion, as well as modulating the activity of cancer cells [101–103].

It should be noted that the metabolomic analysis detected only very small amounts of isothiocyanates compared to glucosinolates in the MOLE sample, including 4-[( $\alpha$ -L-rhamnosyloxy) benzyl]isothiocyanate, 4-[(2'-O-acetyl- $\alpha$ -L-rhamnosyloxy)-benzyl]-isothiocyanate, 4-[(3'-O-acetyl- $\alpha$ -L-rhamnosyloxy)-benzyl]-isothiocyanate, and 4-[(4'-O-acetyl- $\alpha$ -L-rhamnosyloxy)-benzyl]-isothiocyanate (derived from glucomoringin, 4-O-acetyl-rhamnopyranosyloxybenzyl-GS 1, 4-O-acetyl-rhamnopyranosyloxybenzyl-GS 2, and 4-O-acetyl-rhamnopyranosyloxybenzyl-GS 3, respectively). However, as a limitation, we cannot exclude their production from glucosinolate metabolism in myoblasts.

Polyphenols, including subclasses such as flavonoids, flavanols, and phenolic acids [104–106], are well known for their antioxidant properties, which involve scavenging and neutralizing free radicals [107]. They mitigate oxidative stress and cellular damage induced by reactive oxygen species (ROS) [108], modulate the activity of endogenous antioxidant enzymes such as SOD, CAT, and GPx [107,109], and regenerate other antioxidants, such as vitamins C and E [110]. Moreover, these molecules are able to activate specific cellular defense mechanisms and to promote the repair of damaged molecules. This has a significant consequence of enhancing cellular resilience against oxidative damage [111].

Flavanols, such as kaempferol and quercetin, are well-known potent antioxidants capable of inhibiting lipid peroxidation and enhancing endogenous antioxidant defenses through the activation of enzymes like superoxide dismutase (SOD) and catalase (CAT) [112]. Notably, quercetin protects myotubes against tumor necrosis factor (TNF)-induced muscle atrophy under obese conditions by inducing Nrf2-mediated heme oxygenase-1 (HO-1) expression while inhibiting NF- $\kappa$ B activation [113]. Furthermore, it has been demonstrated that quercetin is able to promote mitochondrial biogenesis in skeletal muscles. This has an important impact on improving cellular mitochondrial function, protein content, enzyme activity, and respiratory function [23,114–117].

Additionally, catechin flavonoids present in *Moringa oleifera*, such as epicatechin and epigallocatechin, are recognized for their antioxidant properties [118–122]. Polyphenols also play a crucial role in maintaining cellular proliferative capacity under oxidative stress [123–125]. Among these, phenolic acids, including hydroxybenzoic acids (e.g., gallic acid) and hydroxycinnamic acids (e.g., caffeic acid), exhibit antioxidative properties that contribute to cellular defense against oxidative stress. Specifically, gallic acid and caffeic acid scavenge free radicals, inhibit lipid peroxidation, and preserve cellular health by modulating antioxidant enzyme activity [126–128].

While *Moringa oleifera* leaves (MOLE) contain various bioactive molecules with antioxidant action, it is important to recognize that the biological effects of the extracts likely stem from the synergistic effects of the entire mixture of bioactive compounds rather than a single component [29,43,45]. Key molecules in MOLE include glucosinolates (glucomoringin and 4-O-acetyl-rhamnopyranosyloxybenzyl-GS) and other important molecules, such as flavonoids (e.g., isoquercitrin, astragalin, and rutin), phenolic acids (e.g., chlorogenic acid), and lipids (e.g., omega-3 alpha-linolenic acid). These compounds contribute to the antioxidant protective action of MOLE either independently [129–131] or through synergistic effects [29,43,45].

Interestingly, treatment with MOLE increased proliferation in H<sub>2</sub>O<sub>2</sub>-treated cells, as evidenced by accelerated recovery in the wound scratch assays compared to the control cultures. This assay simulates a damaging situation, and myoblasts at the injury site are evaluated for their migration and proliferation capacity [49]. It mimics the degeneration/inflammation phase preceding muscle regeneration, during which satellite cells/myoblasts migrate to the injury site, proliferate, and begin the differentiation process.

As discussed previously, the production of reactive oxygen or nitrogen species following increased metabolic rate/muscle activity and the increase in inflammatory events following mechanical stress/fiber rupture and inflammatory reaction create oxidative damage, as widely evidenced in the literature with the increase in markers such as malondialdehyde (MDA), thiobarbituric acid reactive substances, and protein carbonyls, which are detected in the blood or muscle cells [132,133].

At the same time, no meaningful differences in antioxidant enzyme activity are reported in the blood [132]. However, in whole muscle lysates, the GSH/GSSG ratio is frequently decreased after the oxidative insult, whereas the levels of antioxidant enzyme activity only slightly increase, most notably when compared with markers of oxidative stress/damage [132,133].

The presence of MOLE in combination with H<sub>2</sub>O<sub>2</sub> facilitated the restoration of wound closure capability. This was attributed to the improvement of the redox state of myoblasts, as demonstrated by the evaluation of the GSH/GSSG ratio and the analysis of total antioxidant capacity, thereby partially restoring the resting condition negatively affected by the oxidative insult from H<sub>2</sub>O<sub>2</sub>. Under our experimental conditions, the GSSG content was higher than that found in vivo [134]. However, these are values that are always found using the experimental model of C2C12 muscle cells.

The evaluation of the intracellular TAC showed that the treatment with MOLE, due to the presence of bioactive molecules with antioxidant action, counteracted the decrease in antioxidant capacity observed after the treatment with hydrogen peroxide.

However, in the analysis of the data, the experimental limitations that this method entails must be taken into consideration [135]. Certainly, more in-depth studies on the modulation of the level of molecules with intracellular antioxidant potential are warranted in the future.

Overall, our results provide important insights into the regeneration process of skeletal muscle, which is a complex event mediated by satellite cells. Maintaining the correct redox balance is crucial for the proliferative and regenerative capacity of myoblasts. A detailed understanding of these mechanisms is essential for comprehending pathological conditions that lead to skeletal muscle degeneration and for identifying targeted therapeutic strategies. In this context, *Moringa oleifera* leaf extract emerges as a protective supplement against oxidative stress and could help muscle regeneration processes.

## 5. Conclusions

This study demonstrated that oxidative stress induced by H<sub>2</sub>O<sub>2</sub> exposure disrupted the redox balance and reduced cell proliferation in C2C12 myoblasts. The combined treatment with MOLE exhibited antioxidant effects by significantly restoring total cellular antioxidant capacity, improving the GSH/GSSG ratio, and enhancing cell viability, leading to the restoration of the proliferative capacity of the cells. Thus, we conclude that MOLE serves as a protective supplement against oxidative stress and can support muscle regeneration processes, making it a significant aid in conditions related to reduced muscle regenerative capacity. Further studies on the molecular mechanisms underlying the effects of MOLE, particularly in degenerative conditions, are warranted.

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## Abbreviations

Moringa oleifera leaf extract (MOLE), reactive oxygen species (ROS), glucosinolates (GLs), reduced (GSH) and oxidized (GSSG) glutathione, reduced-to-oxidized glutathione ratio (GSH/GSSG), total antioxidant capacity (TAC).

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## Review

# Protective Role of Physical Activity and Antioxidant Systems During Spermatogenesis

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**Abstract:** Oxidative stress is a significant factor that contributes to male infertility and sperm dysfunction. In this condition, an increase in ROS production exceeds the body's antioxidant defenses, resulting in a decline in spermatozoa quality and fertilizing capacity. Furthermore, excessive ROS production has been linked to the promotion of genomic damage, lipid peroxidation, inflammation, altered enzyme activity, and ultimately, irreversible alterations, cell death, and a decline in seminal parameters associated with male infertility. It is established that physical activity (PA), acting on inflammatory parameters and improving antioxidant defense, can alleviate the negative effects caused by free radicals, offering numerous health benefits and positively influencing sperm quality. The objective of this review is to highlight the mechanisms of ROS production, the physiological and pathophysiological roles of ROS in relation to the male reproductive system, and recent knowledge on the impact of some protocols of PA on these systems and the molecular mechanisms involved.

**Keywords:** lifestyle; physical activity; sport; oxidative stress; spermatogenesis; steroidogenesis



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## 1. Introduction

Infertility is a significant global issue, affecting over 12% of couples worldwide, with male factors being, in approximately 20% of all cases, solely responsible, while in another 30% to 40% of all infertility cases, they are contributing factors [1,2]. Male fertility relies heavily on spermatogenesis, the process that produces large quantities of sperm cells (spermatozoa) in the testis. Spermatogenesis is a meticulously orchestrated biological process that culminates in the production of haploid spermatozoa capable of fertilizing an oocyte. This process involves three primary stages: proliferation of spermatogonia, the meiotic divisions, and the spermiogenesis [3]. One of the most significant aspects of the final stage is nuclear remodeling, where the compacting of DNA is achieved by replacing histones with protamines with the goal of safeguarding the genetic material, ensuring the optimal compaction of the paternal genomic material and its protection during the journey through the female reproductive tract [4]. Indeed, spermatozoa inherently generate large amounts of reactive oxygen species (ROS), impairing sperm motility and damaging DNA through the oxidation of membrane lipids and nucleic acids [5]. However, their role in spermatogenesis and sperm function is more nuanced, serving as both a

potential disruptor and a key regulator of normal physiological processes. In spermatozoa, ROS mediate capacitation, hyperactivation and acrosome reaction, which are fundamental processes for fertilization, and they take part in intracellular signaling pathways essential for sperm development and function, such as the production of intracellular cAMP, which activates Protein Kinase A (PKA) [6]. Moreover, ROS contribute to cellular balance by regulating processes like vascular tone, oxygen sensing, immune response, and even adhesion properties.

This duality necessitates a robust but carefully regulated antioxidant system, and spermatozoa possess several intracellular antioxidant enzymes to mitigate the effects of ROS and the importance of antioxidants extends beyond endogenous mechanisms [7]. As a matter of fact, exogenous supplementation with compounds such as coenzyme Q10, L-acetyl-Carnitine, vitamin C, and zinc has been widely studied for its potential to counteract oxidative stress in semen. These supplements have demonstrated benefits in improving sperm quality and fertility outcomes, but excessive antioxidant use can lead to a condition known as reductive stress, where the over-suppression of ROS disrupts sperm functionality [8].

Physical activity (PA) is widely recognized for its numerous health benefits, including reduced risks of obesity, diabetes, and cardiovascular disease, pathologies in which oxidative stress-related inflammatory responses is considered a causative process [9]. Interestingly, it also appears to have a role in reducing oxidative stress in sperm, a key factor in maintaining male fertility. However, the relationship between PA and sperm quality is complex, with various levels and types of activity showing differing effects [10]. While moderate PA may provide protective benefits, strenuous exercise has been linked to reduced semen quality in some cases, particularly in activities such as long-distance running and cycling. Animal studies, for example, have shown that running can slow testicular aging by mitigating oxidative stress and it might be possible that regular exercise, improving the redox homeostasis, may lower the generation of ROS, thereby preventing damage to sperm DNA and cellular structures [11].

Indeed, the influence of PA in improving the antioxidant response and redox homeostasis is widely recognized [9], while the regular practice of physical exercise enhances semen quality by means of improving blood circulation and reducing systemic inflammation [10]. In the other hand, PA-induced antioxidant response, counteracting oxidative damage to sperm DNA and lipid membranes, may contribute to the improvements in sperm motility and viability [8].

This narrative review intends to offer an overview of the dual role of reactive oxygen species and antioxidant defense in spermatogenesis, focusing on the potential role of sport and physical activity in maintaining or impairing redox homeostasis control during spermatogenesis or in sperm.

## 2. Spermatogenesis

Spermatogenesis is the mechanism by which spermatozoa are produced from spermatogonial stem cells (SSCs). SSCs proliferate via mitosis to produce spermatogonia and then specialized haploid spermatozoa via meiosis, and spermiogenesis [12,13]. These cells migrate to the gonadal ridge and, after a period of mitotic division to form spermatogonia, enter meiosis. Meiotic divisions and cytological changes transform spermatogonia into spermatozoa, a highly specialized cell equipped with a tail that allows the cell to move. The head of the sperm contains the nucleus, which contains DNA and is located at the periphery, separated from the cell membrane and the acrosome, which contains various substances for penetrating the oocyte. The central part of the sperm contains the mitochondria, organized in different helical arrays around the axoneme. Mitochondria in spermatozoa are crucial

for energy production, redox balance, calcium regulation, and apoptotic pathways, all necessary for sperm motility, capacitation, and fertilization [14]. Mitochondrial dysfunction and quantity can lead to decreased sperm quality and infertility [14].

Spermatogenesis is a complex process involving several hormonal and cells interactions. The hypothalamic release of gonadotropin-releasing hormone (GnRH) stimulates the anterior pituitary gland to secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Sertoli and Leydig cells are two major somatic cells that are pivotal for male reproduction [15]. These cells play critical roles in the process of spermatogenesis, ensuring proper development and maturation of sperm [16]. Sertoli cells, located within the seminiferous tubules of the testes, provide structural support and nourishment to developing germ cells [17]. They form the blood–testis barrier, regulate the environment of the seminiferous tubules, and secrete factors essential for the differentiation of spermatogonia into spermatozoa. Sertoli cells also produce inhibin, a hormone involved in the negative feedback regulation of FSH levels. Leydig cells, located in the interstitial space between the seminiferous tubules, influenced by LH, are responsible for the production of testosterone, which exerts its effects on Sertoli cells, promoting the maturation of germ cells and the progression of spermatogenesis. Consequently, the coordinated actions of GnRH, FSH, LH, Sertoli cells, Leydig cells, and testosterone work in concert to establish a regulated environment that facilitates the sustained production of sperm throughout the male reproductive life.

### 3. Redox Homeostasis and Physio-Pathological Conditions

The concept of “oxidative stress” refers to an imbalance between oxidants and antioxidants, favoring oxidants, which results in disrupted redox signaling, impaired control mechanisms, and/or molecular damage [18]. Originally introduced in 1985 [19], this concept has evolved to encompass advancements in our understanding of redox signaling [20]. Fundamentally, it describes a steady-state redox balance within an open metabolic system, maintained at a specific setpoint that ensures basal redox tone. Any deviation from this balance constitutes stress, triggering a stress response. The definition also acknowledges that (i) a shift to the opposite end of the balance constitutes “reductive stress”, and (ii) deviations can be physiological (“oxidative eustress”) or supraphysiological (“oxidative distress”) [18] (Figure 1).

Oxidative eustress plays a crucial role in physiological redox signaling and control [18,19], aligning closely with the concept of redox homeostasis as the “golden mean” [21].

Metabolic regulation involves a wide array of chemical processes, including the orchestrated modification of proteins, lipids, carbohydrates, and nucleic acids to maintain structure and function. Redox reactions play a significant role in this regulation, with notable interactions between redox modifications and other regulatory mechanisms, such as the phosphorylation and dephosphorylation of proteins. Low-molecular-mass chemically reactive molecules, often referred to as “reactive species”, have been extensively studied for their regulatory roles. These include reactive oxygen species (ROS) [22], reactive nitrogen species (RNS) [23,24], reactive sulfur species (RSS) [25], reactive electrophile species (RES) [26], and reactive halogen species (RHS) [27]. The interplay among these reactive species forms a complex system of checks and balances essential for effective redox regulation [28]. On the opposite side of the redox equilibrium, the defense mechanisms against excessive oxidant levels involve a variety of antioxidant enzymes supported by their auxiliary systems, alongside low-molecular-weight antioxidants, collectively forming an integrated antioxidant network [29]. The expression of these antioxidant enzymes is regulated by key redox signaling pathways as part of the oxidative stress response [18].

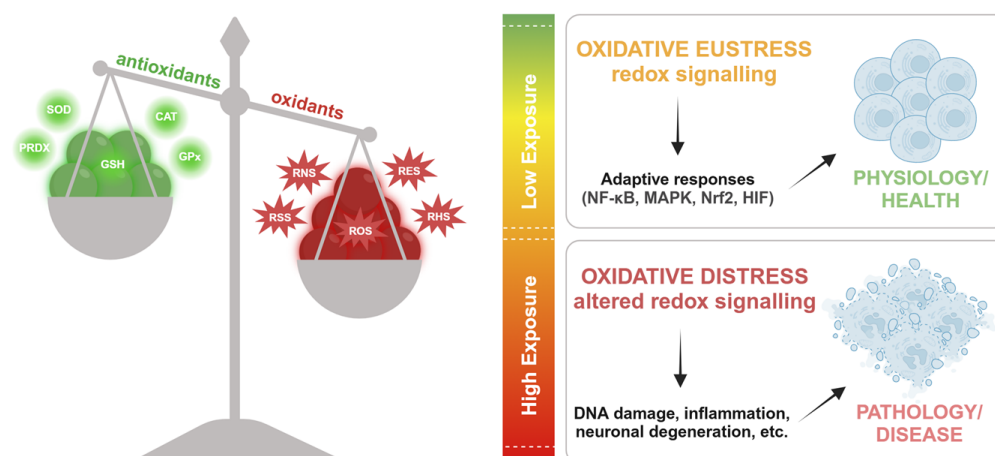


**Figure 1.** Redox balance and its physiological and pathological implications. The equilibrium between antioxidants (SOD, CAT, PRDX, GSH, GPx) and oxidants (RNS, RSS, ROS, RES, RHS) determines cellular redox responses. Low oxidant exposure induces *oxidative eustress*, promoting adaptive signalling (NF-κB, MAPK, Nrf2, HIF) and contributing to physiology and health. In contrast, high oxidant exposure leads to *oxidative distress*, disrupting redox signaling and resulting in pathological consequences such as DNA damage, inflammation, and neuronal degeneration. ROS—reactive oxygen species; RNS—reactive nitrogen species; RSS—reactive sulfur species; RES—reactive electrophile species; RHS—and reactive halogen species.

Maintaining homeostasis is a fundamental aspect of health [30], which is regarded as a dynamic and active biological process [31]. Achieving and sustaining physiological health depends on mechanisms that ensure homeostasis through resistance, tolerance, and resilience, reflecting a homodynamic nature [32,33]. The concept of eustress, as distinct from non-physiological distress, characterizes the body's continuous state of readiness to maintain homeostasis. This distinction, introduced by Selye [34], forms a foundational perspective in understanding stress and its responses [35].

Oxidation–reduction (redox) reactions are integral to life's processes. Research into elements such as oxygen, iron, copper, sulfur, selenium, and nitrogen, alongside studies on free radical oxidation and defense mechanisms, has uncovered the remarkable versatility and applications of redox reactions. These include roles in energy capture, mitigating oxygen toxicity, and producing biochemical defenses against harmful entities. Notably, redox reactions also play a pivotal role in signaling, as they enable fast and reversible processes essential for physiological regulation.

The complex integration of energetically demanding systems makes life in oxygen-rich environments possible. Similarly, this principle applies to the regulation of redox signaling, which governs numerous physiological processes. Ensuring redox homeostasis presents an ongoing challenge, requiring tight biochemical control [18]. For oxidation events that serve as physiological signals, counteractive mechanisms must deactivate the signals and restore the redox balance to maintain health [21].

For over three decades, an understanding of ill health has been associated with the oxidative stress concept in the form of 'an imbalance between oxidants and antioxidants in favor of the oxidants', which developed into the idea of 'disrupted redox signaling' [36]. The refinement of this concept has been hastened by the articulation of a 'Redox Code' in an influential paper describing a set of principles through which biological function is enabled and protected [37]. Many disease processes are attributed to enhanced production of reactive oxygen species (ROS) or 'dysfunctional Redox regulation'. Still, not everything can be explained by excessive ROS production. Many pathophysiological conditions may, in fact, exemplify processes that can be accounted for by other bioactive entities such as

reactive nitrogen or sulfur species (RNS, RSS), or other small signaling molecules such as hydrogen (H<sub>2</sub>), ammonia (NH<sub>3</sub>), and carbon monoxide (CO). Many of these entities can react with each other, with protein thiols or other biomolecules.

These varied interactions modulate the function of ion channels, enzymes, transcription factors, and other biological targets, a scenario defined as the ‘reactive species interactome’ [38]. This reactive species interactome concept provides a useful framework to explain the apparent complexity of adaptive signaling. There is no single marker or process that captures the complexity of these interactions adequately. Rather, it is likely that a combination of readouts from different levels of organization will be required to explain how and why mitochondrial function appears to be so intimately related to chronic disease, inflammation and metabolism.

#### 4. Redox Homeostasis and Spermatogenesis

Spermatogenesis is a highly regulated and continuous developmental process in which undifferentiated spermatogonia proliferate, undergo meiosis, and differentiate into mature spermatozoa [39,40]. This complex process is essential for male fertility and requires precise control to maintain testicular homeostasis. One of the key regulatory factors in spermatogenesis is redox homeostasis, which involves a delicate balance between reactive oxygen species (ROS) production and antioxidant defense mechanisms. Given the high metabolic activity required for sperm production, generating approximately 1000 sperm per second, mitochondrial oxidative phosphorylation is essential but also results in the production of significant levels of ROS [41,42]. Proper regulation of ROS is crucial, as excessive oxidative stress can impair spermatogenesis and male fertility [43].

ROS, including superoxide anions, hydrogen peroxide, and hydroxyl radicals, are natural byproducts of cellular metabolism, particularly mitochondrial ATP production. While low-to-moderate levels of ROS play physiological roles in cell signaling, differentiation, and sperm maturation [44,45], excessive ROS can be detrimental [43,46]. High levels of oxidative stress can lead to lipid peroxidation, DNA damage, and protein oxidation, ultimately disrupting the process of spermatogenesis [46]. An imbalance in testicular redox homeostasis has been associated with reduced sperm count, decreased motility, increased morphological abnormalities, and impaired fertilization capacity [46].

To counteract oxidative stress, cells involved in spermatogenesis are equipped with an array of antioxidant defense systems. Key antioxidant enzymes include superoxide dismutases (SODs), which convert superoxide radicals into hydrogen peroxide; glutathione peroxidases (GPXs) and peroxiredoxins (PRXs), which neutralize hydrogen peroxide; and glutathione S-transferases (GSTs) and thioredoxins (TRXs), which help maintain redox balance. These enzymes are widely expressed in testicular tissue and are regulated by redox-sensitive transcription factors, ensuring that oxidative stress does not reach detrimental levels [47,48].

Recent studies have also identified LanCL2 as a critical antioxidant gene specifically expressed in male germ cells, with high enrichment in spermatocytes and round spermatids [49]. Research using LanCL2 knockout mice has demonstrated that the absence of this gene results in testicular redox imbalance, leading to defects in spermatogenesis. LanCL2 deletion has been linked to premature spermatogonial self-renewal and impaired acrosomal maturation in spermiogenesis, ultimately reducing sperm count and motility. The disruption of testicular homeostasis in LanCL2-deficient mice suggests that this gene plays a crucial role in maintaining redox equilibrium and ensuring proper sperm development [49].

Genetic variations in antioxidant enzymes have been implicated in male infertility. Several polymorphisms in genes encoding key antioxidant proteins have been linked to

compromised sperm function and increased susceptibility to oxidative stress [50–54]. In animal models, the deletion of specific antioxidant genes has resulted in heightened sensitivity to oxidative stress and age-related reproductive decline [55,56]. However, the absence of major fertility defects in most antioxidant-deficient male mice suggests that testis-specific oxidative defense mechanisms have yet to be fully understood. Identifying these unique redox regulatory pathways, including the role of LanCL2, could provide new insights into male reproductive health and potential therapeutic strategies for male infertility.

Redox homeostasis plays a fundamental role in regulating spermatogenesis by balancing ROS production and antioxidant defenses. While physiological levels of ROS are necessary for sperm development and function, excessive oxidative stress can disrupt spermatogenesis and lead to male infertility. The identification of testis-specific oxidative defense mechanisms, such as the LanCL2-mediated pathway, remains an important area of research, with potential implications for understanding and treating male reproductive disorders. Future studies focusing on redox regulation in testicular cells may pave the way for novel therapeutic interventions aimed at preserving male fertility.

## 5. The Role of Oxidative Stress in Male Infertility

Several conditions may possibly alter oxidant/antioxidant balance, which could in turn lead to oxidative stress. These conditions include endogenous factors such as deficiencies in antioxidants, immune system dysfunctions bacterial/viral infections, abnormal spermatozoa, leukocytospermia (LCS) [57,58], and exogenous factors such as smoke, pollution, alcohol, obesity, varicocele or sexually transmitted diseases [59–61] (Figure 2).

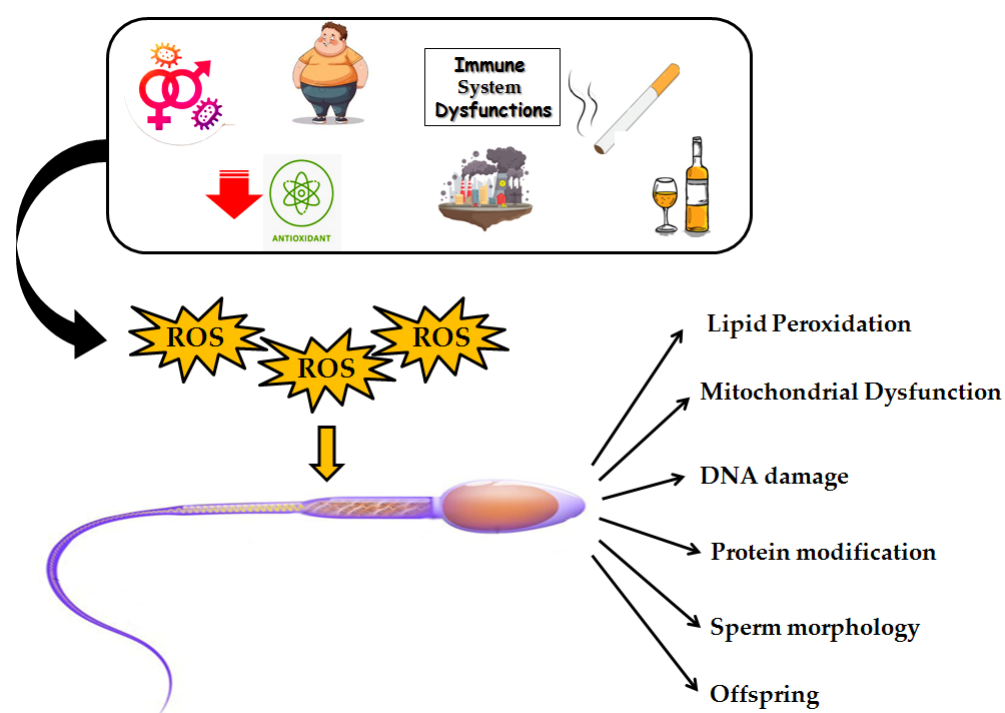


**Figure 2.** Endogenous and exogenous factors that alter oxidant/antioxidant balance, and that lead to oxidative stress and sperm dysfunctions.

Oxidative stress induced by environmental pollution is a critical environmental issue that exerts a significant influence on male fertility [62]. Pollutants, particularly particulate matter (PM), nitrogen dioxide (NO<sub>2</sub>), and ozone (O<sub>3</sub>), generate ROS within the body, resulting in cellular damage. These ROS have been shown to overcome the body's antioxidant defense mechanisms, contributing to inflammation, DNA damage and cellular dysfunction [63]. Several pollutants have been shown to induce oxidative stress in the male

reproductive system [64]. Specifically, endocrine-disrupting chemicals, such as phthalates and bisphenol A (BPA), can mimic or interfere with the action of hormones, thereby disrupting the normal functioning of the reproductive system, impairing sperm development, and decreasing testicular size [65]. These detrimental effects are not limited to immediate consequences but can also have long-term implications for male fertility. In fact, studies have demonstrated that individuals exposed to high levels of environmental pollutants may experience a decline in sperm count and concentration over time [66]. Moreover, the impact of pollution on fertility can extend across generations, as oxidative damage to sperm DNA may result in epigenetic modifications, a DNA fragmentation index, and mitochondrial dysfunction, all of which can lead to male infertility [67].

Indeed, sperm plays a crucial role in intergenerational and transgenerational inheritance, particularly through epigenetic mechanisms [68]. Unlike genetic mutations, epigenetic changes involve modifications, such as DNA methylation, histone modifications, and small non-coding RNAs (ncRNAs) expression, that alter gene expression without changing the underlying DNA sequence [69]. The epigenetic marks respond dynamically to environmental and lifestyle factors, and when modified, they can transmit altered gene expression patterns to the offspring, influencing their health and phenotype [70,71]. Oxidative stress has been significantly implicated in transgenerational sperm effects [72]. As indicated above, mature sperm lack robust DNA repair capabilities due to chromatin compaction, so they are particularly vulnerable to ROS-induced damage [73–75]. Several situations may lead to sperm exposure to ROS, whether it is secondary to aging, environmental exposure, pathological situations, or lifestyle factors, such as unbalanced diet, smoking, alcohol addiction, or physical inactivity [4]. Antioxidant supplementation has been shown to improve sperm function, reduce DNA fragmentation, and maintain sperm motility and fertility, potentially mitigating transgenerational negative impacts [72]. Modification of sperm epigenome by paternal exercise before conception has been demonstrated, significantly impacting metabolic changes in the offspring. Several results indicate that paternal exercise can modify fetal development, placenta inflammation, and tissue-specific gene and protein expression patterns in offspring organs such as the heart, skeletal muscle, tendon, hippocampus, and liver [76]. Although exercise interventions in fathers have shown promising potential to positively shape offspring phenotypes, a notable limitation on this topic is the lack of compelling *in vivo* evidence substantiating a causal link between paternal oxidative stress and transgenerational epigenetic modifications that impact male fertility in humans, so that the precise biological pathways remain unclear [77].

Furthermore, the testicular environment is particularly vulnerable to oxidative damage due to the high metabolic activity of spermatogenic cells [78] and poor vascularization, as well as the presence of a high amount of polyunsaturated fatty acids (PUFAs) in the plasma membrane in conjunction with the absence of cytoplasmic antioxidant enzymes [79–83]. ROS, indeed, can potentially damage sperm membranes organization, mitochondrial activity, and the function of testicular cells [83], consequently affecting germ cells motility and ability to fuse with oocytes, testis steroid hormone synthesis, and steroidogenic capacity [79,84,85]. Mitochondria are the energy powerhouses of the sperm cell, and any disruption in their function can lead to diminished motility, thus reducing the sperm's ability to fertilize an egg [86]. Studies have shown that men with lower sperm motility exhibit decreased glucose-6-phosphate dehydrogenase activity and increased levels of malondialdehyde, a marker of oxidative damage [78]. Furthermore, oxidative stress has been implicated in the disruption of the blood–testis barrier, the key structure that protects the developing germ cells from harmful substances, including ROS [87]. For these reasons, these cells are considered 'ideal' bioindicators of pollution and early sentinels of human health [88].

Interestingly, it has been demonstrated that excessive intake of antioxidants, particularly through high-dose supplements, has been subject to concerns regarding its impact on male fertility [89]. Studies have shown that over-scavenging of ROS can impair essential sperm processes, such as capacitation and the acrosome reaction, reducing fertilization potential [77,90]. Additionally, excessive antioxidants may disrupt sperm maturation and lower sperm quality and motility, as well as interfering with testosterone production, ultimately decreasing sperm production [90]. Consequently, achieving a balanced intake of antioxidants through dietary means rather than relying on supplements is imperative to maintain optimal male fertility. However, all these factors are modifiable and reversible, and hence, by merely changing one's lifestyle, many of these risk factors can be avoided.

#### *Testicular Cells and Oxidative Stress*

The testis is composed of several different cell types, each playing a vital role in the process of sperm production. Sertoli cells, Leydig cells, and germ cells are all integral components of this dynamic environment, and each are influenced by the redox status of the tissue. Sertoli cells, which provide nutritional and structural support to developing germ cells, are particularly sensitive to changes in oxidative balance. Under conditions of chronic oxidative stress, Sertoli cell function is compromised, leading to impaired spermatogenesis and, consequently, a reduction in sperm count and quality [91]. The presence of ROS was associated with significant levels of apoptosis in Sertoli cells; a substantial decrease in connexin-43 (Cx43) expression, a key component of gap junctions, which is pivotal to spermatogenesis regulation; and a failure to maintain the viability of spermatogonial stem cells (SSCs) [92].

Research on Sertoli cells and oxidative stress suggests that various environmental toxins have the potential to induce oxidative damage and apoptosis. It has been suggested that exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) may possibly impair Sertoli cell function through oxidative stress, affecting mitochondrial activity and membrane potential [93]. Similarly, it appears that sodium fluoride exposure may contribute to a decline in cell viability and an increase in oxidative stress and apoptosis. Furthermore, acrylamide and its metabolite glycidamide have been observed to cause oxidative stress and apoptosis in both Leydig and Sertoli cells, affecting cell viability and gene expression of apoptotic markers [94].

Exposure to oxidative stress has been demonstrated to result in a decline in testosterone production, an escalation in apoptosis, and an impairment in steroidogenesis in Leydig cells [94,95]. In 2016, Duan et al. investigated the effects of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) on primary rat Leydig cells and the role of peroxiredoxin 2 (Prdx2), an important antioxidant protein involved in oxidative stress response. The study revealed that H<sub>2</sub>O<sub>2</sub> treatment led to a significant decline in cell viability, inducing apoptosis in a dose-dependent manner and altering Prdx2 protein expression [95]. Subsequently, in 2021, Anak and colleagues explored the impact of aging on testosterone production, conducting a comprehensive literature review that investigated the role of antioxidants in safeguarding Leydig cells against oxidative stress [96]. Two key defects in the steroidogenic pathway have been identified as contributing to age-related reductions in testosterone production: reduced LH-stimulated cAMP production and impaired cholesterol transport to and within the mitochondria.

Increasing oxidative stress appears to play a crucial role in age-related testosterone reduction, and aging is associated with enhanced lipid peroxidation. Leydig cell membranes from older rats exhibit a two- to three-fold increase in basal thiobarbituric acid-reactive substances (TBARSs) formation, indicating increased oxidative damage. They demonstrated that aging leads to a decrease in serum testosterone levels in both humans and rodents.

This decline is not attributable to a loss of Leydig cells, but rather to their reduced capacity to produce testosterone in response to luteinizing hormone (LH) [97].

In spermatozoa, oxidative stress triggers lipid peroxidation of polyunsaturated fatty acids in sperm membranes [42,79], leading to protein aggregation in male germ cells, and potentially disrupting proteostasis [98]. Proteostasis in sperm cells is of pivotal significance for sustaining optimal functionality and fertility. It encompasses regulatory mechanisms that govern protein synthesis, folding, modification, and degradation [99]. Sperm cells are distinguished by their limited capacity for protein synthesis and repair, which renders proteostasis essential for their viability [99]. Disruptions in proteostasis can result in sperm dysfunction, impacting motility and fertilization potential; thus, the interplay between proteostasis and oxidative stress in sperm cells is pivotal to their functionality [98,99]. Proteostasis mechanisms have been shown to mitigate the harmful effects of oxidative stress [100]. Indeed, it has been demonstrated that the sustained production of misfolded proteins can exceed the capacity of the proteostasis network, resulting in its failure and subsequent cell death [101]. Morphological abnormalities in sperm are also frequently observed in men with high oxidative stress levels. These abnormalities, which may include defects in the acrosome, tail, or head, are often a result of lipid peroxidation and protein damage induced by ROS. These structural changes compromise the sperm's ability to penetrate the egg and perform its fertilization function.

Male infertility can be influenced by various endogenous sources of ROS in seminal plasma. The human semen sample contains a variety of cells, including immature and mature spermatozoa, round-shaped cells of different phases of spermatogenesis, epithelial cells, and leukocytes [102]. A significant contributor is leukocytes, particularly polymorphonuclear leukocytes and macrophages, which originate from the seminal vesicles and prostate gland. In the presence of urogenital infections or inflammation, these leukocytes exhibit an enhanced immune response, generating up to 100 times more ROS, thereby leading to oxidative stress [103], while immature, morphologically abnormal spermatozoa also serve as primary sources of ROS [59]. However, the rate of ROS production is up to 1000 times higher in leukocytes (extrinsic source) compared to spermatozoa (intrinsic source) [104].

## 6. Spermatogenesis and Physical Activity: The Role of Oxidative Stress Control

Regular moderate-intensity physical exercise is associated with an increase in antioxidant defense systems and a reduction in systemic oxidative stress [105]. This is largely due to the adaptation of various enzymatic and non-enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), which play key roles in neutralizing ROS [106]. Furthermore, PA has been found to improve mitochondrial function, which may contribute to a more efficient regulation of oxidative metabolism and reduced ROS production [107]. Conversely, intense or prolonged PA, especially when performed in an unconditioned state, can result in an acute increase in oxidative stress. This phenomenon is primarily attributable to the enhanced production of ROS during high-intensity exercise, which can exceed the body's antioxidant defense mechanisms [108,109].

As a result, oxidative damage can occur, leading to muscle fatigue, inflammation, and, potentially, impaired sperm quality. It is important to note that the beneficial effects of physical activity on oxidative stress are dose-dependent, with moderate levels of exercise being most effective in improving antioxidant capacity and mitigating the negative effects of oxidative stress [105,110].

### Impact of Physical Activity on Spermatogenesis

The etiology of male infertility is multifactorial, with physiological factors such as age and lifestyle factors, including physical activity, playing a role. However, the specific impact of sporting activity on semen parameters, and, consequently, on male fertility, remains unclear. Recent studies have explored the impact of physical activity on male fertility, highlighting the complex relationship between exercise and reproductive health [111–114].

Moderate exercise has been shown to improve sperm quality, including count, motility, and morphology, while excessive or severe exercise may have detrimental effects [10,115,116]. Regular physical exercise has been shown to enhance sperm count, motility, and morphology in both human and rat subjects, with a concomitant improvement in testosterone, LH, and FSH levels in rats. PA has been associated with increased serum testosterone levels due to improved circulation and enhanced nutrient delivery to the testes. Moreover, acute and chronic exercise stimulate sex steroidogenesis enzymes expression and activity and sex steroid hormone levels in skeletal muscle tissue [117,118]. Exercise has been demonstrated to have a positive impact on the secretion of FSH, thereby enhancing the support and nourishment of developing sperm cells [119], leading to a higher sperm count and improved sperm quality [120]. It has been demonstrated that exercise can reduce the percentages of sperm with negative tubular differentiation (TDI) and spermiogenesis indices (SPIs) and DNA fragmentation, and can ameliorate diabetes-induced apoptosis and improve sperm apoptosis index in animal models [121]. The positive effects of exercise on male fertility are thought to be mediated by reduced oxidative stress, enhanced antioxidant defense, and improved steroidogenesis [115–117]. Furthermore, the improvement of glucose metabolism that results from an increase in physical activity is often the primary factor that ameliorates sperm parameters in men, since most studies performed involved men affected by metabolic syndrome and type 2 diabetes [117,122–126] (Figure 3).

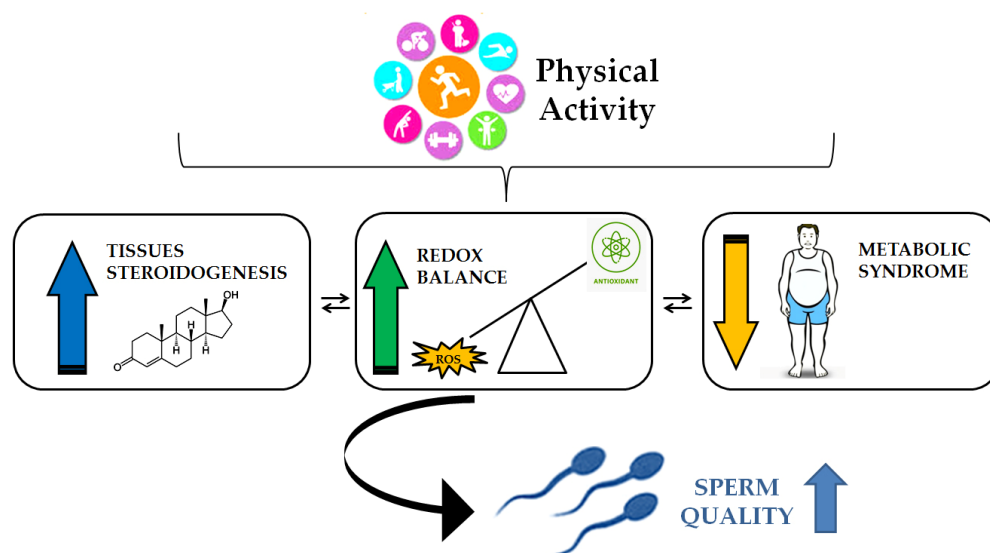


**Figure 3.** Physical activity can improve sperm quality by acting on various aspects of an individual's health. It increases steroidogenesis (in muscles and in the testes), improves redox balance, enhances antioxidants, and reduces metabolic dysfunction. In addition, these factors interact and influence each other to improve sperm quality.

Steroidogenesis, the process of producing steroids such as testosterone, plays a crucial role in regulating various physiological functions, including muscle growth, sexual function, and overall health. In this context, PA has been identified as a potential modulator of steroidogenesis, influencing both testicular and muscular pathways. Acute bouts of

intense exercise, such as heavy resistance training or endurance exercise, lead to transient increases in testosterone secretion [127]. This response is thought to be mediated by the hypothalamic–pituitary–gonadal axis, which stimulates the release of LH and, consequently, the production of testosterone by Leydig cells [127]. Research suggests that the impact of physical activity on male fertility depends on the intensity and duration of the exercise, as well as the profile of the participant [121]. Studies involving recreational athletes demonstrated positive effects of prolonged physical activity. In fact, 433 infertile men training at 70–85% of their maximal oxygen consumption revealed that high-intensity exercise may restrain inflammatory biomarkers, oxidative stress, and antioxidants, while concomitantly enhancing semen parameters and the pregnancy rate [115,121].

While moderate exercise may have beneficial effects on sperm quality, high-intensity or prolonged exercise can negatively affect semen parameters [120,128,129]. Intense physical activity has been associated with decreased sperm concentration, motility, and morphology, particularly in elite athletes [129]. It has been observed that endurance exercise, including activities such as long-distance running or extensive cycling, has the potential to adversely affect seminal parameters [112]. A 16-week low-to-intensive cycling training program resulted in a decline in sperm quality parameters and an increase in seminal inflammatory markers, with some effects persisting even after a 30-day recovery period [130]. The same training regimen has also been shown to result in increased oxidative stress and decreased antioxidant capacity in semen [130]. A further study comparing endurance cyclists to sedentary controls found significantly lower proportions of morphologically normal sperm in cyclists [131]. At the same time, moderate running has been shown to improve cardiovascular health, reduce oxidative stress, and enhance hormone regulation, all of which can potentially benefit male fertility. Moreover, the regulation of insulin sensitivity and the reduction in visceral fat, both outcomes of consistent running, are linked to improved hormonal profiles, including better testosterone levels. However, excessive endurance running or high-intensity training may have the opposite effect, leading to a decrease in sperm quality [129]. This decline in sperm quality is attributed to mechanical impact, gonadal overheating, wearing tight clothes, and increased oxidative stress [59,129]; however, these effects may be reversible with proper rest and recovery periods [112].

Ultimately, the relationship between exercise and male fertility remains complex, with some studies showing conflicting results and difficulties in quantifying physical activity [10]. Further evidence showed the influence of physical exercise on the endocrine system, particularly the hypothalamo–pituitary–adrenal (HPA) axis and stress hormones production, in turn influencing testosterone production. The intensity and duration of exercise have been demonstrated to modulate the HPA axis response, resulting in increased cortisol secretion [132]. While endurance training does not lead to permanent hypercortisolism, it has been observed to result in decreased tissue sensitivity to glucocorticoids [132].

The relationship between cortisol and testosterone during exercise is a complex one, too. Post-exercise, a significant negative correlation between cortisol and total testosterone has been observed, while there is a positive correlation with free testosterone [133]. Prolonged imbalances in cortisol and growth hormone secretion can be detrimental to health [134]. In cases of overtraining, the sympathetic/parasympathetic imbalance and neuroendocrine dysfunction hypotheses have been proposed to explain performance decrements and recovery issues [135]. It is therefore crucial to ensure proper exercise planning with sufficient recovery to prevent overtraining and maintain hormonal balance.

Exercise-induced oxidative stress can be counteracted by the consumption of nutritional strategies [136] and antioxidant-rich foods [137], which can further support the maintenance of sperm quality. Antioxidant supplementation has shown potential in improving sperm quality and fertility outcomes in some individuals [137]. Specific antioxidants such

as selenium, zinc, omega-3 fatty acids, CoQ10, and carnitines have been positively associated with sperm quality [138]. However, excessive antioxidant use may be detrimental to sperm function, resulting in a paradoxical decline in sperm quality [138], which is similar to the effect seen with oxidative stress.

However, an exact cut-off for “excessive” has not been well defined in the existing literature. A balanced diet rich in natural antioxidants from fruits, vegetables, whole grains, legumes, and seeds is recommended as a safe and effective approach to meet antioxidant requirements in physically active individuals and athletes [139].

## 7. Conclusions and Future Perspectives

This review underscores the significant role of exercise in modulating male reproductive health, primarily through its impact on oxidative stress and inflammatory pathways. The findings suggest that engaging in moderate-intensity exercise over a prolonged period can effectively suppress pro-inflammatory cytokine production, improve antioxidant defense mechanisms, and enhance sperm DNA integrity. Such effects are particularly beneficial for individuals experiencing infertility, as oxidative stress is known to impair sperm quality and overall testicular function. High-Intensity Interval Training (HIIT) has also demonstrated potential in improving sperm characteristics in men with fertility issues, further supporting the notion that structured physical activity can be an effective intervention for reproductive health. The testicles are highly susceptible to oxidative stress due to their high rate of cell division, mitochondrial oxygen consumption, and abundance of unsaturated fatty acids. As a result, excessive production of reactive oxygen species (ROS) can disrupt spermatogenesis, impair steroidogenesis, and reduce sperm quality. Exercise exerts a dual effect on male reproductive function—while mild-to-moderate exercise improves testicular steroidogenesis, spermatogenesis, and sexual competence by increasing insulin sensitivity and regulating ROS production, excessive and prolonged exercise can have adverse effects by promoting oxidative stress and impairing testicular function. This highlights the importance of maintaining an optimal balance in exercise intensity and duration to support reproductive health.

Furthermore, research has demonstrated that exercise can improve fertility in men with lifestyle-induced conditions such as obesity and diabetes. By enhancing testicular antioxidant defense, reducing pro-inflammatory cytokine levels, and promoting steroidogenesis, exercise contributes to improved spermatogenesis and semen quality. However, the extent to which exercise benefits male fertility is influenced by multiple factors, including an individual's overall health status, the type, intensity, and duration of exercise, and pre-existing metabolic or hormonal conditions. These factors should be carefully considered when prescribing exercise as a therapeutic strategy for male reproductive health.

Future research should focus on refining clinical guidelines that consider individual health status, exercise volume, intensity, and duration. Further clinical trials are required to validate the underlying mechanisms and establish exercise-based interventions for managing lifestyle-induced infertility. A more comprehensive understanding of the interaction between physical activity and male reproductive health will facilitate the development of evidence-based recommendations that optimize fertility outcomes.

## 8. Review Limitations

This review was designed primarily as a narrative synthesis for providing a flexible overview of the role of physical activity in counteracting oxidative stress-induced sperm alteration by improving redox homeostasis control. The intention was to address broad topics and integrate diverse literature from different fields without restrictive guidelines, so as to facilitate the reading and understanding of such research, especially for non-expert

audiences. It is recognized that the selection of articles leaves room for potential bias and that the analysis might inadvertently overlook key studies. Nevertheless, an informal assessment of methodological quality was performed by examining the clarity of each study's objectives, the description of experimental or clinical methods, and the relevance of the outcome measures.

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## Abbreviations

The following abbreviations are used in this manuscript:

|                               |                                          |
|-------------------------------|------------------------------------------|
| cAMP                          | Cyclic adenosine monophosphate.          |
| CAT                           | Catalase.                                |
| CO                            | Carbon monoxide.                         |
| Cx43                          | Connexin-43.                             |
| FSH                           | Follicle-stimulating hormone.            |
| GPx                           | Glutathione peroxidase.                  |
| GnRH                          | Gonadotropin-releasing hormone.          |
| H <sub>2</sub> O <sub>2</sub> | Hydrogen peroxide.                       |
| H <sub>2</sub>                | Hydrogen.                                |
| LCS                           | Leukocytospermia.                        |
| LH                            | Luteinizing hormone.                     |
| NH <sub>3</sub>               | Ammonia.                                 |
| PKA                           | Protein kinase A.                        |
| PA                            | Physical activity.                       |
| Prdx2                         | Peroxiredoxin 2.                         |
| PUFA                          | Polyunsaturated fatty acids.             |
| RES                           | Reactive electrophile species.           |
| RNS                           | Reactive nitrogen species.               |
| RHS                           | Reactive halogen species.                |
| REDOX                         | Oxidation–reduction.                     |
| ROS                           | Reactive oxygen species.                 |
| SSCs                          | Spermatogonial stem cells.               |
| SOD                           | Superoxide dismutase.                    |
| SPIs                          | Spermiogenesis indices.                  |
| TBARS                         | Thiobarbituric acid-reactive substances. |
| TDI                           | Tubular differentiation.                 |

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## General discussion and conclusions

The body of work presented in this PhD thesis highlights the central role of physical activity in driving cellular adaptations across multiple tissues and organs, with a particular emphasis on skeletal muscle responses, redox-dependent signaling and extracellular vesicles-mediated communication. Although each reported study addresses a different specific question, they collectively underline how reactive ROS generated during muscle contraction act not merely as harmful by-products of energy metabolism, but as essential regulators of physiological processes. Their effects, however, depend critically on their concentration, duration and on the ability of cells to translate redox perturbations into molecular signals, conveying them to both neighbouring and distant cells to promote whole-body homeostasis [34].

Maintaining of redox homeostasis is fundamental for the proper functioning of biological systems, as every cellular process relies on a finely tuned balance between oxidant production and antioxidant defenses. When this equilibrium is preserved, redox signaling supports physiological functions; when it is disrupted, oxidative distress contributes to the onset or progression of a wide spectrum of pathological conditions [122]. An exemple is found in the biology of male reproduction, where redox balance governs essential steps of spermatogenesis and testicular function; however, excessive ROS generation represents one of the leading causes of male infertility, with lipid peroxidation, mitochondrial dysfunction and inflammation representing major mediators of this decline. Importantly, physical activity is a potent regulator of this redox environment. Appropriately dosed exercise enhances endogenous antioxidant defences, increases enzymatic capacity (including SOD, CAT, GPx), reduces inflammatory burden and improves sperm parameters, whereas high-intensity or unregulated exercise amplifies oxidative damage and negatively affects sperm quality and hormonal profiles [123].

Similarly to spermatogenesis, muscle contraction represents one of the most energy-demanding processes in the human body and depends critically on controlled ROS production [124]. Among the various ROS generated during physical activity,  $H_2O_2$  plays a particularly prominent role as redox-sensitive second messenger which target specific cysteine residues within proteins, inducing reversible oxidative modifications that regulate their activity [125,126]. Key pathways such as AMPK, PGC-1 $\alpha$ , NRF2, NF- $\kappa$ B and MAPKs are activated in an  $H_2O_2$ -dependent manner, linking redox changes to gene expression, antioxidant upregulation, mitochondrial dynamics and cell survival [127].  $H_2O_2$  regulatory capacity explains why it is widely used as an experimental stimulus to mimic exercise-induced redox perturbations *in vitro*: when applied at controlled, sub-toxic doses, it reproduces the transient oxidative challenge experienced during muscle contraction. In this thesis work,  $H_2O_2$  was used both to induce oxidative stress in proliferating C2C12 myoblasts and to

modulate small EVs, exosome-like biogenesis in skeletal muscle cells. These applications rely on its biological role as a physiologically relevant redox signal whose effects are strictly dose-dependent: while low concentrations act as adaptive signaling mediators during exercise, excessive or prolonged oxidative exposure shifts the redox environment toward distress, impairing cellular viability, metabolic function and regenerative capacity [128].

Proliferating myoblasts are particularly sensitive to such perturbations and even moderate deviations from homeostatic ROS concentrations can rapidly compromise their ability to proliferate, differentiate and contribute to muscle repair [129]. Our results clearly illustrate this vulnerability: when exposed to supraphysiological concentrations of H<sub>2</sub>O<sub>2</sub>, C2C12 myoblasts experienced reduced glutathione availability, impaired antioxidant capacity and decreased proliferative potential, as extensively demonstrated by our and other groups [130–135]. However, treatment with *Moringa oleifera* leaf extract (MOLE), a rich source of bioactive antioxidant compounds, including glucosinolates, flavonoids, phenolic acids and vitamins, effectively attenuated H<sub>2</sub>O<sub>2</sub>-induced oxidative stress, restored intracellular glutathione levels and preserved myoblast viability and proliferation [136]. The findings of this work also highlight that nutrition operates synergistically with physical activity to modulate the redox status of biological systems [137]. While moderate exercise serves as a physiological stimulus that enhances antioxidant defenses, promotes mitochondrial efficiency and strengthens cellular resilience through hormetic mechanisms, nutritional interventions provide the molecular substrates required to sustain these adaptive processes, thereby complementing the beneficial oxidative changes elicited by exercise [138]. This concept becomes particularly relevant in tissues with high metabolic demands or in cell populations with limited redox buffering capacity, such as proliferating myoblasts or developing germ cells. In these contexts, the integration of targeted nutritional strategies with regular physical activity not only enhances the efficiency of adaptive signaling but also mitigates the risk of oxidative distress when the balance between ROS generation and antioxidant defenses is challenged, reinforcing the idea that optimal redox regulation depends on the coordinated influence of multiple lifestyle factors rather than isolated interventions [139].

Although the health benefits of physical activity are well established, the molecular mechanisms through which exercise elicits coordinated, whole-body effects remain only partially understood. Early studies attributed these systemic effects to the release of soluble factors, collectively termed *exerkines*, which include cytokines, peptides, metabolites and hormones secreted in response to physical activity [51]. Over the past decade, however, this concept has evolved considerably with the recognition that EVs constitute an additional mode of exercise-induced communication [56]. Both acute and chronic bouts of physical activity stimulate the release of EVs into circulation, and their abundance, molecular cargo and functional properties are modulated by the intensity, duration and

modality of the exercise performed [140]. One of the hallmark adaptations induced by exercise and influenced by EV-mediated communication is the enhancement of antioxidant defences [105]. EV cargo reflects the redox state of the cells from which they originate: under physiological redox stress, exercise-trained tissues release EVs enriched in signals that promote antioxidant capacity in recipient cells, contributing to system-wide resilience against oxidative challenges. Conversely, under conditions of excessive or pathological oxidative stress, cells may release EVs containing cargo capable of either propagating damage or triggering protective responses in recipient tissues based on the magnitude of the oxidative stimulus [105,141]. Importantly, the regulatory function of EVs is not limited to the delivery of antioxidant enzymes or proteins: also, miRNAs transported within EVs play a fundamental role in modulating transcriptional programs and cellular behaviour in recipient cells, contributing to long-term redox remodeling [142].

One of the contributions of this thesis lies in the systematic analysis of the role of extracellular vesicle-derived miRNAs as epigenetic regulators of redox homeostasis [26]. By integrating evidence from multiple experimental models, biological contexts and methodological approaches for EV isolation methods, characterization techniques, and quantification strategies, our meta-analysis revealed a remarkably consistent pattern: treatment with EV-derived miRNAs significantly reduced intracellular ROS levels, decreased lipid peroxidation, typically assessed through MDA quantification, and enhanced antioxidant enzyme activity, particularly SOD [143,144]. Mechanistically, EV-embedded miRNAs target multiple nodes of the redox regulatory network. Many of the miRNAs identified in the systematic review are known regulators of pathways involved in ROS detoxification, mitochondrial integrity, inflammation, and apoptotic signalling, including NRF2, KEAP1, NF- $\kappa$ B, forkhead box O (FOXO), and components of the MAPK cascade [145,146]. By modulating these pathways in recipient cells, EVs can enact broad epigenetic reprogramming that enhances oxidative resilience and shifts the cellular state toward a more adaptive, antioxidant phenotype [147]. This has profound implications for exercise biology. Since exercise is a potent modulator of intracellular ROS which shape the cargo of released EVs, it follows that physical activity alters the miRNA repertoire of circulating EVs in ways that influence systemic oxidative balance [148]. Indeed, exercise-induced EVs have been shown to carry miRNAs capable of influencing a broad range of cellular pathways across multiple tissues [149]. In skeletal muscle specifically, a subset of miRNAs, often referred to as myomiRs (including miR-1, miR-133a, miR-206, miR-486), is packaged into EVs following exercise and released into circulation [150–153]. These miRNAs modulate processes central to muscle adaptation such as mitochondrial biogenesis, fibre-type specification, angiogenesis and antioxidant defence [154]. Their export through EVs suggests a coordinated mechanism by which muscle

disseminates its redox state to other organs, thereby promoting systemic adaptation observed with regular physical activity [155].

Increasing evidence indicates that the cellular redox environment critically influences EV secretion rate and molecular identity, as ROS and other redox-active molecules affect multiple stages of the vesiculation process, from endosomal trafficking to selective cargo loading [102]. Given that exercise is a well-known modulator of redox homeostasis [156], it becomes reasonable to consider whether oxidative stimuli generated during muscle contraction act as upstream modulators of EV biogenesis. This possibility is supported by the overlap between the pathways activated during muscle contraction, characterised by ROS-dependent modulation of calcium handling, cytoskeletal dynamics and membrane remodelling, and the molecular machinery governing EV formation [157,158]. An important aspect emerging from the experimental work carried out in this thesis is that redox-dependent modulation of small EV biogenesis differ markedly between myoblasts and myotubes (Sireno et al., *submission in progress*). Proliferating myoblasts, which are characterised by limited antioxidant capacity and high sensitivity to oxidative stress, responded to H<sub>2</sub>O<sub>2</sub> with only minimal changes in EV release and irregular regulation of vesiculation genes, unless exposed to severe stress or driven into senescence as previously documented [159], suggesting that under normal proliferative conditions they prioritise stress protection over intercellular communication. Differentiated myotubes, by contrast, showed a clear and coordinated response: controlled redox stimulation increased EV secretion and upregulated multiple components of the vesicle biogenesis machinery, consistent with their more mature metabolic and redox profile [160,161]. This divergence suggests that only cells with robust antioxidant systems can interpret ROS as physiological signals rather than threats. From a physiological perspective, this supports the idea that mature muscle fibres, not progenitor cells, are the primary source of the EV-mediated communication stimulated by exercise, support a model in which transient ROS elevations are translated into vesicular signals that help coordinate whole-body adaptation (Sireno L. et al., 2025, *submission in progress*).

In summary, this thesis provides an overview of how redox signalling, physical activity and extracellular vesicle-mediated communication regulate both cellular and systemic physiology. Exercise emerges as an important oxidative stimulus that activates adaptive pathways within skeletal muscle and simultaneously modulates the EV-mediated molecular messages released into the extracellular environment. However, several questions remain open. Further work is required to characterise in detail how oxidative stimuli affect the molecular composition of EVs, clarifying which proteins, lipids and regulatory RNAs are selectively packaged under different redox conditions and *in vivo* studies are needed to determine how different exercise modalities influence EV formation, release and cargo selection. Given the growing interest into EV-based therapeutics, addressing the

link between redox signalling and EV dynamics may enable the development of vesicle-based interventions capable of restoring redox balance in conditions characterized by oxidative stress. At the same time, the sensitivity of EV composition to cellular redox state suggests that they could also serve as accessible biomarkers of oxidative stress-related pathologies, offering insights into disease progression and tissue dysfunction and ultimately supporting the development of personalised, exercise-based interventions aimed at restoring redox balance [162].

### **Study limitations**

Some limitations of this PhD study should be acknowledged. A primary limitation is the extensive reliance on *in vitro* models, particularly C2C12 myoblasts and myotubes, which, while widely used and well characterized, cannot fully recapitulate the complexity of the *in vivo* muscle environment or the systemic interactions occurring during exercise. Additionally, the use of H<sub>2</sub>O<sub>2</sub> as a redox stimulus, although physiologically relevant and commonly employed to mimic exercise-induced oxidative perturbations, represents a simplified model of exercise-related ROS production and does not encompass the spatial, temporal, and molecular heterogeneity of ROS generated during muscle contraction *in vivo*. Another limitation concerns EV isolation and characterization where technical variability among isolation methods may influence EV yield, purity, and cargo composition, potentially affecting cross-study comparability and the interpretation of functional outcomes. Finally, although associations between redox modulation, EV release, and adaptive signaling were clearly demonstrated, direct causal links *in vivo* remain to be fully established. Addressing these limitations through integrative *in vivo* studies, standardized EV methodologies, and multi-omics approaches will be essential to strengthen and extend the translational relevance of the findings presented in this thesis.

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