UNIVERSITY OF THESSALY

The Effect of Redox Potential on the Regulation of Satellite Cells and Skeletal Muscle Healing Following Exercise-Induced Muscle Damage

by

Konstantinos Papanikolaou

A Doctoral Dissertation submitted to the Examination Committee in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the School of Physical Education, Sport Science & Dietetics at the University of Thessaly

Trikala

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Η Επίδραση του Οξειδοαναγωγικού Δυναμικού στον Μηχανισμό Ελέγχου των Δορυφόρων Κυττάρων και στην Επούλωση του Σκελετικού Μυ μετά από Ασκησιογενή Μυϊκό Τραυματισμό

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Κωνσταντίνου Παπανικολάου

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Dr. Ioannis Fatouros (Supervisor): Professor, School of Physical Education, Sport Science, & Dietetics, University of Thessaly

Dr. Athanasios Jamurtas: Professor, Professor, School of Physical Education, Sport Science, & Dietetics, University of Thessaly

Dr. Athanasios Chatzinikolaou: Associate Professor, School of Physical Education & Sport Science, Democritus University of Thrace

Members of the Examination Committee:

Dr. Ioannis Fatouros (Supervisor): Professor, School of Physical Education, Sport Science, & Dietetics, University of Thessaly

Dr. Athanasios Jamurtas: Professor, Professor, School of Physical Education, Sport Science, & Dietetics, University of Thessaly

Dr. Athanasios Chatzinikolaou: Associate Professor, School of Physical Education & Sport Science, Democritus University of Thrace

Dr. Dimitrios Draganidis: Assistant Professor, School of Physical Education, Sport Science, & Dietetics, University of Thessaly

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Dedicated to my beloved ones

and especially to my grandfather

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List of Abbreviations

Akt: Protein kinase B
APC: adenomatous polyposis coli protein
ARE: antioxidant responsive element
CAT: catalase
CK: creatine kinase
CK1: casein kinase 1
Cyt C: cytochrome C
DII: Notch delta ligand
Dvl: dishevelled protein
EIMD: exercise-induced muscle damage
Fas: cell death receptor
FGF: fibroblast growth factor
GPX-1: glutathione peroxidase-1
GR: glutathione reductase
GSH: glutathione
GSK-3β: glycogen synthase kinase 3 beta
GTP: green tea polyphenol
HGF: hepatocyte growth factor
IFN-γ: interferone-γ
IGF-1: insulin-like growth factor-1
IGF1-R: insulin-like growth factor 1-receptor
IKKα/β: inhibitor of nuclear factor kappa-B kinase subunit alpha/beta
IL-10: interleukin-10
IL-1β: interleukin-1β
IL-6: interleukin-6
Keap1: Kelch-like ECH-associated protein 1
LRP5/6: Low-density lipoprotein receptor-related protein 5/6
Mrf4: myogenic regulatory factor 4
mTOR: mammalian target of rapamycin
mTORC1: mammalian target of rapamycin complex 1
Myf5: myogenic factor 5
Myf6: myogenic factor 6
MyoD: myoblast determination factor
NAC: N-acetylcysteine
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NICD: Notch intracellular domain
NOX-1: NADPH oxidase 1
NR: nicotinamide riboside
Nrf2: nuclear factor erythroid 2-related factor 2
ORAC: oxygen radical absorbance capacity
Pax7: paired box transcription factor 7
Pax3: paired box transcription factor 3
PI3K: Phosphoinositide 3-kinase
PIP2/PIP3: phosphatidylinositol (4,5)-bisphosphate/phosphatidylinositol (3,4,5)-trisphosphate
PRX: peroxiredoxin
RBP-jk: recombination signal binding protein jk
SCs: satellite cells
SH: cysteine residues
SIPS: stress-induced premature senescence
SO_2H: sulfinic acid
SO_3H: sulfonic acid
SOD: superoxide dismutase

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M1: pro-inflammatory macrophages

M2: anti-inflammatory macrophages

MCs: mast cells

MHC: myosin heavy chain

$MHCn^+/e^+$: myosin heavy chain neonatal/embryonic

MMPs: matrix metalloproteases

SOH: sulfenic acid

TGF-β: transforming growth factor beta

TNFR: tumor necrosis factor receptor

TNF-α: tumor necrosis factor-alpha

TRX: thioredoxin

UA: ursolic acid

VEGF: vascular endothelial growth factor

VEGF: vascular endothelial growth factor

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Abstract

The primary objectives of the present thesis were to (i) assess the efficacy of NAC treatment in upregulating GSH metabolism as well as in mitigating redox status decline in skeletal muscle following EIMD, (ii) evaluate the muscle damage development and regeneration potential as well as strength recovery after EIMD, (iii) monitor the satellite cells and myogenic responses following EIMD and (iv) investigate the redox-dependent regulation of satellite cells activity (via NAC treatment) during regeneration following EIMD in the total sample as well as in individuals with low and high satellite cells content in skeletal muscle. The secondary aim of this thesis was to examine whether young individuals with different levels of redox status differ in terms of (i) satellite cells content, (ii) dietary nutrient intake profile, (iii) physical fitness and (iv) physical activity markers. In this respect we conducted a comprehensive literature review and summarized the available data from in vitro and in vivo studies, supporting the efficacy of NAC and other antioxidant treatments in upregulating redox status and promoting a redox-dependent regulation of satellite cells and skeletal muscle repair following injury. Afterwards, we recorded the complete methodological scheme for the investigation of the redox-dependent regulation of satellite cells and regeneration following EIMD, in a human clinical trial setting and evaluated the relationship between redox status with dietary profile as well as with physical fitness and activity profile. Moreover, molecular, biochemical, histological and functional analyses on GSH metabolism, antioxidant status, muscle damage, regeneration, satellite cells, myogenic potential and muscle function were performed. The results of this work are presented as different chapters of the current thesis. Summarizing, the results of this thesis indicate that NAC treatment successfully upregulates GSH metabolism in skeletal muscle and mitigates redox status decline following EIMD, especially in individuals with low satellite cell content. Regarding muscle damage and regeneration, EIMD elicits a substantial increase in muscle damage and regeneration markers while the effects of NAC treatment are limited to CK and DOMS responses and not to histological parameters. Moreover, individuals with higher satellite cell content demonstrated higher levels of muscle damage but also exhibited an augmented regenerative capacity following injury. Interestingly, at basal state, satellite cell content (Pax7+ cells) was significantly correlated with muscle GSH/GSSG ratio. Furthermore, satellite cells quantity, proliferation and differentiation increased proportionally post-EIMD. Notably, NAC elicited a significant increase in MyoD+ cells only in the low satellite cell content group during the

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restorative phase (8 days) following EIMD. Myogenic programming (mRNA expression) also increased after EIMD with no effects of NAC. Muscle function decreased during the inflammatory and restorative phase with NAC treatment mitigating this decline in the low satellite cell group. Moreover, higher GSH and redox status level is associated with higher dietary cysteine intake, improved muscle strength and increased levels of physical activity in young men. Collectively, the results of the present thesis illustrate the beneficial effect of NAC treatment on GSH metabolism, redox status and muscle strength, especially in individuals with low satellite cell content. Notably, individuals with lower satellite cell content also exhibited a lower redox status level. In individuals with higher satellite cell content, NAC supplementation blunts myogenic differentiation and muscle function recovery. Moreover, our data support a redox-dependent regulation of satellite cells at basal state and partially post-EIMD. Muscle damage and regeneration capacity seem to be largely unaffected by redox alterations but rather rely on the basal satellite cell content of the human skeletal muscle.

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General Introduction

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General Introduction and chapters outline

The impact of ROS-induced cellular signaling on myogenic programming is complex as low levels of ROS are involved in signal transduction and skeletal muscle stem cells (satellite cells) survival and differentiation, while high ROS levels promote oxidative stress and lead to inhibition of satellite cells-mediated myogenesis through apoptotic and necrotic pathways [1]. This redox-dependent regulation of satellite cells has been validated in experimental settings using in vitro and animal models, nevertheless providing conflicting evidence regarding the usage of antioxidant compounds in the regulation of satellite cells viability, activation, differentiation and myogenic potential [2-6]. Moreover, it has been suggested that myogenic potential and satellite cells-mediated growth of skeletal muscle is related to its basal satellite cells content [7, 8]. To this date there is no evidence regarding the redox-dependent regulation of satellite cells in the human skeletal muscle at rest and during regeneration following EIMD as well as to what extent antioxidant treatment can influence these responses. The results of the present thesis aim to decipher the role of redox status on the regulation of satellite cells and skeletal muscle damage/regeneration following injury in an attempt to provide evidence regarding the usefulness of antioxidants for optimal tissue healing in health and disease.

The main objectives of this thesis were to (i) assess the efficacy of NAC treatment in upregulating GSH metabolism as well as in mitigating redox status decline in skeletal muscle following EIMD, (ii) evaluate the muscle damage development and regeneration potential as well as strength recovery after EIMD, (iii) monitor the satellite cells and myogenic responses following EIMD and (iv) investigate the redox-dependent regulation of satellite cells activity (via NAC treatment) during regeneration following EIMD in the total sample as well as in individuals with low and high satellite cells content in skeletal muscle. The secondary aim of this thesis was to examine whether young individuals with different levels of redox status differ in terms of (i) satellite cells content, (ii) dietary nutrient intake profile, (iii) physical fitness and (iv) physical activity markers.

As an introduction, in **Chapter 1**, a literature review was conducted summarizing the available information regarding satellite cells kinetics following aseptic myotrauma, as well as the intrinsic redox-sensitive molecular mechanisms responsible for satellite cells responses. Moreover, the impact of nutritional and pharmacological interventions on myogenic response, regeneration, inflammation and redox status in vitro and in vivo is also discussed. In **Chapter 2**, the detailed experimental methodology of the study is presented. In this technical section, information regarding participants recruitment, inclusion/exclusion criteria, clinical trials design, EIMD protocol,

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antioxidant treatment scheme and analytical procedures is discussed. In **Chapter 3**, by utilizing partial correlation analyses, we present data regarding the relationship between redox status with dietary nutrient intake, physical fitness and physical activity profile. In **Chapter 4**, unpublished data related to muscle damage/regeneration, glutathione metabolism, antioxidant status, satellite cells, myogenic programming, and muscle function are presented.

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Chapter 1

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Redox-Dependent Regulation of Satellite Cells Following Aseptic Muscle Trauma: Implications for Sports Performance and Nutrition

Papanikolaou K., Veskoukis A.S., Draganidis D., Baloyiannis I., Deli C.K., Poulios A., Jamurtas A.Z., & Fatouros, I.G.

Abstract

Skeletal muscle satellite cells (SCs) are indispensable for tissue regeneration, remodeling and growth. Following myotrauma, SCs are activated, and assist in tissue repair. Exercise-induced muscle damage (EIMD) is characterized by a pronounced inflammatory response and the production of reactive oxygen species (ROS). Experimental evidence suggests that SCs kinetics (the propagation from a quiescent to an activated/proliferative state) following EIMD is redox-dependent and interconnected with changes in the SCs microenvironment (niche). Animal studies have shown that following aseptic myotrauma, antioxidant and/or anti-inflammatory supplementation leads to an improved recovery and skeletal muscle regeneration through enhanced SCs kinetics, suggesting a redox-dependent molecular mechanism. Although evidence suggests that antioxidant/anti-inflammatory compounds may prevent performance deterioration and enhance recovery, there is lack of information regarding the redox-dependent regulation of SCs responses following EIMD in humans. In this review, SCs kinetics following aseptic myotrauma, as well as the intrinsic redox-sensitive molecular mechanisms responsible for SCs responses are discussed. The role of redox status on SCs function should be further investigated in the future with human clinical trials in an attempt to elucidate the molecular pathways responsible for muscle recovery and provide information for potential nutritional strategies aiming at performance recovery.

Keywords: Satellite cells, antioxidants, redox status, recovery, sports performance

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1.1 Introduction

Skeletal muscle is a highly dynamic and adaptive tissue which in healthy adults comprises ~40-50% of human body mass and is implicated in multiple mechanical, physiological and metabolic processes including movement, metabolism, and homeostasis [9]. Skeletal muscle is composed of large, multinucleated cells called myofibers, which are subjected to microtrauma and their ability for repair is crucial for proper and life-long muscle function and adaptation [10]. Due to its unique plasticity, skeletal muscle has the ability to regenerate and remodel in response to injury [11].

Muscle microtrauma is present in numerous physiological conditions such as exercise-induced muscle damage (EIMD), which is frequently observed following physical activity and/or strenuous exercise, especially when eccentric contractions are incorporated [12]. Moreover, numerous catabolic and muscle wasting chronic conditions such as myopathies, aging, and cachexia result in a chronic state of muscle microtrauma [13]. Acute or chronic muscle injury results in the initiation of an inflammatory cascade and the mobilization and infiltration of immune cells to the injured muscle tissue. Immune cell subpopulations (neutrophils, macrophages) invade damaged muscle tissue to remove cellular debris, neutralize pathogenic organelles and promote healing [10].

Muscle microtrauma is characterized by elevated inflammation and reactive oxygen species (ROS) which may result in a secondary damage of previously uninjured myofibers [14]. It should be noted that the term ROS will be used throughout the manuscript hereafter since, as it has been insightfully proposed in previous studies, it encompasses the initial species generated by oxygen reduction (superoxide or hydrogen peroxide) as well as their secondary reactive products [15, 16]. During exercise, the main sources of ROS generation include NADPH oxidase enzymes [associated with the sarcoplasmic reticulum (SR), transverse tubules and plasma membrane], phospholipase A2, xanthine oxidase and mitochondrial metabolism whereas an immune cell oxidative burst is active during post-exercise recovery [17-19]. In response to altered levels of ROS, transient and both reversible and non-reversible oxidative modifications occur in the skeletal muscle, including S-nitrosylation, S-glutathionylation and disulfide formation [20]. Specifically, S-glutathionylation of the cysteine residues may result in protein deactivation and activity inhibition [20]. This post-translational redox modification may therefore play important role in the skeletal muscle signaling during exercise and recovery [21].

The aforementioned inflammatory cascade is followed by a healing phase during which skeletal muscle fibers are repaired or regenerated mainly due to the activity of resident skeletal muscle-specific stem cells. Pro-inflammatory (M1) and anti-inflammatory (M2) macrophages seem

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to orchestrate skeletal muscle's myogenic response after microtrauma [22]. Postnatal muscle tissue repair and remodeling following injury is attributed to a heterogenous family of mononucleated myogenic stem cells, called satellite cells (SCs) [23]. In response to injury, SCs are activated, enter the cell cycle, proliferate and give rise to daughter cells that can either return to quiescence (self-renewal) or proceed to terminal differentiation by formatting myoblasts and fuse with damaged myofibers to form new muscle fibers or assist in growing the old ones [24, 25]. This process is accompanied by increased transcriptional and translational capacity at nuclei and ribosomic level, respectively, in muscle cells resulting in the accumulation of contractile proteins (e.g. myosin heavy chain isoforms) and extracellular matrix elements (e.g., proteins, cell adhesion receptors) leading to muscle remodeling and growth [26].

The influence of ROS-induced cellular processes on myogenic programming is complex as ROS present in concentrations below a threshold (that is largely unknown though) are implicated in signal transduction, SCs viability and differentiation while they lead to inhibition of myogenesis through apoptotic and necrotic pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) when their levels surpass this threshold [1]. Recently, in vitro and in vivo studies that used antioxidant compounds to quench excess ROS production in skeletal muscle during the repair process following aseptic myotrauma have provided evidence of accelerated muscle recovery process via upregulation of the myogenic potential, viability, maintenance and activity of the resident SCs [2-4]. However, to this date literature lacks evidence on a redox-dependent regulation of SCs following aseptic myotrauma and inflammation in the human skeletal muscle and to what extent, antioxidant supplementation can affect activity of SCs and muscle's regeneration.

The aim of this review is to present an integrated overview of (i) the mechanisms governing SCs biology following EIMD and its impact on muscle regeneration; (ii) the available evidence demonstrating a redox-dependent regulation of SCs activation and myogenic action; (iii) the implications of a redox-dependent regulation of SCs activation on recovery of skeletal muscle performance following EIMD; and (iv) the potential role of antioxidant supplementation on SCs response to injury.

1.2. Skeletal Muscle Satellite Cells Biology and Tissue Regeneration

Skeletal muscle-specific stem cells (satellite cells – SCs) are the machinery of tissue repair, remodeling and growth. SCs represent approximately ~30-35% of the sublaminal nuclei of myofibers in early postnatal mouse muscle [27, 28]. SCs numbers also depend on muscle fiber type,

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with oxidative (slow-twist, type I) fibers containing more SCs than glycolytic ones (fast-twist, type II) fibers [29]. In addition, SCs can be identified by their location and the expression of several surface and transcriptional factors, with paired box transcription factor Pax7 considered to be the most reliable and reproducible for this cell type [30]. Pax7 has been correlated with SCs self-renewal through the maintenance of an undifferentiated state [31]. Skeletal muscle has a remarkable capacity to preserve muscle structure and integrity by regenerating mature myofibers following injury [30]. Studies that utilized SCs deletion models confirmed that muscle's regenerative capacity relies on their myogenic potential and activity [32]. Intriguingly, Pax7-null mice demonstrated a dramatic reduction of the SCs pool, a completely abolished regenerative capacity following skeletal muscle injury as well as increased fibrotic and fat tissue that resulted in functional impairment and premature postnatal death [33, 34]. Recent advances in transplantational medicine have shown that the detrimental effects induced by the absence of SCs can be reversed via exogenous intramuscular injection of SCs in the injured muscle [35]. Additionally Pax7, Pax3 is also expressed in quiescent SCs, influencing their specification [30]. After birth, SCs express Pax3 and are present in the skeletal muscle in the absence of Pax7 (although in reduced numbers) [36]. Nevertheless, the absence of Pax7 leads to cell cycle impairments and apoptosis resulting in ablation of the resident SCs [37]. Moreover, Pax3 has been shown to play a critical role in orchestrating SCs commitment in the myogenic program by independently regulating MyoD expression in activated SCs during regeneration [38].

Under physiological conditions, SCs reside to the adjunct myofiber mitotically quiescent outside the cell cycle (G0 phase) with a low metabolic rate and RNA content [39]. Microarray transcriptomic analysis has revealed that >500 genes and antioxidant molecules such as thioredoxin-1 (TRX1) and glutathione peroxidase-3 (GPX3) are upregulated during quiescence suggesting a protective mechanism against oxidative damage [40]. Upon muscle injury, SCs are activated by factors released within their microenvironment (also called niche) and enter the cell cycle. These factors include, but are not limited to, immune cells subpopulations which in turn release cytokines, chemokines and growth factors, blood vessels-derived factors, signaling molecules and extracellular matrix cues [41].

The transition from quiescence to activation is accompanied by a shift from fatty acid oxidation to glycolytic metabolism [42]. Activated SCs express both Pax7 and the myogenic factor 5 (Myf5) [28]. In response to muscle injury, the influx of calcium into the cytosol triggers calcineurin and calmodulin kinase through calcium binding to calmodulin, to activate Myf5 expression [43]. SCs activation also involves an IGF-dependent mechanism which induces the upregulation of Myf5

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following injury through the PI3K/Akt and ERK signaling pathways [43]. Activated (Pax7+/Myf5+) SCs undergo several cell divisions, proliferate and follow two distinct fates by either assisting in tissue repair or return to quiescence to replenish the in vivo SCs pool (self-renewal). Activated SCs proceed to proliferation, forming myoblasts, while Pax7+/Myf5- SCs are able to self-renew through symmetric or asymmetric division upon entry into the cell cycle [30].

During the proliferation state, myoblast determination factor (MyoD) is a key regulator of myogenic advancement and subsequent SCs differentiation to myocytes through the induction of cell cycle inhibitors such as p21 and p57 [44, 45]. Proliferative (Pax7+/MyoD+) SCs migrate from their dormant location to the injured area and increase their mitotic activity [46]. Interestingly, MyoD−/− myoblasts exhibit incomplete muscle regeneration as well as downregulated myogenin and myogenic regulatory 4 (MRF4) gene levels [47, 48].

Following differentiation, SCs-derived myocytes downregulate the expression of Pax7 and MyoD and upregulate the expression of myogenin [46]. Differentiating (Myogenin+) myocytes undergo an early (myocyte-to-myocyte attachment) and late (fusion of myocytes to form myotubes) cell-to-cell fusion [49]. Upon terminal differentiation multinucleated myotubes (MRF4+) undergo a maturation process to form functional myofibers, a process which involves the expression of embryonic and neonatal myosin heavy chain isoforms (MHCe/n+) [50]. During this process the Akt/mTOR pathway is the key signaling pathway, activated by IGF-1 which binds to its transmembrane receptor and phosphorylates Akt to subsequently stimulate protein synthesis through the phosphorylation of p70S6/ribosomal protein S6 cascade that results in the accumulation of several contractile proteins such as slow and fast MHC isoforms in the intra- and extracellular matrix (ECM) [51]. Moreover, Akt-induced down-regulation of catabolic pathways [e.g., the Forkhead box protein (FOXO), muscle ring finger-1 (MuRF-1) and atrogin-1 pathways] is also associated with an increased hypertrophic response following myotube formation during myogenesis [52].

The final stage of muscle healing process involves an angiogenesis cascade, ECM remodeling and reinnervation of the regenerated myofibers [53]. During this stage, angiogenesis is driven by the transforming growth factor-β (TGF-β) which is upregulated during regeneration, inducing the physiological fibrosis cascade [54]. ECM components such as laminin, fibronectin, proteoglycans, elastin and various isoforms of collagen accumulate in the regenerative area to provide tissue stabilization, while also serving as a docking station towards the formation of neuromuscular junctions (NMJs) [55]. Importantly, nerve activity directly regulates protein turnover and gene expression within regenerative myotubes, while indirectly affects the activation and proliferation of SCs [56, 57]. The influence of nerve activity in the maturation and functional

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restoration of the regenerating innervated myofibers can be assessed by analyzing the expression of developmental MHC isoforms (i.e. MHCe+/n+) [53]. Collectively, complete muscle restoration requires the recovery of functional performance, which is only beneficial if the regenerated myofibers become efficiently reinnervated (**Figure 1.1**).

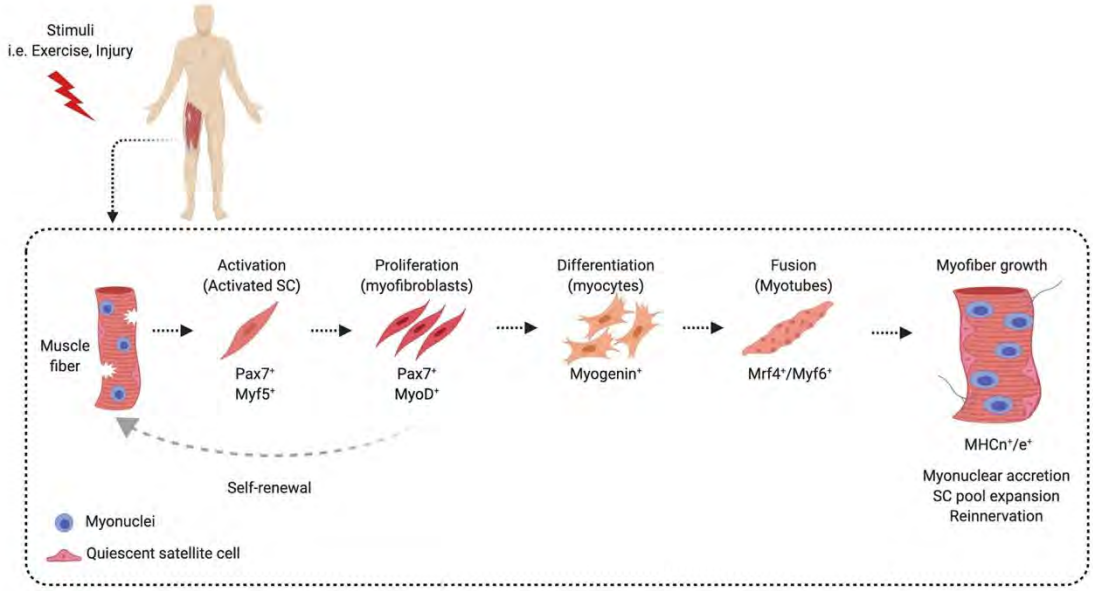

Figure 1.1. The timeframe of skeletal muscle tissue regeneration following acute aseptic myotrauma. In response to stimuli, resident quiescent satellite cells (SCs) are activated and enter the myogenic programming. A part of the proliferating fibroblasts procced to differentiation into myocytes and another part return to quiescence in order to maintain the SCs pool (self-renewal). Differentiated myocytes fuse to form myotubes and repair the injured tissue. Beneath each state are markers to indicate that satellite cells undergo each phase before they get to their terminal phase.

Intensive exercise, particularly eccentric contractions, result in aseptic exercise-induced microtrauma and inflammation characterized by ultrastructural damage of sarcomeres, basal lamina compartments and cell membranes, with a concomitant increase of ROS [58, 59]. The mechanical explanation of sarcomere disruption leads to the dissociation of contractile proteins from the ECM as actin and myosin filaments overstretch along with an increase in titin stiffness [60, 61]. These events attenuate the muscle's excitation-contraction potential and lead to loss of force-production capacity, muscle soreness and functional impairment [10]. Several human studies have examined the SCs response after aseptic myotrauma caused by eccentric exercise, mostly in mixed muscle (Table 1). Overall, SCs number increases from the first hours of the injury with significant changes observed at 24 hours post-injury, peak at 2-3 days, while their number remains elevated even at 8 days post-injury compared to baseline values, especially in type II fibers [62-72]. Noteworthy,

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human studies investigating SCs responses in conjunction with changes in the redox status inside the SCs microenvironment following aseptic microtrauma are lacking.

1.3. The Inflammatory Response Underlies the Evolution of SCs-mediated Tissue Healing

SCs are essential for muscle healing, though proper muscle recovery relies on the interaction with numerous cell types and secreted factors within the SCs niche. In response to muscle injury, cytokines, chemotactic and growth factors are rapidly released propagating an inflammatory cascade that is mainly characterized by infiltration of mast cells (MCs) and leukocyte subpopulations into the injured tissue [73]. In addition, the local MCs are also activated and release pro-inflammatory cytokines such as tumor necrosis factor-a (TNF-a), interleukin-1β (IL-1β) and histamine to attract more neutrophils and other immune cells to the injury site [2]. Both in vitro and in vivo studies have provided evidence that factors secreted by MCs enhance SCs activity and promote myoblast proliferation [74]. Under inflammatory conditions, immune cells recruited by muscle injury promote the induction of matrix metalloproteinases (MMPs) [75]. MMPs cleave collagens, laminin, and other extracellular matrix components release the hepatocyte growth factor (HGF) from the sequestered sites to activate SCs in a nitric oxide-dependent manner [75]. Furthermore, damaged vascular endothelial cells release growth factors such as vascular endothelial growth factor (VEGF) which further promotes SCs activation and proliferation in muscle [41].

The infiltrated neutrophils release pro-inflammatory cytokines including TNF-a, IL-1β and interferon-γ (IFN-γ) to facilitate the removal of necrotic and apoptotic myofibers [59]. Typically, the concentration of neutrophils at the injured site demonstrates its peak within the first 6–24 hours post-injury and returns to baseline values by 72-96 hours [76]. Subsequent to neutrophils, a second wave of immune cells, the macrophages, infiltrate the injured muscle within a time-frame of 24-48 hours after muscle microtrauma [77]. Of note, macrophages are considered a prominent regulator of skeletal muscle myogenesis [78, 79]. Initially, the pro-inflammatory M1 macrophages are recruited, releasing cytokines such as TNF-a, IL-1β and IL-6 [80]. TNF-a promotes the activation of the NF-κB and p38 signaling pathways and upregulates proliferation and differentiation mechanisms [81]. Furthermore, experimental evidence revealed that TNF-a receptor knockout mice exhibit impaired regeneration, highlighting the particularly important role of TNF-a in muscle regeneration [81]. IL-6 is also produced by M1 macrophages and research in animal models has shown that IL-6 ablation results in an attenuated migration, proliferation and differentiation of SCs [82]. In addition, M1 macrophages recruit T cells (CD8+ and CD4+) that infiltrate the injury site at ~72 hours post-injury

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and remain elevated for up to 10 days [83]. T cells promote the release of multiple cytokines that in turn stimulate muscle SCs expansion [84]. Interestingly, the combination of four pro-inflammatory cytokines (IL-1α, TNF-α, IFN-γ) secreted by T cells stimulated SCs proliferation in vivo upon injury and promoted their serial expansion in vitro [70]. Moreover, M1 macrophages-derived fibroblast growth factors (FGFs), regulate SCs proliferation and migration [45, 44, 66]. In vitro experiments suggest that various FGFs elicit a profound SCs proliferation response following injury [85].

During the inflammatory phase, muscle performance deteriorates depending on the type, intensity, duration and total volume of the exercise stimulus [86]. Mild performance deterioration results in minimum strength loss (<20%), limited inflammation and fast recovery (12-48 h) [10]. In moderate performance deterioration (20%–50%) CK increases in serum (>1,000 U/l), the inflammation status is more intense and is characterized by leukocyte infiltration in the injured area and the recovery phase lasts from 5 to 7 days [10]. In severe performance deterioration (>50%), histological analyses have revealed extensive necrosis in myofibers which is accompanied by a pronounced accumulation of neutrophils in the muscle tissue, soreness and swelling. In this scenario, systemic CK levels may exceed 10,000 U/l [10].

In the course of the inflammatory cascade macrophages progressively alter their phenotypic identity from pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages (day 2 to day 4) [79]. The predominant role of M2 macrophages is to resolute the inflammatory milieu by producing anti-inflammatory cytokines (e.g., IL-4, IL-10, IL-13). Additionally, M2 macrophages promote SCs differentiation to myotubes, thus initiating the late stage of myogenesis and muscle healing [59]. At this stage, IL-4 promotes myotube formation during the late fusion of myoblasts into myotubes and IGF-1 stimulates myotube hypertrophy [87, 88]. Previous in vitro observations identified the mechanistic pillar between macrophage phenotype and stages of myogenesis. Specifically, culture of SCs with M1 macrophages increased their proliferative capacity but decreased myogenin levels [83]. On the other hand, culture of SCs with M2 macrophages upregulated myogenin levels and enhanced SCs fusion to form myotubes [89]. During this healing phase, muscle performance is diminished. Particularly in intense EIMD, disruption of the calcium homeostasis leads to muscle protein breakdown even during the early phase of recovery [90]. Of note, 2-4 days post EIMD muscle strength reaches its nadir with concomitant conversion of M1 macrophages to M2 [91].

Altogether, the inflammatory process following muscle trauma is strictly interconnected with SCs function and crucial in promoting muscle regeneration and proper tissue healing (**Figure 1.2**).

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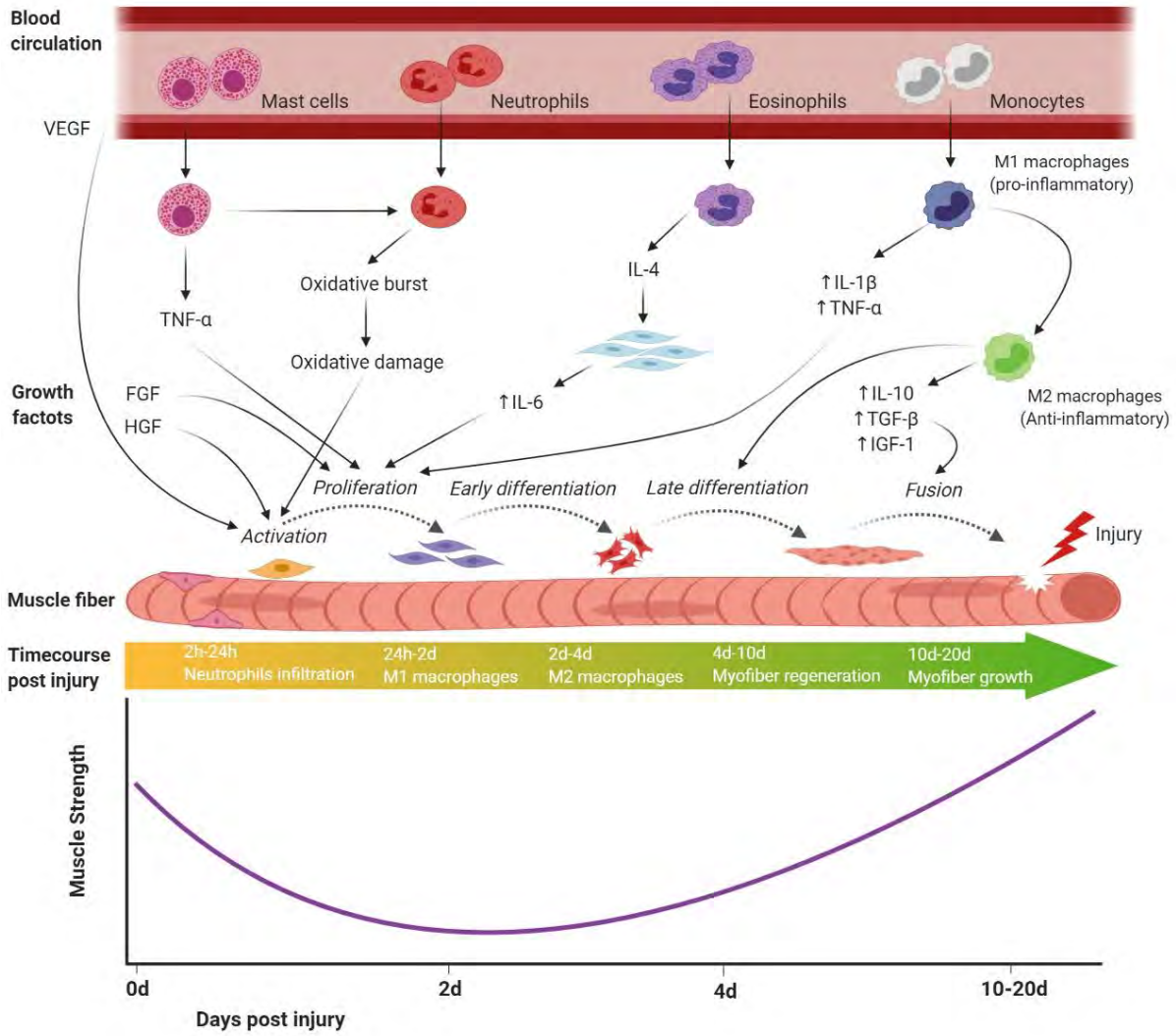

Figure 1.2. The interaction between satellite cells (SCs), inflammation and muscle strength recovery during regeneration. Following injury, immune cells subpopulations are secreted inside the SCs niche. They promote the induction of several cytokines, growth factors and cell types which are closely implicated in the tissue regeneration process by targeting the stages of myogenic advancement. During the early events of the healing phase, muscle strength decreases, however, upon myofiber regeneration and growth muscle strength recovers.

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Table 1.1 The impact of acute damaging exercise on satellite cell response in human skeletal muscle.

Publication [Refs.]	Subjects	Age	Fitness status	Exercise protocol	Biopsy sampling timepoint	SCs related indices
Cermak et al. [62]	8 young men	23 ± 1	Recreationally active	300 KE EC at −180°/s	24-hours post exercise	↑$Pax7^+$ cells (Type II fibers) ↑$PCNA^+$ cells (mixed fibers)
Crameri et al. [63]	8 young men	25 ± 3	Untrained	50 drop-jumps + 80 KE EC at −30°/s + 80 KE EC at −180°/s	4- and 8-days post exercise	↑$N\text{-}CAM^+$ cells (mixed fibers) ↑$FA1^+$ cells (mixed fibers)
Dreyer et al. [64]	10 young men	23 - 35	No resistance training	96 KE EC at – 60°/s	24-hours post exercise	↑$N\text{-}CAM^+$ cells (mixed fibers)
	10 older men	60 - 75	No resistance training	96 KE EC at – 60°/s	24-hours post exercise	↑$N\text{-}CAM^+$ cells (mixed fibers)
Hyldahl et al. [65]	7 young men	23 ± 2	Untrained	196 KE EC at −180°/s	24-hours post exercise	↑$Pax7^+$ cells (Mixed fibers) ↑$MyoD^+$ cells (Mixed fibers)
McKay et al. [71]	8 young men	22 ± 1	No lower-body resistance training	300 KE EC at −180°/s	24-, 72- and 120-hours post exercise	↑$Pax7^+$ cells (mixed fibers) ↑$PCNA^+$ cells (mixed fibers)

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McKay et al. [67]	12 young men	21 ± 2	No lower-body resistance training	300 KE EC at −180°/s	24-hours post exercise	↑Pax7^{+} cells (mixed fibers) ↑N-CAM^{+} cells (mixed fibers) ↑C-Met^{+} cells (mixed fibers)
Mikkelsen et al. [68]	8 young men	23 ± 3	Well trained	200 KE EC at −120°/s	8-days post exercise	↑Pax7^{+} cells (mixed fibers) ↑N-CAM^{+} cells (mixed fibers) ↔Ki67^{+} cells (mixed fibers)
Nederveen et al. [70]	29 young men	21 ± 0.5	Recreationally active	300 KE EC at −180°/s	6-, 24- and 72-hours post exercise 24-hours post exercise 6-, 24-, 72- and 96-hours post exercise	↑Pax7^{+} cells (Mixed and Type II fibers) ↑Pax7^{+}/MyoD^{+} cells (Mixed fibers) ↑MyoD, MRF4 and Myogenin mRNA
O'Reilly et al. [69]	8 young men	21 ± 2	No lower-body resistance training	300 KE EC at −180°/s	24-, 72- and 120-hours post exercise	↑N-CAM^{+} cells (mixed fibers)
Toth et al. [72]	12 young men	21 ± 2	Untrained	300 KE EC at −180°/s	24-hours post exercise	↑Pax7^{+} cells (mixed fibers) ↑Myf5 mRNA (mixed fibers)

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Pax7, paired-box protein 7; Myf5, myogenic factor 5; MyoD, myogenic determination factor; MRF4, myogenic regulatory factor 4; KE, knee extensors; EC, eccentric contractions; CFPE, capillary-to-fiber perimeter exchange index; ↑, increase; ↔, no change.

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1.4. Redox Status in the Satellite Cell Niche

The redox status could be defined as the balance between oxidants (or pro-oxidants) and antioxidants [92]. When this equilibrium is disrupted and tilts towards an oxidized state resulting in excess production of ROS and in the disruption of redox signaling and control, oxidative stress occurs [19]. It has to be stressed, though, that oxidants and pro-oxidants along with antioxidants play fundamental roles in maintaining proper cellular function. ROS, as the main subcellular oxidizing agents, although seemingly act detrimentally on key cellular biological processes, it is established that they also serve as important signaling molecules [93-95]. Therefore, the maintenance of proper cellular function and state is an outcome of a highly sophisticated molecular network, where ROS and antioxidants are equally necessary. Cellular homeostasis relies on a delicate balance between oxidant and pro-oxidant molecules and an optimal level of ROS [96]. Myogenic stem cells are equipped with refined antioxidant systems, extremely sensitive to redox changes inside their niche [97]. Low ROS levels favor cell survival, signal transduction, mitochondrial function and biogenesis, SCs differentiation and muscle growth whereas high ROS levels are detrimental, promoting cell apoptosis, autophagy, mitochondrial disfunction and impaired SCs differentiation, muscle healing and myofiber growth [98-100]. Low ROS levels (e.g., H_2O_2) may activate the ERK and c-Jun N-terminal kinase (JNK) intracellular signaling pathways to promote myoblast proliferation in a dose- and time-dependent manner [101]. Excess ROS can lead to abnormal differentiation and apoptosis of stem cells through the activation of p38 and p53 signaling pathways and compromise hematopoietic stem cells self-renewal capacity and engraftment [102]. Additionally, following transplantation, increased ROS in mesenchymal stem cells reduce their engraftment potential and induce apoptotic mechanisms [102]. Although the precise effects of ROS on signaling pathways during the myogenic process have not been fully elucidated, ROS levels appear to increase during reprogramming and to cause DNA damage in pluripotent stem cells, a phenomenon that can be mitigated by the addition of the non-specific antioxidant N-Acetylcysteine (NAC) [103].

Evidence emerged during the past two decades suggests that ROS produced physiologically by cells are important signaling molecules, acting through mechanisms such as post-translational redox modifications of cysteine thiols on proteins [18]. Post-translational redox modifications such as S-glutathionylation (most common) involves the formation of mixed disulphides between GSH and cysteine thiol groups of proteins that results in activation or deactivation [104] (**Figure 1.3**). GSH is crucial for SCs antioxidant defense under oxidative

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conditions, mainly by promoting cell survival mechanisms [105]. Collectively, endogenous antioxidants, which include enzymes and small molecules such as TRX, glutathione reductases, PRX and GSH are considered fundamental for the control of redox signaling networks and myogenic programming (**Figure 1.4**).

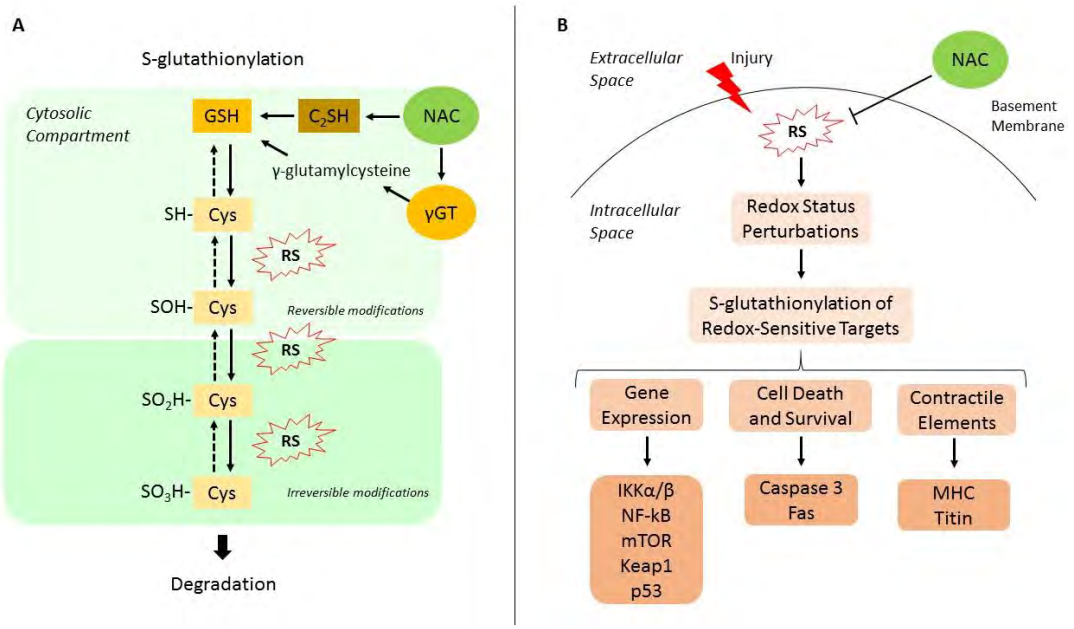

Figure 1.3. Post-translational S-glutathionylation biochemistry and its impact in redox-sensitive cellular processes. Thiol oxidation of redox-sensitive cysteine residues results in reversible and/or irreversible modifications in multiple targets promoting their degradation, depending on the degree of oxidation. N-acetylcysteine (NAC) replenishes glutathione (GSH) bioavailability mainly through the γ-glutamylcysteine. Acute muscle injury promotes the production of reactive oxygen species (ROS) and the induction of S-glutathionylation of several redox-sensitive targets however, NAC supplementation may mitigate the rise of ROS and protect the cysteine residues of proteins from oxidation, thus enhancing their functionality.

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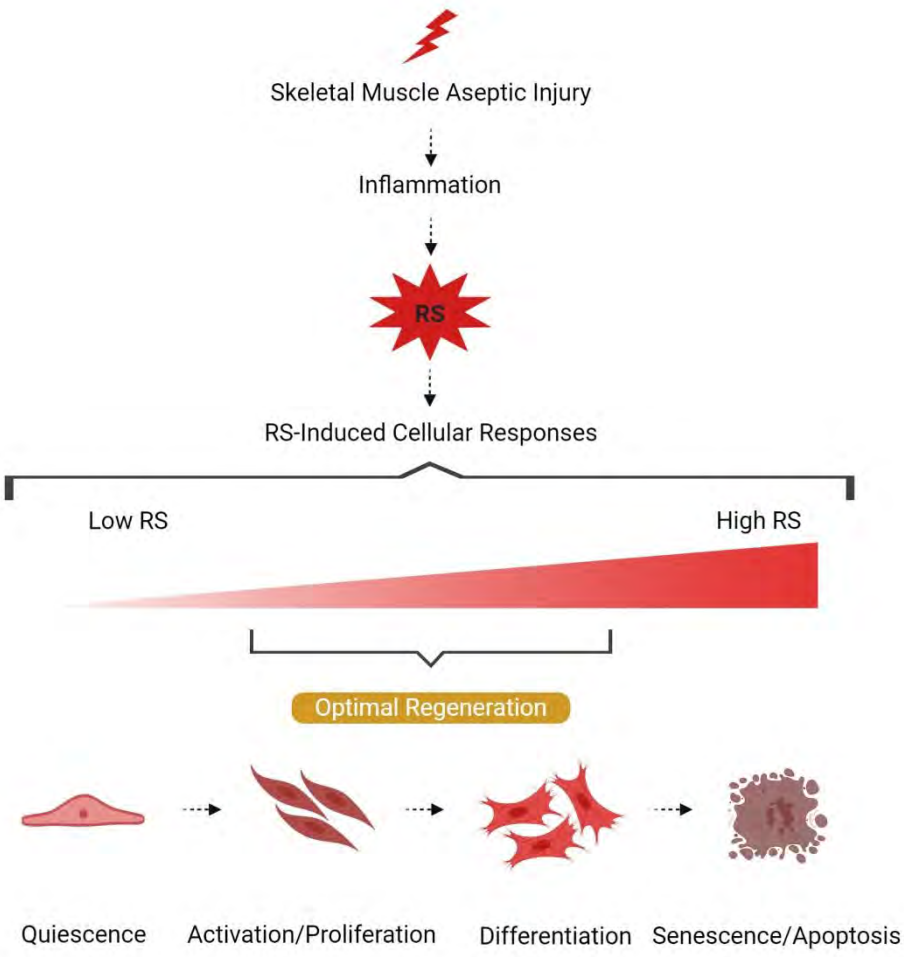

Figure 1.4. The effect of redox status on satellite cells (SCs) homeostasis and function. Exercise-induced muscle damage (EIMD) results in the onset of aseptic inflammation and the production of reactive oxygen species (ROS). Proper muscle regeneration relies in the capacity of SCs to proliferate, differentiate and fuse with damaged myofibers. Low ROS promote SCs survival and self-renewal, while promoting proliferation and differentiation mechanisms. On the other hand, high ROS lead to premature, abnormal differentiation and cell death. ROS balance inside the SCs niche is mandatory for appropriate tissue regeneration and remodeling following injury

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1.5. Evidence of a Redox-Dependent Regulation of Satellite Cells

Intrinsic and extrinsic cues in the SCs microenvironment are redox-dependent and decide the fate of myogenic precursor cells during the stages of muscle regeneration [106]. Redox-sensitive signaling pathways directly regulate redox sensors which then affect downstream factors involved in cellular homeostasis [107]. They include the forkhead homeobox type O family (FoxO), nuclear factor erythroid 2-related factor 2 (Nrf2), apurinic/apyrimidinic (AP), endonuclease1/redox factor-1 (APE1/Ref-1) and ataxia telangiectasia mutated (ATM) which monitor redox homeostasis under low ROS levels [105]. High ROS levels induce abnormal differentiation and/or senescence by redox-sensitive molecules such as the hypoxia-inducible factors and p38 [106]. Skeletal muscle SCs reside in a redox-sensitive niche as ROS levels mediate their status and activity [106]. Increasing evidence in the literature supports the notion that redox homeostasis is an important modulator in the self-renewal and differentiation process of stem cells [108, 109]. Notably, ROS are thought to negatively affect myogenesis in numerous pathological conditions (e.g., muscular dystrophy, sarcopenia and muscle wasting) characterized by muscle microtrauma, persistent inflammation and impairment of SCs activity [110-112]. Depletion of intracellular GSH raised ROS levels immensely and promoted NF-κB activation that led to MyoD downregulation and decreased myogenic potential [113]. Specifically, in H2O2-treated C2C12 cells, ROS production was markedly elevated accompanied by a decrease in GSH stores which resulted in a significant reduction in MyoD mRNA levels during myoblasts proliferation and of myogenin and MRF4 at the later stages of differentiation [113]. Furthermore, the redox-sensitive factor PIXT2 act as key regulator of the intracellular redox state, preventing DNA damage as cells undergo differentiation [114, 115]. Accordingly, Vallejo et al. [116] showed that the c-isoform of PITX2 transcription factor can modify the myogenic potential of SCs of a dystrophic-deficient mouse by increasing cell proliferation and the number of myogenic committed cells by regulating micro-RNA 31 (miR-31). Altogether, the combined effects of ROS elevation and GSH depletion resulted in mitochondrial degradation and apoptosis [113]. Similarly, an extensive mitochondrial disruption was observed in oxidatively injured C2C12 murine myoblasts [117].

Previous studies have shown that increased ROS could lead to decreased myogenesis by reducing MyoD protein expression and loss of viability and stemness of SCs following a rise in NF-κB signaling in SCs and myoblasts [118]. However, it must be noted that the ROS/NF-κB axis can regulate both positively and negatively the myogenic process as low amounts of ROS are needed for the initiation of the regeneration phase [119]. In this context, ROS impact cellular

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signaling via post-translational modifications and more specifically protein oxidations (intramolecular disulfide bridges, sulfonylamide bond formation, activation of tyrosine kinases, inhibition of phosphatases) of multiple target proteins. Oxidation of the cysteine thiol group residues is the most extensively characterized type of protein modification and cysteine oxidation, accounting for approximately 1.9% of all protein modifications by ROS [102]. In turn, the catalytic activity of antioxidant enzymes, such as PRX, catalase (CAT), and glutathione peroxidase (GPX) can also be modified by signaling molecules indicating an influential balance between cellular signaling and redox status [120]. Yet, antioxidant enzymes like SOD, CAT, GPX and heme oxygenase-1 (HO-1) seem to regulate myogenic proliferation and differentiation [1]. Numerous in vivo studies have reported beneficial protective effects for GPX, SOD1, HO-1 and CAT regarding oxidative damage and maintenance of myogenic precursor cells function [118, 121].

Two major pathways in SCs biology, which are well-characterized as redox-sensitive and act antagonistically are the Notch and Wnt signaling pathways [122, 123]. The Notch signaling pathway is instrumental for the maintenance of the quiescent state of SCs and regulates the proliferation and differentiation of myogenic precursor cells [123]. The Notch pathway is partly modulated by the ROS -producer NADPH oxidase 1 [124, 125]. Dysregulation of this pathway leads to premature differentiation of myoblasts and reduction in SCs pool via impaired self-renewal [126]. Downstream events in this pathway lead to the expression of Hes1, Hey1 and HeyL genes which regulate quiescence and stemness sustainability through the upregulation of PAX7 and inhibition of activation genes [126]. Canonical Wnt signaling is implicated in SCs differentiation, while non-canonical signals regulate self-renewal and myofiber growth [127]. Wnt proteins bind to transmembrane Frizzled receptors (Fzd) which cooperate with Dishevelled (Dvl) and heterotrimeric G-proteins for downstream signaling. During canonical Wnt signaling phosphorylation of β-catenin by casein kinase I (CK1) leads to its translocation into the nucleus where it forms complexes with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors promoting the expression of MyoD protein and downregulating Pax7, thus promoting myogenic proliferation and differentiation [127]. Non-canonical Wnt signaling stimulation, especially Wnt7a/Fzd7 signaling, can lead to the PI3K (Phosphoinositide 3-kinase) activation, of which then activates the AKT/mTOR pathway resulting in increased protein synthesis [128, 129]. The Wnt pathway can be modulated by TRX [interacts with Dishevelled (Dvl) inside the pathway] and selenium which influences several Wnt proteins through GPX1 [124].

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TNF-α/NF-κB axis is known to inhibit myogenesis, an effect that is widely attributed to the oxidative activation of NF-κB and subsequent gene expression [119]. The redox regulation of the NF-κB family of transcriptional activators plays a central role in differentiation, adaptation, and apoptosis of muscle cells. Muscle damage, inflammation and oxidative stress can activate NF-κB through phosphorylation and release of its inhibitory protein IKKα/β, while ROS can regulate NF-κB via multiple mechanisms [130].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is also implicated in redox control of SCs [131]. Under normal conditions, Nrf2 is localized in the cytosol, bound to its inhibitor Kelch-like ECH-associated protein 1 (Keap1). ROS oxidize Keap1 and it dissociates from Nrf2, which then translocate to the nucleus, heterodimerizes with Maf proteins and binds to antioxidant responsive element (ARE) in order to induce expression of ARE-dependent genes [132]. ROS-dependent Nrf2 signaling plays a critical role in the regulation of SCs self-renewal and proliferation as it can upregulate MyoD and downregulate myogenin levels [133]. In corroboration, Takahata et al. [134] reported protective effects of Nrf2 in response to oxidative damage in undifferentiated mesenchymal C3H10T1/2 stem cells.

IGF-1, a peptide hormone with a complex post-transcriptional regulation, is known to promote muscle protein synthesis and is also correlated with myoblast survival through a protective mechanism against H2O2-induced cell death via PI3K/Akt and ERK1/2 MAPK pathways [135, 136]. Acting in a synergistic manner with Sirtuin 1 deacetylase, it initiates myoblast proliferation [137]. In vitro experiments have demonstrated that during late differentiation phase, ROS-induced IGF-1 activation promotes myotube hypertrophy likely through the upregulation of the Akt/mTOR pathway [138]. Myofiber growth and tissue regeneration may be affected by antioxidant enzymes not only through changes induced in SCs viability and proliferation but also by regulation of the differentiation process per se. Notably, increased myotube formation was observed following GPX upregulation [139], whereas a reverse effect was evident in GPX-1-deficient mice [121] and in response to reduced GSH concentration [113]. Collectively, this piece of evidence suggests the presence of a strong interaction and association between redox-regulated molecules and pathways involved in skeletal muscle SCs fate during both quiescence and the regeneration process (**Figure 1.5**).

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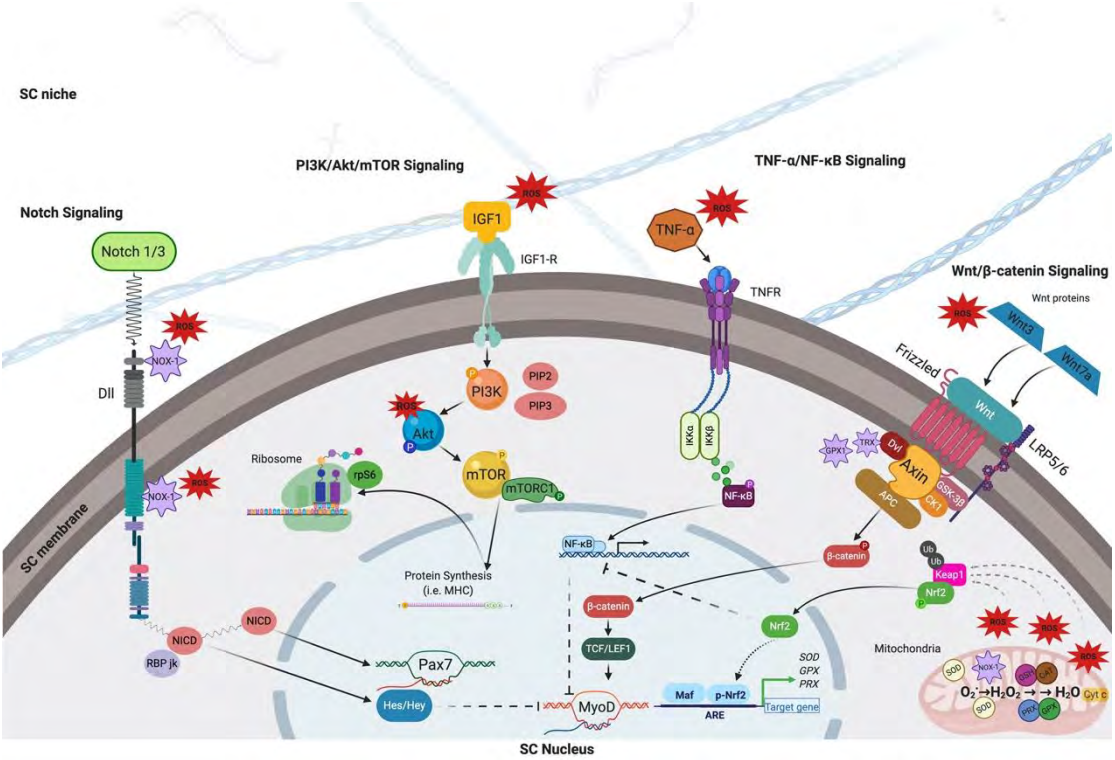

Figure 1.5. Redox-sensitive signaling pathways involved in satellite cells (SCs) homeostasis and recovery following acute aseptic muscle microtrauma. Intracellular signaling pathways inside the SCs microenvironment orchestrate their activity and myogenic potential. Redox sensors and proteins sensitive to alterations in redox status can modulate quiescence, proliferation and differentiation through the induction of specific genes inside the SCs nucleus. Notch signaling promotes a quiescent state through the upregulation of paired box protein 7 (Pax7) and downregulation of the myoblast determination factor (MyoD). Antagonistically, several Wnt proteins (Wnt/β-Catenin cascade) induce the expression of MyoD and the proliferation of SCs. TNF-α/NF-κB pathway inhibits SCs proliferation by blocking MyoD expression. Nuclear factor erythroid 2-related factor 2 (Nrf2) dissociation from Kelch-like ECH-associated protein 1 (Keap1) and its translocation into the SCs nucleus induces the expression of antioxidant enzymes responsible for SCs maintenance and function. Phosphoinositide 3-kinase/Protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway is crucial during the late stages of tissue regeneration and remodeling, mainly via the induction of a hypertrophic response, thus promoting skeletal muscle growth.

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1.6. The Influence of Pathophysiological Conditions on the Redox-Dependent Regulation of Satellite Cells

Several pathophysiological conditions such as aging, muscular dystrophies, sarcopenia and muscle wasting have been shown to significantly impair SCs homeostasis and function, while inflammation and excess ROS also negatively affect SCs responses [140-145].

During aging, the regenerative capacity of skeletal muscle is compromised, due to a decline in SCs number and impaired sensitivity to the damage stimuli [146]. In aged mice, it has been shown that ~10% of the resident SCs are susceptible to senescence following mitogen exposure, thus limiting the ability of aged SCs for regeneration [143]. In humans, advanced age is often accompanied by the development of a low-grade, chronic, systemic inflammation (termed inflammaging), which is characterized by a marked elevation in circulating inflammatory molecules (i.e., TNF-α, IL-6) and overproduction of ROS [147, 148]. These inflammatory cues inside the SCs niche propagate a cytokine-mediated upregulation of several redox-sensitive targets such as NF-κB pathway, further mitigating SCs activation capacity (through MyoD inactivation) and regeneration potential [149]. Furthermore, redox-mediated signaling pathways such as p38, p16, JAK/STAT and Notch/Wnt are largely affected during aging and impact SCs function. Inhibition of p38 in aged SCs increase their proliferative capacity and self-renewal potential [150], while high ROS levels can cause DNA damage in SCs, leading to a p16-induced cellular senescence response [151]. Furthermore, upregulation of JAK/STAT signaling during aging contributes to altered SCs phenotypes and aging-associated impairments that can be reversed by transient downregulation of JAK/STAT pathway activity [152]. In aged muscle, Notch ligand Delta1 is reduced and SCs ability to induce Notch signaling is compromised, causing defects in maintenance, survival and proliferation of aged SCs [153]. Additionally, Wnt signaling upregulation during aging, via the complement protein C1q, perturbates its dynamic balance with Notch, and thus favors misdifferentiation at the expense of self-renewal, contributing to the age-associated exhaustion of the SCs pool [140]. Interestingly, this age-related reduction in SCs pool has been characterized as a major hallmark of sarcopenia [140]. Altogether, the molecular redox-mediated alterations in the microenvironment and within the SCs during aging seem to impair their maintenance and function, leading to aberrant skeletal muscle regeneration.

In muscle diseases such as muscular dystrophy and muscle wasting, characterized by muscle microtrauma, SCs function and regeneration potential are also diminished. The increase in ROS production and alterations in redox balance have been shown to correlate with the severity

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of the pathology in several muscular dystrophies, mitigating skeletal muscle healing potential [1]. Cellular mechanisms related to ROS signaling have been recently associated with SCs defects in disease models. Experiments in mice have shown that dystrophin-deficient SCs-derived myotubes are more susceptible to oxidative damage and that this susceptibility was associated with the severity of the disease [154]. Interestingly, it has been reported that the susceptibility of mdx muscle to oxidative stress was accompanied by the alteration of p38 and JNK signaling pathways, both redox-sensitive and involved in myogenic programming [155]. SCs derived from facioscapulohumeral muscular dystrophy (FSHD) patients also present increased susceptibility to oxidative damage compared to that from nonaffected muscles, a finding that was accompanied by morphological differentiation defects [156]. Epigenetic control has also been reported to interact with redox signaling and SCs function. It was shown that upregulation of the Polycomb group protein Bmi1 in mdx-derived SCs triggers a protective antioxidative response, limits DNA damage and enhances regeneration, thus improving the pathology [157]. Collectively, evidence suggests that disturbances in redox signaling negatively affect SCs intrinsic responses in the context of muscle diseases, and as such mitigate skeletal muscle's regeneration and healing potential.

1.7. Is Muscle Performance Under Redox Regulation?

As mentioned, SCs are crucial regulators of muscle performance recovery kinetics following intense exercise. Therefore, a question may arise here as to whether muscle performance is redox-dependent. So far, supplementation with vitamins (predominantly C and E) has been extensively utilized to alter redox status and improve performance in athletes; however, their effectiveness in enhancing performance and promoting molecular adaptations to exercise training is to date ambiguous (**Table 2**). In humans, Yfanti et al. [158] reported that vitamins C (500 mg/day) and E (400 IU/day) have no impact on endurance adaptive responses, mitochondrial biogenesis and antioxidant enzyme activity. In contrast, animal studies revealed that supplementation with vitamin C, E or their combination down-regulates biomarkers of mitochondrial biogenesis and the intramuscular antioxidant content [159]. In line with these findings, Ristow et al. [160] reported that supplementation with 1000 mg/day vitamin C combined with 400IU/day of vitamin E following 4 weeks of endurance training attenuated the expression of several mitochondrial biogenesis-related mRNAs (PGC-1α, PGC-1β and PPARγ) and reduced the levels of TBARS in previously untrained male individuals. Similarly, in

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recreationally endurance trained men and women, supplementation with vitamin C and E (1000 mg/day and 235 mg/day, respectively) for 10 weeks, reduced mitochondrial biogenesis markers (PGC-1α, COX4, CDC42 and MAPK1), though no impact on redox status was observed [161]. In addition, several studies investigated the impact of vitamin C and E supplementation on redox status and performance adaptations induced by resistance exercise training. The work by Theodorou et al. [162] indicated that supplementation with vitamin C during 4 weeks of eccentric training had no effect either on performance or on redox status in recreationally trained young men. Conversely, Paulsen et al. [163] reported that administration of vitamins C and E ameliorated the increase in muscle strength and hypertrophy during a 10-week resistance exercise intervention in trained individuals. Likewise, vitamin C and E supplementation decreased muscle thickness and blunted the increase in lean mass following resistance training in healthy older males [164]. Nevertheless, other studies have reported positive effects of vitamin C and E supplementation. Characteristically, short-term ingestion of combined vitamin C and E supplementation (vitamin C: 1000mg/d; vitamin E: 400 IU/d) not only attenuated the levels of CK and muscle soreness, but also enhanced muscle protection through an improved oxygen radical absorbance capacity (ORAC) following a second bout of exercise [165].

To this end, although indications exist that antioxidants can prevent protein oxidation during exercise [166], there is little or no evidence in vivo to suggest that antioxidant supplementation is sufficient to attenuate EIMD and/or accelerate recovery during the succeeding days. It is yet to be investigated whether changes in muscle performance appear in conjunction with alterations in redox status and SCs-mediated responses during the healing phase following myotrauma. Thus, we propose that data from interventional studies utilizing supplementation with antioxidants should be interpreted with caution as baseline redox status, the dose and length of the antioxidant supplementation as well as the choice of the redox biomarkers measured are critically implicated in the controversial cellular and physiological responses observed [167].

NAC, a thiol-based supplement and a precursor molecule for GSH, has been shown to exert positive effects on performance and skeletal muscle recovery [91]. NAC supplementation results in a significant preservation of muscle performance, via the upregulation of intramuscular GSH levels, and thus, it might be a useful strategy to enhance performance during short-term competitive situations in young recreationally trained men [168]. NAC may attenuate muscle strength decline, in part via improved K+ regulation suggesting a role for ROS in muscle fatigue [169]. In a double-blind, randomized study, NAC supplementation improved cycling performance via an improved redox balance and promoted adaptive processes in well-trained

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athletes undergoing strenuous physical training [170]. These results are in agreement with previous observations in endurance athletes where NAC improved performance probably through an enhanced availability of muscle cysteine residues and GSH, possibly promoting an optimal redox environment [171]. Only few studies have investigated the effects of redox status alterations on the recovery and healing processes following intense exercise in human muscle. In a double blind, crossover, randomized trial, NAC supplementation improved muscle GSH/GSSG ratio throughout recovery and attenuated the elevation of inflammatory markers of muscle damage (CK activity, C-reactive protein, proinflammatory cytokines), NF-κB phosphorylation, and the reduction of muscle strength during the first 2 days of recovery [91]. Of particular interest, NF-κB is associated with the early phase of regeneration and its down-regulation is required for proper SCs activation (via MyoD activation) [172]. In another study, NAC supplementation efficiently altered the redox status following a very intense eccentric exercise protocol, as evident by the attenuated decline of reduced glutathione in erythrocytes, a diminished rise of plasma protein carbonyls as well as an enhanced antioxidant activity observed during recovery [77]. The authors also reported that NAC reduced biomarkers of muscle damage and inflammation (CK activity, CRP, pro-inflammatory cytokines, adhesion molecules) and protected muscle strength during recovery [77]. Furthermore, the rise of HLA+ and 11B+ macrophages, as well as intramuscular macrophage infiltration have been shown to decrease in response to NAC supplementation under conditions of EIMD [77, 91]. Altogether, these findings provide strong evidence for a mechanistic pillar between inflammation, redox status and performance in human skeletal muscle following EIMD.

Recent reports provided evidence for a redox individuality, suggesting that responses to antioxidant supplementation may be individualized [167]. Specifically, in the investigation by Paschalis et al. [173], thirty-six participants were classified in three experimental groups according to their basal GSH levels in blood (low, moderate and high GSH group), and were supplemented with NAC daily (2×600mg), for 30 consecutive days. NAC supplementation restored baseline glutathione levels and redox homeostasis, reduced systemic oxidative stress and improved performance significantly in the low glutathione group [173]. Similarly, previous work by the same group revealed that low vitamin C levels were linked with decreased physical performance and increased oxidative stress. However, daily vitamin C supplementation (3×333mg) for 30 days, reduced oxidative stress and increased exercise performance only in those with low basal vitamin C levels [174]. These findings may explain the discrepancies often observed between studies investigating the effects of antioxidant supplementation on muscle

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performance. Although in this section we predominantly present indirect evidence of a redox-dependent regulation of performance recovery, we assume that these findings provide valuable information regarding the interconnection between muscle performance, redox status and the role of SCs. Studies that are directly investigating the relationship between redox status, SCs biology and performance in humans are currently lacking.

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Table 1.2 The effects of antioxidant administration on muscle performance, inflammation and redox status in humans.

Publication [Refs.]	Subjects	Fitness status	Supplementation protocol	Exercise regime	Effects on performance	Effects on inflammation	Effects on redox status
Bjornsen et al. [164]	34 elderly men	Untrained	12 weeks: 1000 mg Vt C + 235 mg Vt E per day	Resistance training	↓Muscle thickness ↑Muscle strength ↓Lean mass	N/A	N/A
Cobley et al. [168]	12 young men	Recreationally trained	6 days: 50 mg/kg/day NAC	Damaging exercise	↑YIRT-L-1 ↑Sprint	↑CK	N/A
Gomez-Cabrera et al. [159]	14 young men	Sedentary	8 weeks: 1000 mg/day Vt C	Endurance training	↔	N/A	N/A
He et al. [165]	22 young men	Moderately trained	2 weeks: 1000 mg Vt C + 400 IU Vt E per day	Downhill running	↓DOMS	↓CK	↑ORAC
McKenna et al. [169]	8 young men	Well-trained	125 + 25 mg/kg/h NAC before and during exercise	Endurance exercise	↑Na^+, K^+ pump activity ↑Performance	N/A	N/A

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Medved et al. [171]	8 young men	Endurance trained	125 + 25 mg/kg/h NAC before and during exercise	Submaximal exercise	↓Fatigue	N/A	↑Muscle NAC ↔Blood GSH ↑TGSH/GSH ↑Muscle Cys
Michailidis et al. [91]	10 young men	Recreationally active	8 days: 20 mg/kg/day NAC	Damaging exercise	↑Short-term recovery	↓CRP, IL-6, CK	↑GSH/GSSG
Paschalis et al. [173]	36 young men	Recreationally active	30 days: 2 × 600 mg/day NAC	Performance testing	↑Performance	N/A	↓Isoprostanes F_2 ↑NADPH ↑GSH ↑SOD ↓PC
Paschalis et al. [174]	20 young men	Recreationally trained	30 days: 1000 mg/day Vt C	Endurance exercise	↑VO_{2max}	N/A	↓Isoprostanes F_2 ↓PC

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Paulsen et al. [161]	54 young men and women	Recreationally trained	11 weeks: 1000 mg Vt C + 235 mg Vt E per day	Endurance training	↓PGC-1α ↓MAPK1 ↓CDC42 ↓COX4	N/A	↔
Paulsen et al. [163]	32 young men and women	Recreationally trained	10 weeks: 1000 mg Vt C + 235 mg Vt E per day	Resistance training	↓Muscle hypertrophy ↓Muscle strength	N/A	N/A
Ristow et al. [160]	40 young men	Trained and untrained	4 weeks: 1000 mg Vt C + 400 IU Vt E per day	Complex training	↓PGC-1α ↓PGC-1β ↓PPARγ	N/A	↓SOD1SOD2, GPX1 mRNA ↓TBARS
Sakelliou et al. [77]	10 young men	Recreationally active	8 days: 20 mg/kg/day NAC	Damaging exercise	N/A	↓HLA^+, ↓$11B^+$ cells ↓Neutrophils ↓Leukocytes	↓TBARS ↓GSSG ↓PC

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Slattery et al. [170]	10 young men	Well-trained	9 days: 1.2 g/day NAC	Endurance exercise	↑Sprint	↓IL-6	↓Isoprostanes F_2 ↓TBARS ↑TAC
Theodorou et al. [162]	28 young men	Recreationally trained	9 weeks: 1000 mg Vt C + 400 IU Vt E per day	Eccentric training	↔	↔	↔
Yfanti et al. [158]	21 young men	Moderately trained	16 weeks: 500 mg Vt C + 400 IU Vt E per day	Endurance training	↔	N/A	↔

Vt, vitamin; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPARγ, peroxisome proliferator-activated receptor gamma; TBARS, thiobarbituric reactive substances; SOD1/1, superoxide dismutase 1,2; GPX1, glutathione peroxidase 1; COX4, cytochrome c oxidase 4; CDC42, cell division control protein 42 homolog; MAPK1, mitogen-activated protein kinase 1; DOMS, delayed onset of muscle soreness; CK, creatine kinase; ORAC, oxygen radical absorbance capacity; YIRT-L-1, Yo-Yo intermittent recovery test level 1; IL-6, interleukin 6; TAC, total antioxidant capacity; GSH, glutathione; GSSG, oxidized glutathione; TGSH, total glutathione; CRP, c-reactive protein; Cys, cysteine; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; ↑, increase; ↓, decrease; ↔, no effect; N/A, not applicable.

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1.8. The Impact of Nutritional and Pharmacological Interventions on Satellite Cells-Mediated Skeletal Muscle Regeneration

In an attempt to ameliorate the detrimental effects of ROS-induced oxidative damage in muscle tissue and to enhance the myogenic potential as well as the capacity of skeletal muscle to promote healing, a number of in vivo and in vitro studies have utilized antioxidant and anti-inflammatory supplementation both in basal conditions and following myotrauma (**Table 3**). In regards to antioxidant manipulation two studies showed that administration of 20 mg/kg/day of proanthocyanidolic oligomer (PCO) orally for 14 days pre- and post-contusion injury resulted in increased SCs number and fetal myosin fibers (MHCf+), improved muscle ultrastructural recovery, enhanced antioxidant capacity and reduced TNF-α levels compared to placebo in adult male Wistar rats [3, 4]. In vitro and in vivo ursolic acid (UA) supplementation at rest (in vitro: 10μM UA+DMSO; in vivo: 200 mg/kg) for 7 days resulted in increased Pax7+ and myogenin+ cells, increased myonuclei and myofiber content and reduced SCs apoptosis while cell death was evident [2]. In aged mice, Trolox (Vitamin E analog) supplementation (in vitro: 100 μM 20% FBS containing Ham's F10 medium; in vivo: 30 mg/kg/day for 2 weeks intraperitonially) at rest, led to decreased autophagy and senescence of SCs and lower myostatin mRNA expression levels [175]. Interestingly, improved SCs viability was accompanied by a decrease in mitochondrial ROS production in SCs following supplementation with Trolox [175]. Furthermore, Zhang et al. [176] demonstrated a beneficial effect of nicotinamide riboside (NR) supplementation (400 mg/kg/day for 6 weeks) at rest and following cardiotoxin-induced muscle damage including a pronounced increase in the muscle regeneration and SCs number (Pax7+ cells) 14 days post-injury. In the same study, mdx mice treated with the same dosage of NR also exhibited improved muscle regeneration and upregulated SCs content (Pax7+ cells) [176]. Likewise, Hollinger et al. [177] reported that quercetin (plant flavonoid)-enriched diet (0.2% quercetin for 6 months) improved muscle morphology and reduced fibrosis and TNF-α levels, at basal state, in mdx mice. In a model of stress-induced premature senescence (SIPS) induced by H2O2, tocotrienol-rich-fraction (Vitamin E isomer) treated CHQ5B cells (50 mg/ml for 24 hours post-SIPS) demonstrated increased myoblast proliferation and self-renewal and decreased myostatin mRNA expression levels [178]. Moreover, primary cell cultures (from mdx mice) treated for 48 hours with green tea polyphenol (GTP) blend and epigallocatechin gallate (EGCg), both classes of catechins, exhibited improved myogenisity (desmin+ immunoreactivity) and cell viability with concomitant upregulation in GSH availability and GSH/GSSG ratio [179].

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A few human studies have investigated the effect of anti-inflammatory based supplementation on SCs kinetics (the propagation from a quiescent to an activated/proliferative state) after muscle damaging stimuli, providing equivocal results. Indomethacin supplementation (100 mg/day orally for 4 days) prior to a 36-km race (intense exercise) decreased NCAM+ cells during recovery while no effect was observed in newly formed myofibers and central nuclei count [180]. Muscle infusion with indomethacin (50 mg/ml, 2 μl/min, 7.5 h) prior to and after 200 maximal eccentric contractions of the quadriceps muscles resulted in decreased Pax7+/NCAM+ cells at day 8 of recovery and no alteration in the number of activated myogenic cells (Ki67+ cells), while CD16+ cells increased at day 8 post-exercise [68]. In contrast to these observations, supplementation with ibuprofen (1200 mg/day orally, 14 days before injury and for 30 days post-injury) increased Pax7+ and Notch1+ cells (in all fiber types) at day 7 following electrical stimulation-induced muscle injury, while no alteration observed in Ki67+ cells (activated SCs) [181]. Ibuprofen failed, however, to alter CD68 mRNA levels, CK, LDH and myoglobin responses, whereas embryonic (MHCe+) and neonatal (MHCn+) fibers decreased at 30 days post-injury [181].

Interestingly, studies examining the effect of antioxidant and/or pharmaceutical compounds on SCs activity following intense exercise protocols in skeletal muscle are lacking. It appears that antioxidant and anti-inflammatory treatment ameliorates ROS production, preserves short-term muscle performance following EIMD and expedites muscle healing possibly via upregulation of SCs activation, proliferation and differentiation. Refined intrinsic and extrinsic mechanisms related to redox status in SCs seem to coordinate this process and the redox-specific molecular mechanisms are yet to be determined.

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Table 1.3 The impact of nutritional and pharmacological interventions on myogenic response, regeneration, inflammation and redox status *in vitro* and *in vivo.*

Publication [Refs.]	**Species**	**Condition**	**Compound administration protocol**	**Effects on myogenic response**	**Effects on muscle damage/regeneration**	**Effects on inflammation**	**Effects on redox status**
Bakhtiari et al. [2]	Isolated SCs	Culture	7 days: UA+DMSO in serum	↑Myogenin^{+} cells ↓SCs apoptosis ↑Pax7^{+} cells	N/A	N/A	N/A
	Male C57BL/6 mice	Resting	7 days: 200 mg/kg/day UA	↑Pax7^{+} cells	↑Myofibers ↑Nuclei ↑Mb	N/A	N/A
Dorchies et al. [179]	*Mdx* primary cells	Oxidative-induced damage	48 hours: GTP + EGCg in serum	↑Myogenisity ↑Cell viability	N/A	N/A	↑GSH/GSSG ↑GSH

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Hollinger et al. [177]	*Mdx* mice	Resting	6 months: 0.2% quercetin/day	↔Myf5, MyoD myogenin, mRNA	↑Muscle morphology ↔MHCe^{+} mRNA ↓Muscle fibrosis	↓TNF-α	N/A
Kruger et al. [3]	Male Wistar rats	Contusion-induced damage	14 days: 20 mg/kg/day PCO	↑Pax7^{+} cells	↑Regeneration ↔CK	↑Macrophage ↓Neutrophils ↔ TNF-α ↔IL-1β ↓IL-6	↑ORAC
Laura Garcia-Prat et al. [175]	Aged mice	Resting	2 weeks: Trolox, 30 mg/kg/day (in vivo), 100 μM in serum (in vitro)	↓Myostatin mRNA ↓SCs senescence ↓SCs autophagy	N/A	N/A	↓SCs mt-ROS
Lim et al. [178]	CHQ5B cells	SIPS induction	24 hours: 50 mg/ml TRF	↑SCs Proliferation ↓Myf5, Myf6, myostatin mRNA	N/A	N/A	N/A

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				↑SCs renewal			
Mackey et al. [180]	Endurance-trained athletes	36-km race	4 days before race: 100 mg/day Indomethacin	↓NCAM^{+} cells ↔FA1^{+} cells	↔Central nuclei ↔MHCe^{+}	N/A	N/A
Mackey et al. [181]	Young adults	ES–induced injury	14 days: 1200 mg/day Ibuprofen	↑Notch1^{+} cells ↔Ki67^{+} cells ↑Pax7^{+} cells	↔CK, LDH, Mb ↔Regeneration ↓MHCe^{+}	↔CD68 mRNA	N/A
Messina et al. [112]	*Mdx* mice	Resting	5 weeks: 8,5 mg/kg/day flavocoxid	↑Myogenin^{+} SCs	↑Muscle morphology ↓CK	↓JNK-1, TNF-α ↓P-p38	N/A
Mikkelsen et al. [68]	Young adults	Damaging exercise	7.5 hours: 50 mg/ml indomethacin	↓Pax7^{+}, NCAM^{+} cells ↔ Ki67^{+} cells	↔Central nuclei	↑CD16^{+} cells	N/A
Myburgh et al. [4]	Male Wistar rats	Contusion-induced muscle damage	14 days: 20 mg/kg/day PCO	↑Pax7^{+}, CD56^{+}, CD34^{+} cells	↑MHCf^{+} myofibers	↑Macrophages ↓Neutrophils	↑ORAC

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						↓TNF-α ↔IL-6	
Whitehead et al. [130]	*Mdx* mice isolated myofibers	Stretched contractions	20Mm NAC in serum	N/A	↓Central nuclei ↑Muscle force ↑β-dystroglycan ↑Utrophin	↓Muscle damage ↓NF-κB	↓ROS
Zhang et al. [176]	Aged C57BL/6J mice	Resting and CTX-induced damage	6 weeks: 400 mg/kg/day NR	↑ $Pax7^+$	↑Regeneration	N/A	N/A
	Mdx mice	Resting and CTX-induced damage	6 weeks: 400 mg/kg/day NR	↑$Pax7^+$ cells ↑Beta-Gal^+ cells	↑Regeneration	N/A	N/A

SCs, satellite cells; PCO, proanthocyanidolic oligomer; UA, ursolic acid; CK, creatine kinase; H&E: hematoxylin and eosin staining; IL-1β, interleukin-1β; IL-6, interleukin-6; FA1, fetal antigen 1; JNK, c-Jun N-terminal kinases; TNF-α, tumor necrosis factor alpha; ORAC, oxygen radical absorbance capacity; MHCe, embryonic myosin heavy chain; mt-ROS, mitochondrial reactive oxygen species; LDH, lactate dehydrogenase; Mb, myoglobin; i.p., intraperitonially; TRF, tocotrienol-rich fraction; NR, nicotinamide riboside; CTX, cardiotoxin; SIPS, stress-induced premature senescence; ES, electrical stimulation; GTP, green tea polyphenol blend; EGCg, epigallocatechin gallate; eMHC, embryonic myosin heavy chain; NAC, N-acetylcysteine; DHE, Dihydroethidium; P-p38, phosphorylated-p38; ↑, increase; ↓, decrease; ↔, no effect; N/A, not applicable.

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1.9. Conclusions

Strong evidence from in vivo and in vitro experiments suggests an interplay between skeletal muscle SCs, inflammation and ROS in response to acute and/or chronic muscle microtrauma characterized by an elevated inflammatory status. Although low levels of ROS are beneficial and necessary for proper cell function through redox-sensitive signaling cascades, excessive ROS production leads to irreversible post-translational modifications (mainly thiol oxidation) and deactivation of key signaling molecules which may be detrimental for the successful tissue healing during regeneration. Taking into consideration the complexity of redox regulations, human studies investigating the effect of redox disturbances and ROS-induced SCs kinetics in conditions characterized by increased inflammation and deterioration of muscle performance are lacking. Evidence from cell lines and animal models indicate that the use of substances with antioxidant properties (i.e., vitamins, polyphenol extracts, NAC, NR) increase SCs viability and function under physiological stress or in pathological conditions such as aging and muscular dystrophy. It is therefore plausible to speculate that antioxidant treatment may offer a valuable nutritional and/or therapeutic strategy in order to enhance skeletal muscle SCs kinetics (by up-regulating key survival and action mechanisms) under inflammatory conditions and potentially lead to improved muscle regeneration and performance. Future studies need to examine the SCs response in humans using aseptic models of muscle trauma, in combination with nutritional/pharmaceutical supplements able to alter redox status, in order to elucidate the redox-dependent regulation of SCs kinetics [182]. Although the redox-regulated pathways involved in SCs responses are limited to observations in animal studies it would be of great importance to examine these redox-sensitive molecular pathways related to SCs responses (i.e., Notch/Wnt, Nrf2, TNF-α/NF-κB and IGF-1/Akt/mTOR signaling) using randomized, well-controlled, human clinical trials. Moreover, key redox sensors, antioxidant molecules and protein/lipid oxidation-modification (i.e., DNA methylation) must be assessed in relationship to SCs kinetics following myotrauma. Results from such studies will provide valuable insight into the impact of redox status during recovery from aseptic muscle trauma/injury and potential nutritional/pharmaceutical strategies to enhance muscle repair and remodeling in health and disease.

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Chapter 2

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The redox-dependent regulation of satellite cells following aseptic muscle trauma (SpEED): Study protocol for a randomized controlled trial

Papanikolaou K., Draganidis D., Chatzinikolaou A., Laschou V.C., Georgakouli K., Tsimeas P., Batrakoulis A., Deli C.K., Jamurtas A.Z., Fatouros I.G.

Abstract

Muscle satellite cells (SCs) are crucial for muscle regeneration following muscle trauma. Acute skeletal muscle damage results in inflammation and production of reactive oxygen species (ROS) which may be implicated in SCs activation. Protection of these cells from oxidative damage is essential to ensure sufficient muscle regeneration. The aim of this study is to determine whether SCs activity under conditions of aseptic skeletal muscle trauma induced by exercise is redox-dependent. Based on their SCs content in their vastus lateralis skeletal muscle, participants will be classified as either high or low respondents. In a randomized, double-blind, crossover, repeated measures design, participants will then receive either Placebo or N-acetylcysteine (alters redox potential in muscle) during a preliminary 7-day loading phase, and for 8 consecutive days following a single bout of intense muscle-damaging exercise. In both trials, blood samples and muscle biopsies will be collected, and muscle performance and soreness will be measured at baseline, pre-exercise, 2- and 8-days post-exercise. Biological samples will be analyzed for redox status and SCs activity. Between trials, a 4-week washout period will be implemented. This study is designed to investigate the impact of redox status on SCs mobilization and thus skeletal muscle potential for regeneration under conditions of aseptic inflammation induced by exercise. Findings of this trial will provide insight into i) molecular pathways involved in SCs recruitment and muscle healing under conditions of aseptic skeletal muscle trauma present in numerous catabolic conditions and ii) if skeletal muscle's potential for regeneration depends on its basal SCs content.

Trial registration: ClinicalTrials.gov, NCT03711838.

Keywords: muscle stem cells, redox potential, antioxidants, cell signaling, tissue regeneration.

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2.1. Background

Skeletal muscle stem cells (Satellite cells - SCs) are required for muscle repair, remodeling and growth [183]. Under conditions of increased transcriptional activity, such as in response to skeletal muscle injury, SCs are activated to promote healing [184]. SCs can be identified by their location (beneath the basal lamina of myofibers) and by the expression of transcriptional proteins, such as Pax7. Each stage of the regeneration process is partly controlled by myogenic regulatory factors (MRFs) which include myogenic factor 5 (Myf5), myogenic determination factor (MyoD), myogenin and myogenic factor 4 (Myf4), all orchestrating skeletal muscle's myogenic programming [50, 185].

Recent data suggest a redox regulated link between SCs and myogenesis [186]. Muscle injury, triggers the NF-kB-mediated apoptosis to control stem cell survival and maintain stemness [187]. SCs' antioxidant reserves protect these cells from oxidative damage whereas antioxidant enzymes glutathione peroxidase 3 (GPX-3) and thioredoxin (Trx-1) regulate the homeostatic programming of quiescent SCs [188, 189]. Redox-sensitive signaling pathways such as Notch and Wnt/β-catenin are also critical for multiple stages of myogenesis including activation and proliferation of SCs. Interestingly, several Wnt proteins have been shown to regulate SCs activation, proliferation and differentiation [125, 128].

Skeletal muscle trauma is observed in numerous catabolic conditions, characterized by elevated proteolysis and muscle wasting such as cancer, cachexia and muscular dystrophy, which result in physical capacity impairment and a deteriorated quality of life [80, 190]. In the absence of SCs, injured skeletal muscle abolishes its ability to regenerate or regenerates very poorly in response to muscle injury [191]. Flow cytometric analysis in isolated SCs revealed that the number of Pax7+ cells/mg of muscle in G2/M, G0/G1 and S phases of the cell cycle increased by 202%, 32% and 59% respectively at 24 h post-injury [67]. Under these conditions, SCs rapidly migrate to the injured area, differentiate into mature myoblasts and contribute to myofiber repair [192]. Exercise-induced muscle damage (EIMD) results in an aseptic muscle microtrauma and a pronounced inflammatory cascade characterized by leukocyte immobilization, macrophage infiltration, cytokine production and increased oxidative stress (OXS) [10, 193]. A study incorporating a muscle damage protocol (300 maximal eccentric contractions) on an isokinetic dynamometer, showed an increase in SCs content even at 180 h post-exercise [69]. These marked similarities between aseptic EIMD and trauma makes eccentric exercise a valuable model to investigate the redox-dependent intracellular regulation of SCs involvement in skeletal muscle's healing potential.

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Only two studies have examined the effectiveness of antioxidant supplementation on skeletal muscle healing and inflammatory response in primary cultured cells and rat gastrocnemius muscle demonstrating that polyphenol administration enhances SCs activation and evokes an earlier appearance of type M2 macrophages promoting an anti-inflammatory phenotype [4, 194]. However, no studies investigated so far, the effect redox status perturbations on SCs responses and myogenic potential following aseptic trauma in human skeletal muscle. A powerful antioxidant that has been in clinical and sports performance practice for several decades is N-acetylcysteine (NAC). NAC is a thiol donor, non-specific antioxidant with a binary antioxidant role: Firstly, NAC directly scavenges a number of reactive oxygen species (ROS) and secondly, it produces reduced cysteines (Cys) by deacetylation, which supports the biosynthesis of endogenous reduced glutathione (GSH) via the activity of γ-glutamylcysteine synthase [195]. GSH plays a pivotal role in cellular metabolism and especially in terms of stem cells oxidative defense, activity, survival and self-renewal under conditions of increased OXS and ROS production [106]. Low to moderate ROS levels control physiological cellular signaling pathways and facilitate muscle growth and development while high ROS levels impair myogenesis and cause cell death through apoptotic and/or necrotic mechanisms (e.g., NF-kB signaling) [196]. We hypothesize that NAC administration following aseptic muscle trauma characterized by severe inflammation and high levels of ROS, will alleviate the increase in oxidative stress by decreasing ROS activity levels, resulting in an optimal intracellular redox environment, favoring the activity (activation, proliferation, differentiation and fusion) of SCs and thus facilitating muscle's regenerative process and adaptation following muscle injury. Thus, the objectives of this clinical trial are to examine the effects of redox status perturbation (via NAC administration) on intracellular pathways responsible for SCs responses at rest and following aseptic muscle trauma induced by damaging exercise.

2.2. Methods/design

2.2.1 Study overview and design

The methods and ethics of the present study have been approved by the Institutional Review Board of the University of Thessaly (ref. number 1387) and procedures are in accordance with the Declaration of Helsinki, as revised in 2013. The SpEED study incorporates a randomized, two-trial (NAC vs placebo), cross-over, double-blind, repeated measures design. Participants who will fulfill the inclusion criteria will have their body mass and height, resting

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metabolic rate (RMR), body composition, muscle strength, soreness and maximal oxygen consumption (VO2max) measured. Since protein and antioxidant consumption as well as systematic physical activity and/or exercise may affect SC responses and/or redox status, participants' daily nutrient intake (via a 7-day diet recall with emphasis on protein and antioxidant intake) and physical activity levels (via accelerometry instrumentation) will be monitored for a week before the inception of the study [8, 197, 198]. Then, a 2-week adaptation period (only before the first trial) will be applied to adjust (through a balanced diet, using RMR values and daily energy expenditure measurements) protein and antioxidant intake at the levels required by current Recommended Dietary Allowances (RDAs) (0.8-1 g of protein/kg/day; 900 mg/day of vitamin A; 90 mg/day of vitamin C; 600 IU/d of vitamin D; 15 mg/day of vitamin E; 11 mg/d of Zinc; 400 mg/d of Magnesium and 55 mg/d of selenium) [199, 200]. This dietary protocol will be designed and implemented by a registered dietician. During the same time-frame, familiarization with experimental procedures will be provided.

Evidence exists that skeletal muscle growth and myogenic potential is directly related to its basal SCs content [7, 8]. As such, volunteers will provide a resting (they will abstain from any exercise or intense physical activity for at least five days prior to sampling) muscle biopsy sample (baseline sampling) from their vastus lateralis muscle (of their dominant limb) and based on its SCs content will be stratified to either high (HR) or low (LR) respondents. Participants will be stratified as high (HR) or low (LR) respondents using a K-means cluster analysis. Blood sampling and performance measurements will also be performed at baseline.

Following stratification to HR and LR group, volunteers of each group will then perform two trials in a randomized order: a) NAC ingestion and b) placebo ingestion. NAC or placebo will be consumed before (a 7-day loading phase) and after (immediately post-exercise and for eight consecutive days thereafter) an intense eccentric exercise protocol. During both trials, participants will follow the balanced daily dietary protocol of the adaptation period. However, daily dietary intake will be recorded and analyzed during each trial in an attempt to minimize deviations from the prescribed diet. A 4-week washout period will be implemented between trials (dietary intake during this period will also be adjusted according to that applied during the adaptive period). During the entire experimental period, participants will be asked to abstain from any strenuous physical activity or exercise. Muscle biopsies and blood samples will be collected after overnight fasting before the exercise protocol (pre-exercise sampling) as well as at 2- and 8-days post-exercise. Muscle strength and delayed onset of muscle soreness (DOMS) will be evaluated at the same time points. All measurements and collection of biological samples will be

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performed at the same time of day, in both trials, to prevent circadian rhythm variations. **Figure 2.1** shows the CONSORT diagram of the study and **Figure 2.2** illustrates the experimental flowchart for the clinical trials.

The primary outcomes of the study are the satellite cell markers measured in muscle (Pax7+, MyoD+ cells per type I/II muscle fibers), the macrophages' markers measured in muscle (CD11b+, CD206+ cells per muscle fiber), the myogenesis markers measured in muscle (Myf5, MyoD, myogenin, MRF4, myostatin mRNA expression levels), the cell signaling markers measured in muscle (GPX-3, SOD-2, Trx-1, IGF-1, Notch1, Wnt3 protein expression levels), and the oxidative stress markers measured in muscle (GSH, GSSG, PC, MDA). The secondary outcomes are the oxidative stress markers measured in blood [total antioxidant capacity (TAC) in serum, GSH, GSSG, CAT, and hemoglobin in red blood cell lysates], inflammatory markers measured in blood (CRP and cytokines TNF-α, IL-6, IL-8, IL-10), blood creatine kinase activity (CK) as a marker of muscle damage, and serum cortisol. Other outcomes include body mass, height, body mass index (BMI), total and regional fat mass, lean mass and body fat, resting metabolic rate, number of steps/day and time spent at sedentary, light, moderate, vigorous and moderate-to-vigorous PA, total daily energy intake and expenditure, daily intake of carbohydrate, fat, protein, vitamin A, vitamin C, vitamin D, vitamin E, selenium, zinc, and magnesium, maximal oxygen consumption (VO2max), knee extensors' maximal eccentric and concentric peak torque, delayed onset of muscle soreness (DOMS).

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CONSORT

TRANSPARENT REPORTING of TRIALS

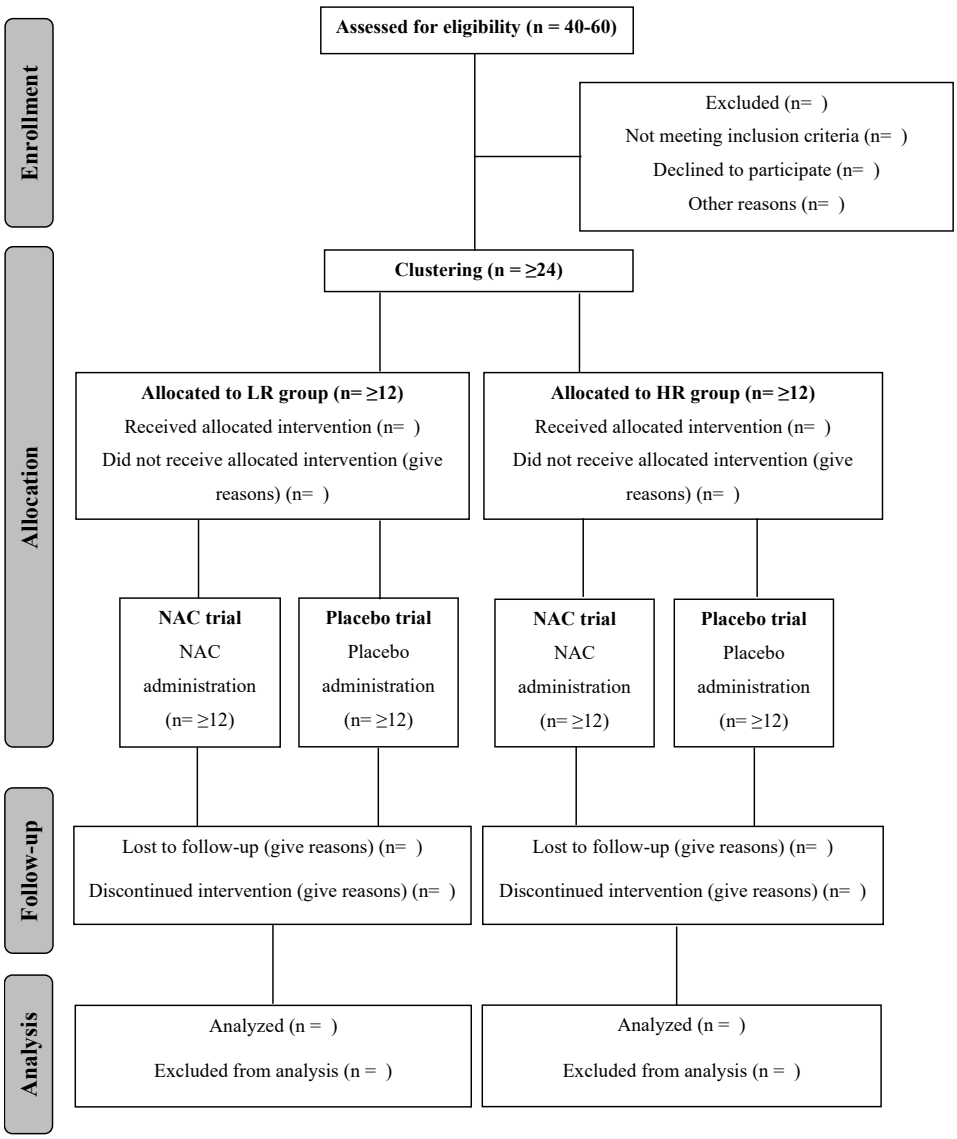

Figure 2.1. The Consolidated Standards of Reporting Trials (CONSORT) diagram of the study.

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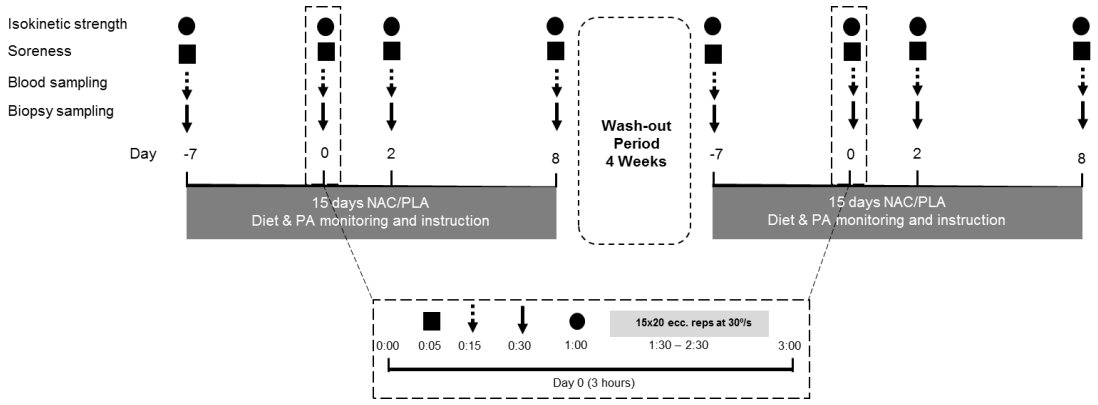

Figure 2.2. The experimental flowchart for the clinical trials.

2.2.2. Participants recruitement and screening

We will initially recruit 40-60 young males. In organized meetings, participants will be informed by the investigators about the purpose of the study, the experimental procedures and all the possible risks and benefits associated. Participants will be recruited via media advertisements and posters. All volunteers will complete a health history questionnaire and a written consent form will be acquired from each participant by the investigators. All personal information and data obtained will be confidential and only the researchers of the study will have access to the database. Participants will be included in the study if they (i) are healthy, non-smokers, aged 18-30 years; (ii) have a BMI of 18.5-24.9 kg/m2; (iii) abstain from any vigorous physical activity during and ≥4 weeks prior to the study; (iv) have no recent history of musculoskeletal injury, lower limb trauma and metabolic diseases; and (v) they refrain from consumption of alcohol, caffeine, any type of nutritional supplements, NSAIDs and medication before (≥6 months) and throughout the experimental period.

Exclusion criteria

- Allergies or intolerance to NAC
- Recent febrile illness
- Use of anti-inflammatory medication

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- Use of medication interacting with muscle metabolism

2.2.3. Exercise protocol

Participants will perform a protocol consisting of 300 eccentric unilateral maximal contractions (20 sets, 15 repetitions/set, 30-second rest between sets) of the quadriceps muscles on an isokinetic dynamometer (Cybex 770, USA) at a speed of 30 °/s. A different limb will be used in each trial. Before the protocol a standard warm up will precede involving 8-min cycling on a cycle ergometer (Monark 834, 154 ERGOMED C, Sweden) at a speed of 70 rpm/min and at 50 W, followed by 5-min stretching exercises. Knee extensors will be isolated using straps in the shoulders, hips and thigh. This protocol has been described in the literature to effectively induce a significant level of skeletal muscle damage and myofibrillar disruption as documented with electron microscopy and immunohistochemistry [14, 91].

2.2.4. Supplementation protocol

Participants will consume either NAC or placebo in a random order according to a double-blind, crossover design. A dosage of 40 mg NAC/kg/day will be administered orally in 3 daily doses (equally distributed), in order to maximize cysteine levels (for glutathione synthesis) in the circulation and skeletal muscle, primarily due to NAC fast rate of clearance (~6 h post-ingestion) [201]. According to a model proposed by Reid in 2001, there is an optimal intracellular redox status required for optimum muscle function and force production [96]. Hence, when NAC is supplemented orally as an exogenous antioxidant (glutathione precursor), under conditions of increased oxidative stress and ROS production, it is plausible to administrate a moderate dose for optimal scavenging of ROS, which according to recent studies, represents an absolute dose of ~1.2 to 5 g/day and evokes a pronounced improvement in muscle function and performance [170, 202-204]. Larger doses of NAC supplementation may buffer physiological levels of cellular oxidants and may result in impaired muscle force production and performance deterioration [205]. With our dose of 40 mg/kg/day we will reach an absolute dose of approximately ~2.8 g of NAC for an average participant weighted ~70 kg. Additionally, this dosage has been shown to successfully increase total thiols levels in plasma [206]. NAC in a powder form will be diluted in a 250-mL drink containing 248 ml water and 2 ml of natural, non-caloric, flavoring-sweetener containing sucralose (Flavdrops, My Protein, UK). The placebo supplement will be prepared to

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be identical to NAC in terms of taste and smell apart from the NAC content. In both trials, each participant will be asked 15 times (once a day) if they realize whether the drink they consumed was the placebo or the experimental one. Responses will be recorded, and correct or incorrect answers will be measured. A research assistant will perform the randomization and assign participants to interventions (NAC vs placebo) during the clinical trials using an online, computer-based, third-party, semi-automatic randomization system. The same research assistant will have full access to this list and will monitor the presence of any adverse side effects in both trials via questionnaires [91]. Both investigators and participants will be blinded to supplementation condition. Possible adverse reactions to oral NAC supplementation include an upset stomach, nausea, stomach and/or intestinal gas, sleepiness, metallic taste, light-headedness, redness of eye, face, or hands, and cough [206].

2.2.5. Anthropometric measurements

Standing body mass and height will be measured on a beam balance equipped with stadiometer (Beam Balance-Stadiometer, SECA, Vogel & Halke, Hamburg, Germany) while participants wearing light clothing as described previously [207]. Body mass index will be calculated as mass per height squared. Dual energy X-ray absorptiometry scanner (GE Healthcare, Lunar DPX-NT) will be utilized for body composition assessment. On each testing day the equipment will be calibrated using a LS phantom in accordance to standard procedures. Participants will be asked to remain still and they will be scanned in the supine position using the total body analysis under scanning conditions automatically selected by the software (standard, thick, thin). Total and regional fat mass (g), lean mass (kg) and body fat (% and kg) values will be obtained. GE enCORE software will be utilized for all DXA scans and analyses.

2.2.6. Resting metabolic rate

For RMR assessment, resting VO2/CO2 values will be measured in the morning (07:00–09:00) after overnight fasting utilizing an open-circuit type indirect calorimeter with a ventilated hood system (Vmax Encore 29, BEBJO296, Yorba Linda, CA, USA) and the 24-h RMR will be calculated as previously described [208].

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2.2.7. Physical activity asseement

Habitual physical activity (PA) will be monitored over a 7-day period using the ActiGraph, GT3X+ accelerometers (ActiGraph, Pensacola, FL, USA). Participants will be taught, by an experienced researcher, how to wear the adjustable belt on the waist with the accelerometer monitor on the right side of the hip and they will be asked to wear it throughout the day for seven consecutive days, apart from bathing, swimming and sleep. To be included in the analysis, participants will have to complete four full days of wearing time (i.e., ≥four days with ≥10 wear hours/day). From the data obtained, non-wear time will be calculated and daily activity levels and sedentary time will be expressed as steps per day and time spent at sedentary, light, moderate, vigorous and moderate-to-vigorous PA [209, 210]. ActiLife 6 software will be used to initialize accelerometers and download data using 60-s epoch length.

2.2.8. Dietary intake analysis

Participants will be instructed by a registered dietitian on how to estimate food/fluid servings and sizes and how to complete a 7-day diet recall during both trials and the washout period to ensure that they will follow the same dietary regimen. Specifically, participants will be provided with colored images showing different food portions and detailed instructions that they will use to weight their food. When possible, the name of the brand and/or manufacturer will be recorded. Diet recalls will be analyzed using the Science Fit Diet 200 A (Science Technologies, Athens, Greece) dietary software for data regarding total energy (kcal), carbohydrate, fat, protein (g/kg/day & g/day), vitamin A (mg/day), vitamin C (mg/day), vitamin D (IU/day), vitamin E (mg/day), selenium (mg/day), zinc (mg/day), and magnesium (mg/d).

2.2.9. Maximal oxygen consumption

Maximal oxygen consumption (VO2max) will be measured using open-circuit spirometry with an automated pulmonary gas exchange system (Vmax Encore 29, BEBJO296, Yorba Linda, CA, USA) via the breath-by-breath analysis during a graded exercise test on a treadmill (Stex 8025 T, Korea) until volitional fatigue, according to procedures previously described [211]. Briefly, following a standard warm-up (8 minutes of low intensity running on a treadmill) each participant will complete a graded exercise test protocol at a starting speed of 8-10 km/h (depending on participants' fitness training history), with an increase of 1 km/h in the running

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speed every 2 minutes. During the test VO2/VCO2 values will be measured in 20-second intervals. Criteria for terminating the test include: i) Participant reached a level of volitional fatigue, ii) predicted maximum heart rate reached and/or surpassed, iii) respiratory quotient values ≥1.10 and iv) plateau in VO2 values. VO2max will be calculated from the averaged VO2 measures during the final minute of the test.

2.2.10. Muscle strength and soreness

Knee extensors' (KE) maximal eccentric and concentric peak torque of the exercised limb will be measured on an isokinetic dynamometer (Cybex 770, USA) at 60 °/s as described elsewhere [212]. Delayed onset of muscle soreness (DOMS) of KE of the exercised limb will be evaluated by palpation of the belly and distal region after participants have performed three full squat repetitions. Then, participants will rate their soreness level on a scale from 1 to 10 (1 = no pain, 10 = extremely sore). DOMS assessment will be carried out by the same investigator [213].

2.2.11. Blood sampling and biochemical assays

Following an overnight fasting blood samples will be drawn from the antecubital vein by venipuncture with a 20-gauge disposable needle equipped with a Vacutainer tube holder (Becton Dickinson) with the participants in a supine position. For serum separation, blood samples will be allowed to clot at room temperature and then will be centrifuged (1370 g, 10 min, 4 C). The supernatant will be aliquoted into eppendorf tubes for subsequent analysis of CRP, TNF-α, IL-6, IL-8, IL-10 (inflammation), creatine kinase (CK) activity (muscle damage), total antioxidant capacity (TAC) (oxidative stress) and cortisol (hormonal response). Another blood portion will be collected in EDTA-containing tubes and will be centrifuged at 1370 g, 10 min, 4 C to collect the plasma. Plasma samples will be used for the measurement of protein carbonyls (PC) (protein oxidation) and malondialdehyde (MDA) (lipid peroxidation). Packed erythrocytes (RBCs) will be obtained after lysis of the plasma samples for the measurement of reduced and oxidized glutathione (GSH; GSSG), catalase (CAT) and hemoglobin (Hb) (RBCs' redox status). All samples will be aliquoted in multiple eppendorf tubes and stored at – 80 °C until analysis. A small portion of whole blood (2 ml) will be collected in tubes containing EDTA for a complete blood count analysis on an automated hematology analyzer (Mythic 18, Orphee SA, Geneva, Switzerland). All assays will be performed in duplicate.

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2.2.12. Muscle biopsy sampling

Percutaneous needle muscle biopsies will be obtained after an (~10 h) overnight fast (baseline) using the Bergstrom technique with the application of manual suction from the mid-portion of the vastus lateralis muscle under local anesthetic (xylocaine 1%), by a registered surgeon [214]. After the biopsy, no antibiotics, pain killers or anti-inflammatories will be administered to participants. Volunteers who will receive any type of pharmaceutical drugs and/or analgesics in the biopsy site, in the rare case of excess bleeding or pain, will be excluded from the analyses. Subjects will be asked to refrain from any physical activity at least 96 hours prior to muscle biopsy sampling. Subsequent muscle biopsies (Pre, 2- and 8-days post-exercise) will be spaced 5 cm apart to diminish a repeated biopsy effect. Upon excision, adipose tissue and blood will be carefully removed and muscle samples suited for histology will be aligned and immediately be mounted in optimal cutting temperature (OCT) compound, immersed in nitrogen-cooled isopentane and stored at –80°C. Embedded samples will be sectioned (7 μm) at –20°C using a cryostat and stored at –80°C. Muscle samples suited for western blotting, mRNA, muscle thiols and oxidative stress analyses will directly be frozen in liquid nitrogen, and stored at –80°C.

2.2.13. Histological analyses

Sections will be stained with hematoxylin and eosin (H&E) in order to quantify damaged myofibers [181]. Fibers indicating loss of the physiological outline, sarcolemma damage, mononuclear cells infiltration and central nuclei will be expressed as a percentage of the total number of fibers.

2.2.14. Immunofluorescence

Muscle cross sections (7μm) will be allowed to air dry at room temperature for 30 minutes. For fiber-type-specific SCs analyses samples will be stained with appropriate primary and secondary antibodies against specific antigens such as, Pax7, MyoD, myosin heavy chain type II, and laminin as described previously [215-217]. For fiber-type-specific M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophage quantification, muscle cross sections will be stained with appropriate primary and secondary antibodies against CD11b+, CD206+ and laminin as described previously [218]. Nuclei will be visualized with 4',6-diamidino-2-

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phenylindole (DAPI) contained in the mounting media prior to cover slipping. The specificity of staining will be verified using negative controls. Slides will be viewed using the Olympus BX2 microscope equipped with a high-resolution fluorescent camera. Images will be captured and analyzed using the manufacture's software. All images will be obtained with the 20X objective. Fiber cross sectional area (CSA), fiber type distribution (% Type I and II fibers) myonuclei content (DAPI+ cells), fiber-type-specific satellite cell content and activation status (i.e., Pax7+/MyoD− and Pax7+/MyoD+) and M1 (CD11b+ cells) and M2 (CD206+ cells) macrophage content will be determined. The satellite cells content and activation will be determined via the colocalization of Pax7+ and DAPI and/or the colocalization of Pax7, MyoD and DAPI within the laminin border. Macrophage content will be determined via the colocalization of CD11b+ and DAPI for M1 macrophages and CD206+ and DAPI for M2 macrophages.

2.2.15. Quantitative RT-PCR

Total RNA will be isolated from 10-20 mg of frozen muscle tissue using the NucleoSpin RNA Plus kit (MACHERY-NAGEL), according to the manufacturer's instructions, at a final volume of 80-120 μl. RNA concentration (ng/mL) and purity (260/280) will be measured spectrophotometrically (Hitachi UV/VIS; Hitachi Instruments Inc.). Then samples will be reverse transcribed using a PrimeScript 1st strand cDNA synthesis kit (Takara) in 20 μl reaction volumes, according to the protocol of the manufacturer. Quantitative RT-PCR reactions will run in triplicates containing RT SYBR Green qPCR Master Mix. Primers for Myf5, MyoD, myogenin, MRF4, myostatin and GAPDH will be purchased and mRNA expression levels will be calculated using the 2 - ΔΔCt method. Fold changes from baseline will be calculated using the ΔΔCt method and normalization will be performed using the housekeeping gene GAPDH [219].

2.2.16. Western blotting

Changes in protein expression levels of glutathione peroxidase 3 (GPX-3), superoxide dismutase 2 (SOD-2) and thioredoxin (Trx-1) (related to SCs homeostasis), IGF-1, Notch1 and Wnt3 (related to SCs mobilization) will be analyzed by immunoblotting. Muscle samples will be homogenized in lysis buffer and then centrifuged (13,000 rpm, 4°C, 10 min) and the supernatant will be collected. Total protein concentration will be determined using the Bradford method (Bradford Protein Assay; Bio-Rad). 20 mg of protein will be loaded in gradient precast gels

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(Mini-PROTEAN TGX Gels; Bio-Rad) and will be subjected to SDS-PAGE electrophoresis at room temperature. Afterwards, proteins will be transferred to transblot stacks using the Trans-blot Turbo transfer system (Bio-Rad), blocked for 1 hour and incubated with primary antibodies overnight at 4 °C. Membranes will be washed in TBS-T solution and will be incubated with appropriate secondary antibodies for 1 hour at room temperature. Following another washing step (in TBS-T), membranes will be visualized by chemiluminescence and quantified using densitometry. Normalization will be performed with the housekeeping protein GAPDH.

2.2.17. Muscle thiols and OXS markers

Muscle samples will be homogenized in PBS containing protein inhibitors as described previously [162]. After homogenization, the samples will be centrifuged (12000 g, 4°C, 30 min) and the supernatant will be collected. Muscle reduced and oxidized glutathione (GSH, GSSG), protein carbonyls (PC), and malondialdehyde (MDA) will be measured as indices of muscle's redox status. All measurements will be performed spectrophotometrically (Hitachi UV/VIS; Hitachi Instruments Inc.) as described elsewhere [162]. All assays will be performed in duplicate.

2.2.18. Statistical analyses and power calculation

A preliminary power analysis (based on previous studies that used NAC administration to investigate its effects on EIMD), using the G*Power 3.0.10 program, showed that a minimum number of 10 participants per group is needed to obtain statistical meaningful results among repeated measurements [91]. Specifically, power calculation was performed for a Two-way repeated measures ANOVA, within-between interaction test and input variables included: Effect size, 0.55; α error, 0.05; power, 0.95; number of groups (LR and HR), 2; correlation among repeated measures, 0.5 and non-sphericity correction, 1. However, the total number of participants depends also on potential dropouts according to the following formula: $n' = n/(1-d)$ [220]. Thus, the final number of participants to be recruited with a dropout rate of 15% would be $n' = 10 / (1-0.15) = 11.8$. Therefore, ≥12 participants per group (LR vs. HR) will be selected from the initial sample (N = 40-60) via k-means clustering to participate in the clinical trial (NAC vs placebo).

A k-means cluster analysis will be utilized to efficiently define two separate groups of subjects (LR and HR group) from the total sample (N=40-60), based on the SCs content of their

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vastus lateralis muscle of their dominant leg [8]. This type of analysis requires a relatively large initial sample size (N = ≥40), is a form of partitional clustering and is a multivariate method used to identify homogeneous groups (i.e., clusters) of cases based on a common trait [7, 221].

All analyses and reporting of the results will comply with the SPIRIT statement for reporting RCTs [222]. Results of participant's baseline characteristics and outcome variables (primary, secondary and other) will be summarized using descriptive statistics and will be expressed as mean (standard deviation) or median (range) for continuous variables. Data normality will be examined using the Kolmogorov-Smirnov and the Shapiro-Wilk test. If our data sets follow normal distribution, parametric tests will be applied. Baseline comparisons on the LR and HR group (anthropometrics, body composition, strength, VO2max, dietary profile, physical activity) will be performed using a One-Way ANOVA test. Time- and trial-effect comparisons within and between trial (NAC or placebo) in the LR and HR group will be analyzed using a Two-way repeated measures ANOVA test with a Bonferonni correction for pairwise comparisons. By utilizing a Two-way repeated measures ANOVA test, the type I error (among the 19 primary outcome variables) will reduce significantly as each variable will be analyzed separately and no multiple comparisons will be made. If the data normality is violated, non-parametric tests will be applied. Baseline comparisons on the LR and HR group will be performed using a Kruskal–Wallis test. Time-effect comparisons within trial (NAC or placebo) in the LR and HR group will be analyzed using a Friedman test accompanied by Wilcoxon signed-rank test for pairwise comparisons. Trial-effect comparisons between trials (NAC vs placebo) in the LR and HR group will be analyzed using a Kruskal–Wallis test accompanied by a Mann-Whitney test for pairwise comparisons. As stated for the parametric statistics, all variables will be analyzed separately to reduce the type I error. Pearson's correlation analysis will also be used to examine possible relations among variables. Correlation coefficients of $r < 0.2$, $0.2 < r < 0.7$ and $r > 0.7$ will be defined as small, moderate, and high, respectively. The level of statistical significance will be set at $p < 0.05$. Effect sizes (ES) and confidence intervals (95% CI) will be calculated on results of all dependent variables using the Hedge's g method, corrected for bias. ES will be interpreted as none, small, medium-sized, and large for values 0.00–0.19, 0.20–0.49, 0.50–0.79, and ≥0.8, respectively. Multiple-imputation analysis will also be utilized to handle missing data during data collection and sensitivity analyses will be executed to evaluate the robustness of the results [223]. Statistical analyses will be performed using the SPSS 20.0 software (IBM Corp., Armonk, N.Y., USA).

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2.3. Discussion

The present study is designed to assess the impact of redox status on SCs responses and the mechanisms (hormonal regulation, M1 and M2 macrophages, intracellular signaling) associated with their mobilization and function following aseptic skeletal muscle trauma induced by exercise. Nutritional supplementation or medications have been shown to affect SCs biology under traumatic conditions. Hydrolyzed whey protein supplementation and anti-inflammatory medication (ibuprofen) results in increased SCs response and expedites skeletal muscle recovery [181, 197]. Antioxidant supplementation (vitamin C, vitamin E, NAC or combined antioxidants) may attenuate loss of muscle force production and reduce muscle soreness and lipid peroxidation levels but it may also delay recovery suggesting a potential redox-associated mechanism involved in muscle healing [162, 224]. However, this possibility has not been explored in human skeletal muscle. More specifically, there is no information regarding the redox-dependent mechanism of SCs mobilization and action in human skeletal muscle. It is well established in the literature that thiol oxidation is a major post-translational oxidative modification affecting the cysteine residues in multiple proteins [225]. On the other hand, increased ROS can oxidize GSH leading to irreversible modification [226]. In this sense, low levels of GSH may not only attenuate the antioxidant defense leading to satellite cell damage but it may also alter cellular redox status drastically. Furthermore, SCs activity is also determined by redox-sensitive cues (cytokines, immune cells, signaling molecules) emerging from the surrounding microenvironment [227]. Supplementation with a powerful GSH precursor such as NAC could not only foster GSH levels and thus muscle's antioxidant potential but also will change myofiber redox balance which is crucial for redox-dependent regulation of intracellular signaling pathways mediating pro- and anti-inflammatory response to muscle trauma [91]. Our research hypothesis states that NAC-induced change of muscle's redox status will upregulate SC availability and mobility under conditions of increased oxidative stress and inflammation in human skeletal muscle. This is of great importance as muscle injury is present in several clinical conditions characterized by increased muscle wasting, atrophy and sepsis that result in physical deterioration and poor quality of life such as many types of cancer, cachexia, muscular dystrophies etc. [80, 190]. Consumption of NAC, a potent thiol-based antioxidant, upregulates GSH/GSSG and reduces the respiratory burst and MAPK- and NF-kB–mediated proinflammatory cytokine release during inflammation produced by muscle injury [91, 228]. Information derived from this study will elucidate the redox-dependent regulation of intracellular signaling pathways involved in SCs regulation and muscle healing in human skeletal muscle. Therefore, the results of the proposed study will

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provide information about possible nutritional and/or pharmaceutical interventions to promote SCs function and skeletal muscle's healing potential.

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Chapter 3

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Skeletal Muscle and Erythrocyte Redox Status is Associated with Dietary Cysteine Intake and Physical Fitness in Healthy Young Physically Active Men

Papanikolaou K., Jamurtas A.Z., Poulios A., Tsimeas P., Draganidis D., Margaritelis N.V., Baloyiannis I., Papadopoulos C., Sovatzidis A., Deli C.K., Rosvoglou A., Georgakouli K., Tzatzakis T., Nikolaidis M.G., Fatouros I.G.

Abstract

To investigate the association between redox status in erythrocytes and skeletal muscle with dietary nutrient intake and markers of physical fitness and habitual physical activity (PA). Forty-five young physically active men were assessed for body composition, dietary nutrient intake, muscle strength, cardiorespiratory capacity and habitual PA. Blood and muscle samples were collected to estimate selected redox biomarkers. Partial correlation analysis was used to evaluate the independent relationship of each factor with redox biomarkers. Dietary cysteine intake was positively correlated ($p<0.001$) with both erythrocyte ($r=0.697$) and muscle GSH (0.654, $p<0.001$), erythrocyte reduced/oxidized glutathione ratio (GSH/GSSG) ($r=0.530$, $p=0.001$) and glutathione reductase (GR) activity ($r=0.352$, $p=0.030$) and inversely correlated with erythrocyte protein carbonyls (PC) levels ($r=-0.325$; $p=0.046$). Knee extensors eccentric peak torque was positively correlated with GR activity ($r=0.355$; $p=0.031$) while, one-repetition maximum in back squat exercise was positively correlated with erythrocyte GSH/GSSG ratio ($r=0.401$; $p=0.014$) and inversely correlated with erythrocyte GSSG and PC ($r=-0.441$, $p=0.006$; $r=-0.413$, $p=0.011$ respectively). Glutathione peroxidase (GPx) activity was positively correlated with step count ($r=0.520$; $p<0.001$), light ($r=0.406$; $p=0.008$), moderate ($r=0.417$; $p=0.006$), moderate-to-vigorous ($r=0.475$; $p=0.001$), vigorous ($r=0.352$; $p=0.022$) and very vigorous ($r=0.326$; $p=0.035$) PA. Muscle GSSG inversely correlated with light PA ($r=-0.353$; $p=0.022$). These results indicate that dietary cysteine intake may be a critical element for the regulation of glutathione metabolism and redox status in two different tissues pinpointing the independent significance of cysteine for optimal redox regulation. Musculoskeletal fitness and PA levels may be predictors of skeletal muscle, but not erythrocyte, antioxidant capacity.

Keywords: glutathione, cysteine, antioxidant enzymes, redox regulation, muscle function, physical activity

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3.1. Introduction

Glutathione is a ubiquitously distributed antioxidant consisting of the three amino acids cysteine, glycine and glutamic acid, exerting its biological antioxidant activity via the thiol group of cysteine residues [229]. The tripeptide is mainly present in its reduced (GSH) and oxidized form (glutathione disulfide, GSSG), and the ratio between these (GSH/GSSG) is used as an index of cellular redox status [230]. Glutathione acts as a direct scavenger of free radicals (reactive oxygen and nitrogen species, RONS) as well as a substrate for the glutathione peroxidase (GPx) enzyme family [231]. GPx catalyzes the reduction of hydrogen peroxide (H_2O_2) to water through the oxidation of GSH to GSSG, which is reduced back to GSH by the activity of glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate (NADPH) [232]. Through its antioxidant properties, GSH is essential for numerous intracellular and physiological processes including redox signaling, immunoregulation, muscle metabolism and function and, thus, low glutathione availability may have important physiological implications [77, 233, 234].

Evidence suggests that genetics play an important role in GSH deficiency or sufficiency as mutations in the GSH synthase gene could severely impact its concentration [235, 236]. Furthermore, nutrition and especially cysteine intake is also a significant determinant for GSH bioavailability and redox status. Human and animal studies have revealed that inadequate sulfur amino acid intake may result in low cysteine and GSH levels [237, 238]. In fact, reduced dietary cysteine intake is associated with decreased erythrocyte GSH concentration and elevated plasma lipid peroxidation levels in young individuals [239]. Furthermore, recent studies indicate that individualized antioxidant interventions targeting in reversing specific antioxidant inadequacies (i.e., N-acetylcysteine for glutathione deficiency or vitamin C for vitamin C deficiency) could result in improved redox profile and physiological function [240], while the presence of oxidative stress per se does not justify the general use of antioxidants [241].

Beyond the dietary regulation, physical activity (PA) has been also considered a crucial determinant of redox status. Increased PA levels have been associated with enhanced systemic redox status and reduced oxidative stress both in youth [242] and elderly [243] populations. Nevertheless, the relationship between tissue-specific (i.e., skeletal muscle) redox status and PA remains to be elucidated. Studies in humans have shown that low levels of GSH and other antioxidants (i.e., vitamin C, vitamin E) are linked to increased oxidative stress and impaired muscle performance, a phenomenon that is reversible via specific antioxidant supplementation [173, 174]. However, previous reports suggest that general use of antioxidants should be treated with caution as it may interfere with physiological adaptations to exercise (i.e., mitochondrial

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biogenesis and glucose uptake) [244, 245]. Moreover, under physiological conditions, oxidative stress has been shown to promote a signalling cascade, nessecary for optimal muscle regeneration following exercise-induced muscle damage [246].

Despite the pivotal role of redox status in cellular homeostasis and muscle function, there is lack of data regarding the relationship between tissue-specific (particularly in skeletal muscle) redox status constituents (i.e., GSH state, antioxidant enzyme activity) and dietary cysteine intake, as well as physical fitness and PA profile. Therefore, the aim of this investigation was to examine whether erythrocyte and skeletal muscle redox status is associated with (i) dietary nutrient intake, (ii) lower-limb muscle strength, (iii) cardiorespiratory capacity and (iv) habitual physical activity levels in healthy young physically active men. We hypothesized that decreased redox status in erythrocytes and skeletal muscle would be independently associated with lower dietary cysteine intake, physical fitness and PA levels.

3.2. Methods

3.2.1 Participants and experimental design

This study is part of a primary randomized controlled trial, with its purpose and methodology described in detail elsewhere [182]. In this work, data upon screening/baseline secondary variables (redox status, dietary nutrient intake, physical fitness and PA) are presented. **Figure 3.1** depicts the timeframe diagram of the study. A total of 48 healthy young men 18-30 years volunteered and underwent baseline medical screening. Participation in this investigation was secured if participants: (a) were non-smokers; (b) refrained from unaccustomed and/or heavy exercise ≥4 weeks before the study; (c) had no recent history of musculoskeletal injury, febrile illness, lower limb trauma and metabolic diseases and (d) abstained from the consumption of alcohol, caffeine, nutritional supplements, and medication (i.e., non-steroidal anti-inflammatory drugs) before (≥6 months) and throughout the study. Following recruitment, participants who met the inclusion criteria (n=45) underwent an assessment of body mass, body height, body composition, lower-limb muscle strength and cardiorespiratory fitness. Thereafter, participants were given accelerometers and diet recalls to assess their habitual PA and nutrient intake, respectively, during a 7-day period. Thereafter, participants were instructed to refrain from any strenuous PA and/or exercise for 5 days and a resting blood and muscle biopsy sample was collected early in the morning following overnight fasting. Participants' anthropometric characteristics, dietary intake, muscle strength, cardiorespiratory capacity, PA and redox status

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are shown in **Table 3.1**. The study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The overall study was approved by the Institutional Review Board of the University of Thessaly (#1387/2018) and was pre-registered at clinicaltrials.gov (ID: NCT03711838).

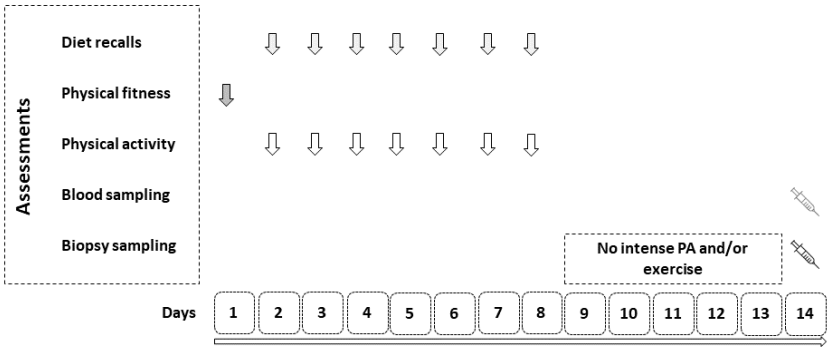

Figure 3.1. The timeframe diagram of the study. *PA* physical activity

3.2.2 Body composition

Body height was measured with a stadiometer (Stadiometer, SECA, Vogel & Halke, Hamburg, Germany) as described [14] while body mass and composition (total, fat and lean mass) were measured by dual emission X-ray absorptiometry (DXA, GE Healthcare, Lunar DPX NT, Diegem, Belgium) as described before [207].

3.2.3 Dietary nutrient intake

Participants were instructed by a registered dietician on how to complete a 7-day diet recall by precisely estimating food/fluid servings and sizes. Colored food images were also provided, illustrating different portion amounts that they could use to estimate their food volume [247]. Participants were asked to describe their dietary intake in as much detail as possible (i.e., the name of the manufacturer for commercially available products). Diet recalls were analyzed using the Science Fit Diet 200A (Science Technologies, Athens, Greece) dietary software and the following parameters were estimated: Energy (kcal/day), protein (g/day and % of total energy/day), carbohydrates (g/day and % of total energy/day), fat (g/day and % of total

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energy/day), cereals (g/day), legumes (g/day), vitamin C (mg/day), vitamin E (mg/day), selenium (μg/day), zinc (mg/day), cysteine (g/day), glutamic acid (g/day), glycine (g/day) and methionine (g/day). From the estimated parameters, the following variables were also calculated: Total cysteine, glutamic acid and glycine intake (Cyst-GA-Gly in g/day), sulfur amino acid (SAA) intake (cysteine and methionine in g/day), cysteine/energy (g/kcal/day), cysteine/protein (g/g/day) and cysteine/SAA (g/g/day). Unusual foods in participants' diets, not established in the database, were analyzed by substituting these foods with appropriate equivalents (according to their nutrient category, composition and quantity) which were designed and validated by a registered dietician, to produce complete dietary analysis reports.

3.2.4 Muscle strength

Maximal knee extensors eccentric peak torque (Nm) at 60°/s was measured using an isokinetic dynamometer (Cybex Norm 770, Ronkonkoma, New York) after a familiarization session as previously described [248]. Maximal strength (kg) in back squat (one-repetition maximum, 1RM) was determined using standardized procedures as previously described [249] and following familiarization.

3.2.5 Cardiorespiratory capacity

Maximal oxygen consumption (VO2max) was assessed during a graded exercise test on a treadmill (Stex 8025 T, Korea) by open-circuit online spirometry, using an automated pulmonary gas exchange system (Vmax Encore 29, BEBJO296, Yorba Linda, CA, USA) via breath-by-breath analysis as previously described [198] and expressed as ml/kg/min.

3.2.6 Habitual physical activity

PA was assessed using the tri-axial GT3X+ accelerometers (ActiGraph, Pensacola, FL, USA). Participants were instructed on how to wear the elastic belt containing the accelerometer around their waist with the device aligned on the right hip and they were asked to wear it throughout the day, except for bathing and sleep, for seven consecutive days. Data were analyzed if participants had five days with ≥10 wear hours/day. Non-wear time was calculated according to the algorithms developed by Choi et al. [209] for vector magnitude (VM) data and categorized as intervals of counts/minute (cpm). Daily activity and sedentary time were estimated according

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to VM data and expressed as steps/day and time/day in sedentary (<199 cpm), light (200–2689 cpm), moderate (2690–6166 cpm), vigorous (6167–9642 cpm), very vigorous (>9643 cpm) and moderate-to-vigorous (2690-6167 cpm) PA [210]. ActiLife 6 software was used to set up accelerometers and download data in 60-s epoch length intervals.

3.2.7 Blood sampling and redox assays

Blood samples were collected from an antecubital arm vein using a 20-gauge disposable needle equipped with a vacutainer tube holder after overnight fasting. Samples were collected into tubes with ethylenediaminetetraacetic acid (EDTA) that were immediately centrifuged for plasma separation (1370 g, 4°C, 10 min). Subsequently, plasma was discarded and an equal volume of distilled water was added to the packed erythrocytes. Tubes were vigorously mixed, centrifuged (4000 g, 4°C, 15 min) and the resultant erythrocyte lysate was collected for subsequent analysis of GSH, GSSG, protein carbonyls (PC) and hemoglobin (Hb). Erythrocyte lysates were stored at −80°C in multiple aliquots until assayed. Hb was measured spectrophotometrically using a commercially available kit (Zafiropoulos, Greece). Erythrocyte GSH, GSSG and PC were determined spectrophotometrically as described previously [250] and normalized to Hb concentration.

3.2.8 Muscle biopsy sampling and redox assays

Muscle biopsy samples were obtained from the middle portion of vastus lateralis muscle under sterile conditions and application of local anesthetic (xylocaine 1%), using the Bergstrom needle technique modified for manual suction, as previously described [14]. Muscle samples were separated from any visible fat and connective tissue residues, immediately frozen in liquid nitrogen and stored at -80°C for further analysis of GSH and GSSG concentration as well as GPx, and GR enzyme activity.

Prior to analyses, muscle samples were homogenized in ice-cold phosphate-buffered saline (PBS, 0.01 M, 1 mM EDTA, pH: 7.4) containing a protease inhibitors cocktail (1 mM leupeptin, 1 mM aprotinin, 1 mM phenylmethanesulfonylfluoride) and the lysate was centrifuged (15000 g, 4°C, 15 min) to obtain a clear supernatant. Total protein concentration was determined using the Bradford assay (Bradford Protein Assay, Bio-Rad).

Muscle GSH and GSSG concentrations were determined spectrophotometrically as described previously [250]. Briefly, for GSH, 50 μl of muscle homogenate were treated with 5%

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trichloroacetic acid (TCA), centrifuged (15000 g, 4°C, 5 min) and the supernatant was collected. Then, 20 μl of the supernatant were mixed with 660 μl of phosphate buffer (PB, 67 mM, pH 7.95) and 330 μl of 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB, 1 mM). The samples were incubated in the dark at room temperature for 15 min, and the absorbance was read at 412 nm. GSH concentration was normalized to total protein content.

For GSSG, 50 μl of muscle homogenate were treated with 5% TCA, centrifuged (15000 g, 4°C, 5 min) and neutralized to pH 7.0–7.5 with NaOH. Thereafter, 1 μl of 2-vinyl-pyridine was added, and the samples were incubated for 2 h at room temperature with vortexing every 15 min. 5 μl of sample were mixed with 600 μl of PB (143 mM, 6.3 mM of EDTA, pH 7.5), 100 μl of nicotinamide adenine dinucleotide phosphate (NADPH, 3 mM), 100 μl DTNB (10 mM) and 194 μl of distilled water and the samples were incubated for 10 min at room temperature. Following the addition of 1 μl of glutathione reductase, the change in absorbance at 412 nm was read for 1 min. GSSG concentration was normalized to total protein content.

GPx activity was determined as previously described [251]. Briefly, 500 μl PB (100 mM, 1 mM EDTA, pH 7), 100 μl GR (0.24 U), 100 μl GSH (10 mM) and 100 μl of the muscle homogenate (diluted 1:10 in PBS) were mixed and the solution was incubated at room temperature for 10 min. Then, 100 μl NADPH (1.5mM in 0.1% NaHCO3) solution was added and the samples were incubated at room temperature for 3 min. Afterward, 100 μl of tert-Butyl hydroperoxide were added to the samples and the decrease in absorbance at 340 nm was monitored for 5 min. GPx activity was calculated based on the molar extinction coefficient of NADPH (6200 l/mol/cm) and normalized to total protein concentration.

GR activity was determined as previously originally described [252] and modified [253]. Briefly, for the samples, 700 μl PB (200 mM, 1 mM EDTA, pH 7.5), 250 μl 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, 3 mM in PBS), 50 μl NADPH (2 mM in PBS) and 50 μl GSSG (20 mM in PBS) were mixed. The reaction started by the addition of 25 μl of the muscle homogenate (diluted 1:2 in PBS) and the increase in absorbance was monitored at 412 nm for 1 min. For the standard, muscle homogenate was replaced by 25 μl of GR (1 U/ml) solution. GR activity was calculated based on the absorbance change of the standard sample and normalized to total protein concentration.

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3.2.9 Statistical analyses

Data normality was evaluated using the Shapiro-Wilk test. The relationship between selected variables was tested using bivariate or partial correlation analysis in order to exclude the effects of confounding variables in the relationships examined. Specifically, (i) the relationship between redox status and dietary nutrient intake was controlled for physical fitness and PA levels, (ii) the relationship between redox status and physical fitness was controlled for dietary nutrient intake and PA levels and (iii) the relationship between redox status and PA was controlled for dietary nutrient intake and physical fitness level. For partial correlation analysis, the variables included in the dietary nutrient intake, physical fitness and PA sets were the following: Dietary nutrient intake (cysteine, glutamic acid and glycine), physical fitness (knee extensors peak torque, 1RM squat and VO2max), PA (step count, sedentary time, light PA, moderate PA, vigorous PA, very vigorous PA and moderate-to-vigorous PA). When at least one of the variables (i.e., in each pair of variables that were examined for relationship) violated the assumptions of parametric analyses, non-parametric (Spearman) partial correlation analysis was utilized. This type of analysis has been used to assess the relationship between non-normally distributed variables, while controlling for confounding factors [254, 255]. The variables in which normal distribution was violated were the following: Age, energy, protein (g/kg/day), carbohydrates (g/day), fat (g/day and % of total energy/day), legumes, selenium, vitamin C, vitamin E, cysteine, glutamic acid, Cyst-GA-Gly, cysteine/energy, cysteine/protein, 1RM back squat, vigorous PA, very vigorous PA, erythrocyte protein carbonyls and muscle oxidized glutathione. The magnitude of the correlations was considered as minimal, small, medium- and large-sized for values 0.1-0.2, 0.2-0.5, 0.5-0.8 and >0.8, respectively, according to Cohen's d criteria. Linear regression analysis was also conducted and 95% confidence bands of the best-fit line were estimated. Differences in cereal and legume intake were examined by using an independent samples T-test and a non-parametric Mann-Whitney test respectively. The level of statistical significance was set at $p<0.05$. Data are presented as means ± SD for normally distributed variables and as median with 25%/75% intervals in parenthesis for non-normally distributed variables. Statistical analyses were performed using the SPSS software (IBM SPSS Statistics, version 25.0).

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3.3. Results

3.3.1 Participants' anthropometric characteristics, dietary profile, physical fitness, physical activity and redox status

Overall, participants' daily dietary macro- and micro-nutrient intake was within the safe and recommended range (**Table 3.1**) for the age, gender and PA level [200, 256]. From the total sample, the proportion of underweight, normal and overweight participants was 2.2, 68.9 and 28.9% respectively. Moreover, participants' PA level was at the upper limit of the current recommendations adopted by WHO for adult population aged 18-64 (150-300 min of moderate PA per week) [257]. However, it should be noted that participants' PA profile exhibited a substantial level of variability and in some cases, it was twice as high as some measures of average PA in this age group.

Table 3.1. Participants' anthropometric characteristics, dietary nutrient intake, physical fitness, physical activity and redox status

Anthropometrics		
Age (years)	21.40 (19.95/22.80)	[18.00-28.10]
Body mass (kg)	73.66 ± 10.32	[50.70-93.40]
Height (m)	1.76 ± 0.07	[1.60-1.93]
BMI (kg/m^2)	23.65 ± 2.37	[17.75-28.33]
Body fat (%)	19.48 ± 7.57	[7.10-35.30]
Lean mass (kg)	54.99 ± 6.43	[39.12-69.60]
Dietary intake		
Total energy (kcal/day)	2245.05 (1842.41/2636.59)	[1201.49-4753.59]
Protein (g/kg/day)	1.27 (1.06/1.65)	[0.60-3.00]
Protein (% of total energy/day)	17.30 ± 2.96	[10.98-25.00]
Carbohydrates (g/day)	255.47 ± 84.47	[100.31-502.68]
Carbohydrates (% of total energy/day)	44.10 (40.60/47.90)	[26.16-58.36]
Fat (g/day)	88.92 (71.64/114.94)	[43.40-227.30]
Fat (% of total energy/day)	37.22 (33.87/40.52)	[26.16-71.95]
Cereals (g/day)	373.90 ± 13.52	[215.60-589.40]
Legumes (g/day)	21.40 (0.00/37.50)	[0.00-84.00]
Vitamin C (mg/day)	89.83 (65.62/132.76)	[8.68-646.52]
Vitamin E (mg/day)	7.63 (5.81/10.10)	[1.35-20.35]

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Selenium (μg/day)	125.76 (101.06/150.67)	[67.05-227.81]
Zinc (mg/day)	13.15 ± 4.44	[6.50-23.40]
Methionine (g/day)	2.47 ± 0.94	[0.40-4.80]
Cysteine (g/day)	1.50 (1.05/2.20)	[0.28-3.30]
Glutamic acid (g/day)	16.55 (14.95/20.38)	[4.32-36.57]
Glycine (g/day)	3.69 ± 1.16	[0.48-6.60]
SAA (g/day)	4.10 ± 1.63	[0.68-8.10]
Physical fitness		
KE eccentric peak torque (Nm)	274.11 ± 55.24	[180.00-400.00]
1RM back squat (kg)	105.00 (100.00/116.25)	[64.70-170.00]
VO_{2max} (ml/kg/min)	50.18 ± 4.82	[34.60-60.80]
Physical activity		
Steps count (n)	7248.56 ± 2553.15	[818.00-11771.00]
ST (min/day)	459.20 ± 132.45	[101.00-751.00]
LPA (min/day)	256.33 ± 94.12	[46.50-505.00]
MPA (min/day)	45.85 ± 18.58	[3.80-81.20]
VPA (min/day)	2.60 (0.67/6.60)	[0.00-17.20]
VVPA (min/day)	0.00 (0.00/0.80)	[0.00-12.80]
MVPA (min/day)	51.56 ± 21.44	[4.60-101.40]
Erythrocyte redox status		
GSH (μmol/g Hb)	4.48 ± 1.16	[2.44-7.07]
GSSG (μmol/g Hb)	0.25 ± 0.09	[0.11-0.49]
GSH/GSSG ratio	20.37 ± 7.69	[5.63-38.49]
PC (nmol/g Hb)	2.10 (1.04/3.53)	[0.29-5.61]
Skeletal muscle redox status		
GSH (nmol/mg protein)	19.07 ± 5.70	[8.80-37.21]
GSSG (nmol/mg protein)	0.82 (0.56/1.30)	[0.32-2.36]
GSH/GSSG ratio	24.77 ± 12.83	[5.24-57.75]
GPx (U/mg protein)	0.0117 ± 0.0023	[0.0070-0.0170]
GR (U/mg protein)	0.0018 ± 0.0005	[0.0010-0.0030]

Data are presented as mean ± SD or median and 25%/75% intervals in parenthesis with ranges in brackets (*n*=45). *BMI* body mass index, *GSH* reduced glutathione, *GSSG* oxidized

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glutathione, *GPx* glutathione peroxidase, *GR* glutathione reductase, *KE* knee extensors, *LPA* light physical activity, *MPA* moderate physical activity, *MVPA* moderate-to-vigorous physical activity, *PC* protein carbonyls, *SAA* sulfur amino acids, *ST* sedentary time, VO_{2max} maximal oxygen consumption, *VPA* vigorous physical activity, *VVPA* very vigorous physical activity, *1RM* 1 repetition maximum

3.3.2 The independent relationship between redox status and dietary nutrient intake

Figure 3.2 depicts the correlation between redox status and dietary nutrient intake. Dietary cysteine intake was positively correlated with both erythrocyte and muscle GSH (r=0.697, p<0.001 and r=0.654, p<0.001 respectively), erythrocyte GSH/GSSG ratio (r=0.530, p=0.001), and muscle GR activity (r=0.352, p=0.030), while it was inversely correlated with erythrocyte PC (r=-0.325, p=0.046). No other significant correlations were observed between redox status and dietary nutrient intake. Glutamic acid and glycine intake as well as the total intake of cysteine, glutamic acid and glycine (Cyst-GA-Gly) showed no significant correlation with redox biomarkers (**Table 3.2**). Total SAA intake was positively correlated only with erythrocyte and muscle GSH (r=0.566, p<0.001 and r=0.374, p=0.027 respectively) (**Table 3.2**). The correlations between dietary cysteine intake and redox biomarkers with and without the adjustments for confounders are presented in **supplementary file 3.3**.

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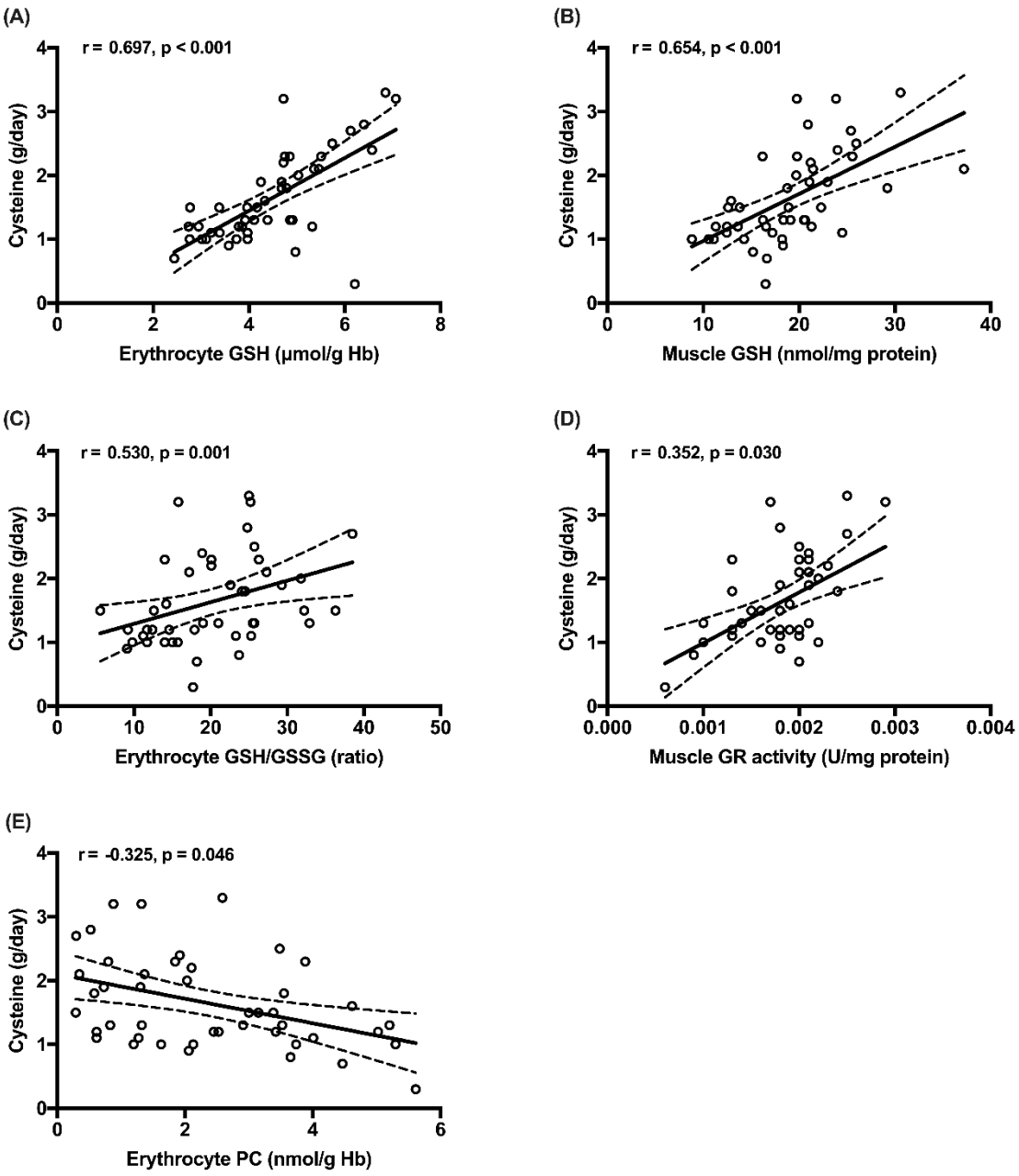

Figure 3.2. Partial correlation and linear regression analyses between dietary cysteine intake vs erythrocyte (A) and muscle (B) glutathione, erythrocyte reduced/oxidized glutathione ratio (C), muscle glutathione reductase enzyme activity (D) and erythrocyte protein carbonyls (E), (n=45). Correlations were adjusted for physical fitness and PA variables. GSH glutathione, GR glutathione reductase, GSSG oxidized glutathione, PC protein carbonyls

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Table 3.2. Partial correlations between glutamic acid, glycine and Cyst-GA-Gly dietary intake with redox biomarkers

Glutamic acid vs redox biomarkers	
Erythrocytes	
Glutamic acid (g/day) and GSH (μmol/g Hb)	r = -0.007; p = 0.965
Glutamic acid (g/day) and GSSG (μmol/g Hb)	r = 0.123; p = 0.475
Glutamic acid (g/day) and GSH/GSSG (ratio)	r = 0.011; p = 0.951
Glutamic acid (g/day) and PC (nmol/g Hb)	r = 0.194; p = 0.258
Skeletal muscle	
Glutamic acid (g/day) and GSH (nmol/mg protein)	r = -0.031; p = 0.857
Glutamic acid (g/day) and GSSG (nmol/mg protein)	r = 0.206; p = 0.228
Glutamic acid (g/day) and GSH/GSSG (ratio)	r = -0.204; p = 0.233
Glutamic acid (g/day) and GPx activity (U/mg protein)	r = 0.246; p = 0.148
Glutamic acid (g/day) and GR activity (U/mg protein)	r = -0.002; p = 0.992
Glycine vs redox biomarkers	
Erythrocytes	
Glycine (g/day) and GSH (μmol/g Hb)	r = -0.029; p = 0.867
Glycine (g/day) and GSSG (μmol/g Hb)	r = 0.204; p = 0.226
Glycine (g/day) and GSH/GSSG (ratio)	r = -0.162; p = 0.339
Glycine (g/day) and PC (nmol/g Hb)	r = 0.205; p = 0.231
Skeletal muscle	
Glycine (g/day) and GSH (nmol/mg protein)	r = -0.186; p = 0.270
Glycine (g/day) and GSSG (nmol/mg protein)	r = 0.252; p = 0.138
Glycine (g/day) and GSH/GSSG (ratio)	r = -0.307; p = 0.064

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Glycine (g/day) and GPx activity (U/mg protein)	r = -0.079; p = 0.641
Glycine (g/day) and GR activity (U/mg protein)	r = -0.113; p = 0.507
Cyst-GA-Gly vs redox biomarkers	
Erythrocytes	
Cyst-GA-Gly (g/day) and GSH (μmol/g Hb)	r = 0.084; p = 0.624
Cyst-GA-Gly (g/day) and GSSG (μmol/g Hb)	r = 0.149; p = 0.386
Cyst-GA-Gly (g/day) and GSH/GSSG (ratio)	r = 0.057; p = 0.741
Cyst-GA-Gly (g/day) and PC (nmol/g Hb)	r = 0.182; p = 0.288
Skeletal muscle	
Cyst-GA-Gly (g/day) and GSH (nmol/mg protein)	r = -0.001; p = 0.995
Cyst-GA-Gly (g/day) and GSSG (nmol/mg protein)	r = 0.209; p = 0.221
Cyst-GA-Gly (g/day) and GSH/GSSG (ratio)	r = -0.199; p = 0.245
Cyst-GA-Gly (g/day) and GPx activity (U/mg protein)	r = 0.213; p = 0.211
Cyst-GA-Gly (g/day) and GR activity (U/mg protein)	r = -0.018; p = 0.917
SAA vs redox biomarkers	
Erythrocytes	
SAA (g/day) and GSH (μmol/g Hb)	**r = 0.566; p < 0.001**
SAA (g/day) and GSSG (μmol/g Hb)	r = 0.268; p = 0.119
SAA (g/day) and GSH/GSSG (ratio)	r = 0.227; p = 0.189
SAA (g/day) and PC (nmol/g Hb)	r = -0.145; p = 0.405
Skeletal muscle	
SAA (g/day) and GSH (nmol/mg protein)	**r = 0.374; p = 0.027**
SAA (g/day) and GSSG (nmol/mg protein)	r = 0.194; p = 0.265
SAA (g/day) and GSH/GSSG (ratio)	r = 0.035; p = 0.842

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SAA (g/day) and GPx activity (U/mg protein)	r = -0.104; p = 0.553
SAA (g/day) and GR activity (U/mg protein)	r = 0.319; p = 0.062

Data are presented as correlation coefficients and p values (*n*=45). Correlations were adjusted for physical fitness and PA profile variables. *Cyst-GA-Gly* total intake of cysteine, glutamic acid and glycine intake, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *GPx* glutathione peroxidase, *GR* glutathione reductase, *PC* protein carbonyls, *SAA* total dietary intake of cysteine and methionine. Significant correlations and the corresponding p-values are presented in bold

3.3.3 The independent relationship between redox status and physical fitness

Figure 3.3 illustrates the correlation between redox status and physical fitness markers. Knee extensors eccentric peak torque was positively correlated with muscle GR enzyme activity (r=0.355, p=0.031). 1RM in back squat exercise was positively correlated with erythrocyte GSH/GSSG ratio (r=0.401, p=0.014) and inversely correlated with erythrocyte GSSG (r= -0.441, p=0.006) and PC (r= -0.413, p=0.011). No other significant correlations were detected between redox status and physical fitness variables. The correlations between physical fitness and redox biomarkers with and without the adjustments for confounders are presented in **supplementary file 3.3**.

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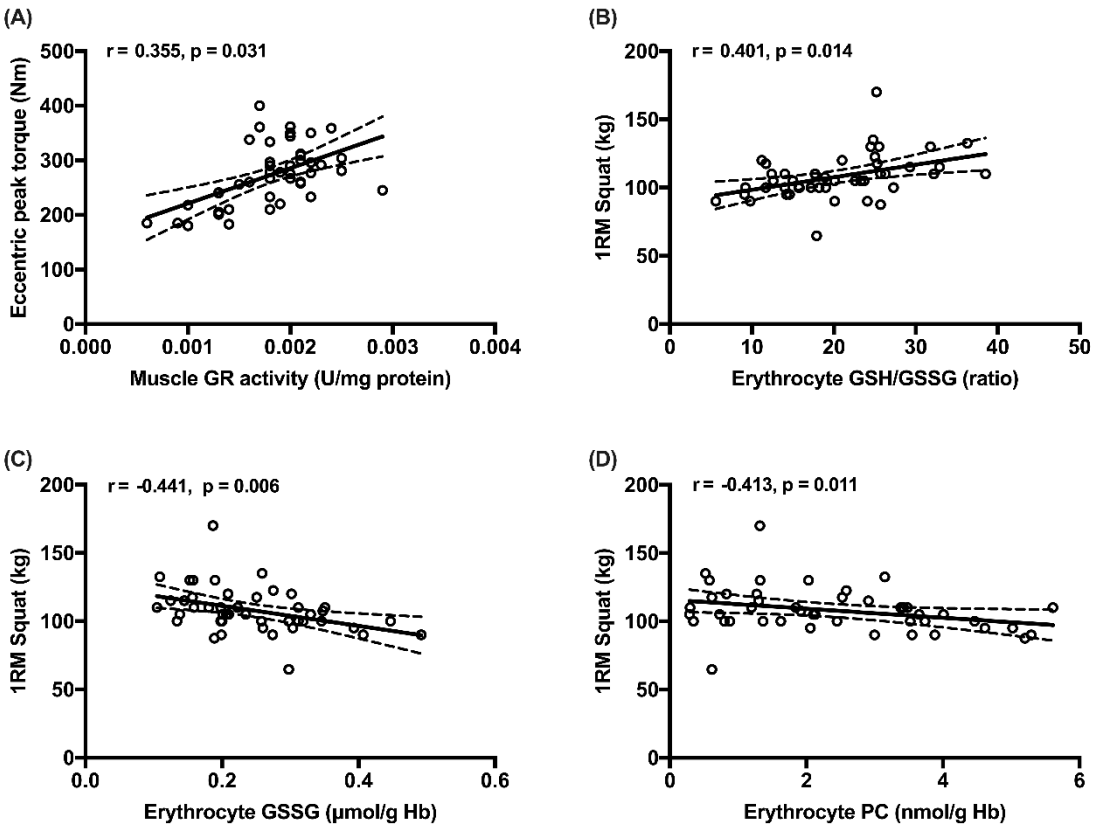

Figure 3.3. Partial correlation and linear regression analyses between muscle glutathione reductase enzyme activity vs knee extensors eccentric peak torque (A), 1-repetition maximum in squat exercise vs erythrocyte reduced/oxidized glutathione ratio (B), erythrocyte oxidized glutathione (C) and erythrocyte protein carbonyls (D), (n=45). Correlations were adjusted for dietary nutrient intake and PA variables. GSH glutathione, GR glutathione reductase, GSSG oxidized glutathione, PC protein carbonyls, 1RM one-repetition maximum

3.3.4 The independent relationship between redox status and physical activity

Figure 3.4 presents the correlation between redox status and PA levels. Muscle GPx enzyme activity was positively correlated with step count (r=0.520, p<0.001) light PA (r=0.406, p=0.008), moderate PA (r=0.417, p=0.006), moderate-to-vigorous PA (r=0.475, p=0.001), vigorous PA (r=0.352, p=0.022), and very vigorous PA (r=0.326, p=0.035) while muscle GSSG inversely correlated with light PA (r= -0.353, p=0.022). No other significant correlations were noted among redox status and PA variables. The correlations between physical activity and redox biomarkers with and without the adjustments for confounders are presented in **supplementary file 3.3**.

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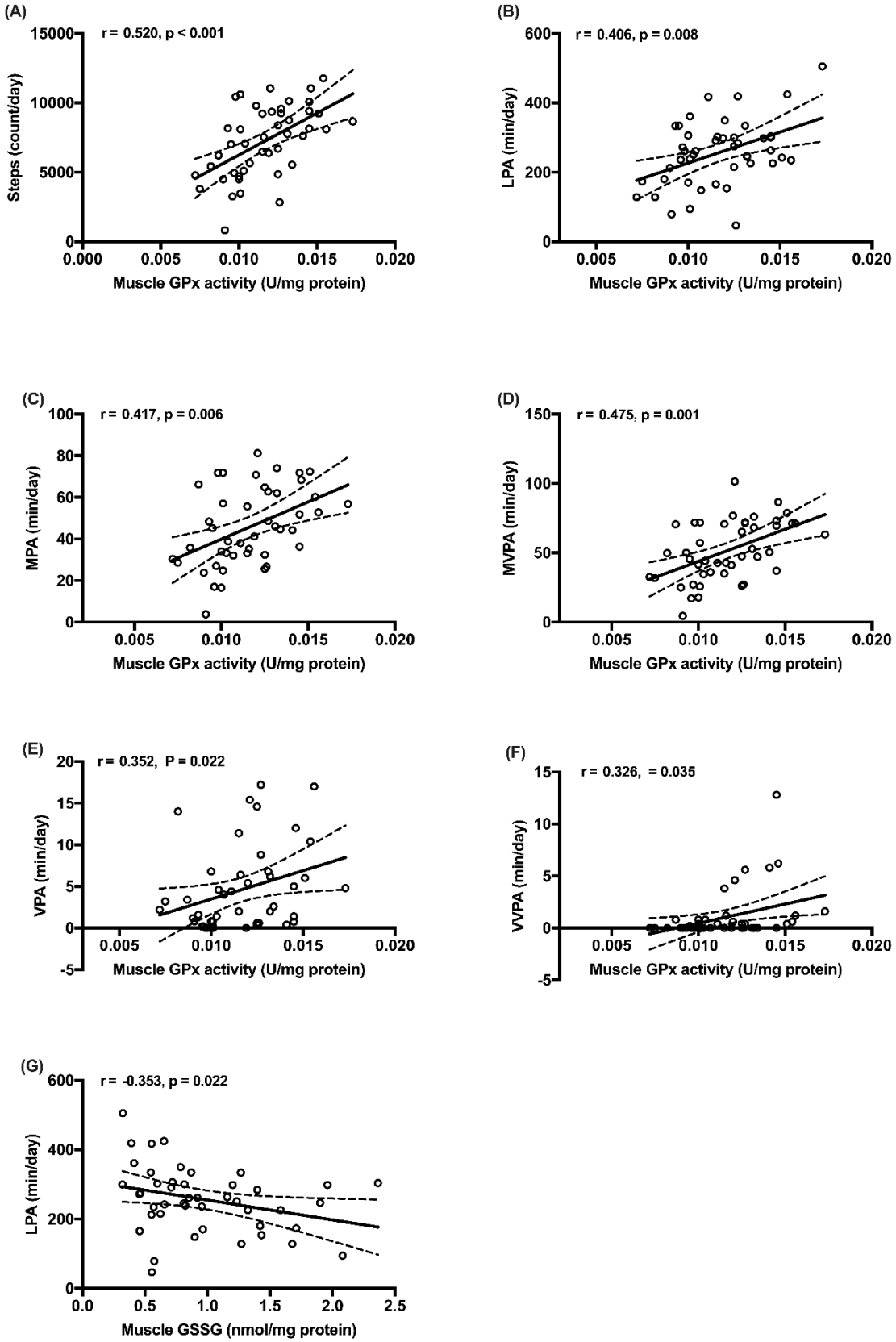

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Figure 3.4. Correlation and linear regression analyses between muscle glutathione peroxidase enzyme activity vs steps count (A), light physical activity (B), moderate physical activity (C), moderate-to-vigorous physical activity (D), vigorous physical activity (E) and very vigorous physical activity (F) as well as muscle oxidized glutathione vs light physical activity (G), (n=45). Correlations were adjusted for dietary nutrient intake and physical fitness variables. GPx glutathione peroxidase, GSSG oxidized glutathione, LPA light physical activity, MPA moderate physical activity, MVPA moderate-to-vigorous physical activity, VPA vigorous physical activity, VVPA very vigorous physical activity

3.4. Discussion

In this investigation we examined for the first time, the independent relationship between dietary nutrient intake, muscle strength, cardiorespiratory capacity and PA profile with erythrocyte and skeletal muscle redox status in healthy young physically active men. Our findings suggest that (i) reduced dietary cysteine intake is linked to impaired glutathione metabolism and elevated protein oxidation in erythrocytes and skeletal muscle, (ii) upregulated redox status is associated with increased lower-limb muscle strength, (iii) higher physical activity levels are associated with enhanced antioxidant enzyme activity in skeletal muscle and (iv) redox status markers in both tissues were not associated with either VO2max or any other nutrient.

Dietary cysteine intake exhibited a meaningful correlation with erythrocyte and skeletal muscle GSH concentration as well as with overall redox status (GSH/GSSG ratio). Similarly, previous human studies showed a positive association between dietary cysteine intake and erythrocyte GSH concentration [239, 258]. In contrast, no association was detected between glutamic acid, glycine (both constituent amino acids for GSH synthesis) and the total dietary intake of cysteine, glutamic acid and glycine (Cyst-GA-Gly) with GSH metabolism, which corroborates previous observations showing that cysteine is the "rate-limiting" amino acid for GSH synthesis [239]. It seems that, in young physically active adults, increased dietary cysteine intake is associated with higher intramuscular GSH concentration and turnover (via GR enzyme activity). Previous in vitro and animal studies indicated that GSH levels are linked to cysteine availability in a dose-dependent manner [238, 259]. Reduced cellular GSH/GSSG ratio in cysteine-depleted media of HT29 cell culture was reversed upon re-addition of the amino acids to the culture media [260]. Moreover, in healthy young men, sulfur amino acid depletion over a 4-day period negatively affected plasma levels of free cysteine, while sulfur amino acid repletion

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immediately recovered cysteine/cystine redox status [237]. To date, there is no consensus statement regarding the recommended dietary intake (RDI) for cysteine intake, nevertheless, a previous study proposed the consumption of ~21 mg/kg/day of dietary cysteine for adult healthy men [261]. In our study, participants' average dietary cysteine intake corresponds to approximately 22,3 mg/kg/day which is slightly above the recommended value indicated in the aforementioned study. However, it should be noted that the significant trends observed in the present study may also refer within the "theoretical normal range" of dietary cysteine intake. Additionally, it has been suggested that SAA intake surplus (>2-3 times of RDA) is considered safe and may confer health and/or performance benefits, via upregulation in GSH synthesis and turnover [262]. The literature on human SAA intake consistently refers to the combination of methionine and cysteine intake, as methionine conversion to cysteine through the methionine cycle and the transsulfuration pathway can supply ~100% of the requirements under resting conditions [263, 264]. In accordance, erythrocyte GSH fractional synthesis rate and concentration did not change with increasing cysteine intakes in healthy young men receiving an adequate diet (protein intake of 1 g/kg/day and methionine intake of 14 mg/kg/day) [265]. However, in some cases, defects in enzyme and metabolite activity interacting with methionine metabolism may compromise the methionine-cysteine conversion [266]. Moreover, it has been suggested that dietary cysteine has a sparing effect of ~64% on methionine requirement in adult men [261, 266]. Nevertheless, cysteine has been characterized as a "conditionally indispensable" amino acid [267], requiring a dietary source to provide enough precursor when basal endogenous synthesis cannot meet the metabolic need [268]. Thus, it could be speculated that in physically active and/or trained individuals the metabolic requirement for cysteine may exceed the endogenous conversion rate from methionine as a result of an adaptive response to systematic PA and upregulation of the cysteine-dependent GSH enzymes [269]. Interestingly, our results revealed a significant independent correlation between dietary cysteine intake with GSH metabolism and overall redox status, while in contrast, we did not detect similar partial associations when substituting cysteine with total SAA intake, except for GSH in both tissues (**Table 3.2**). A possible explanation for this discrepancy in the observed associations (cysteine vs. SAA intake), which warrants further investigation, may be the increased fitness status of our cohort, as a result of a higher PA level, which may have resulted in an upregulated metabolic need for cysteine, due to a chronic effect of PA, despite the fact that samples were collected at a resting state.

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Based on these observations, dietary cysteine intake seems to represent the major dietary determinant factor of overall GSH metabolism, justifying its physiological relevance and assessment separately from total SAA intake in young physically active men.

It is evident that future clinical studies with more participants, incorporating advanced statistical and analytical tools (e.g., isotope amino acid tracers) and controlling for relevant factors such as sex, age and fitness status are needed to confirm the validity of the associations observed in the present investigation in the context of dietary cysteine monitoring, in an attempt to distinguish between possible redox deficiencies accompanied by impaired GSH metabolism.

Regarding protein intake, previous reports showed that low protein diets may alter GSH synthesis rate and decrease antioxidant capacity as a result of a limited amino acid dietary intake [258]. Here, participants' median dietary protein intake (1.27 g/kg of body weight) was within the safe levels for physically active young men [256, 270]. However, contrary to a previous report [239], we did not detect a significant correlation between total protein intake and redox biomarkers, which may be attributed to some participants' high degree of variability in total protein intake (ranging from 0.6 to 1 g/kg/day) during the 7-day assessment period, as a result of a more plant-based diet protocol. Indeed, 18% of our cohort (8 out of 45 participants), when compared to the total sample, consumed a higher amount of legumes (median values: 47.5 vs 15.7 g/day, $p<0.001$), mainly including beans, chickpeas and lentils and lower amounts of cereals (mean values: 304.4 vs 391.3 g/day, $p=0.002$), mainly comprising of pasta, rice, oats and quinoa. Of note, such dietary plans (high legume/low cereal plant-based diet) may result in high concentrations of saponins, isoflavones and other phytochemicals which can potently exert redox effects by improving the antioxidant profile and reducing oxidative damage in men and women [271, 272] through direct (Nrf2 activation) as well as indirect (citric acid cycle enzymatic activity) mechanisms [273]. In our study, participants with lower cysteine intake (plant-based diets) exhibited redox status imbalances. Moreover, we did not detect any significant correlation between legume intake and redox biomarkers (**supplementary table 3.4**), thus the possible increased amount of phytochemicals in the participants' diet may not have influenced antioxidant status and the validity of the presented correlations.

To provide further insight into the causal role of dietary cysteine consumption in GSH metabolism, we performed additional analyses and corrected its consumption with energy, protein and SAA intake. Following correction, associations between cysteine intake and GSH status remained significant (with the exception of antioxidant enzymes), thus substantiating, for the first time, the functional/causal role of dietary cysteine intake in redox regulation, irrespective

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of other macro- and micro-nutrient consumption (**supplementary table 3.4**). Interestingly, cysteine intake was positively correlated with muscle GR enzyme activity, which may be attributed to a cysteine-dependent increase in GSH synthesis and/or turnover. GR is an essential component of the intracellular redox system, reducing GSSG back to GSH, thus assisting in optimal redox balance [274]. Indeed, defects in the enzyme activity are linked with several pathologies [275, 276]. A cysteine-induced increase of GSH levels through diet may positively impact skeletal muscle antioxidant enzyme capacity and redox regulation. Moreover, higher cysteine intake was inversely correlated with erythrocyte protein oxidation, which is in line with a negative association between cysteine intake and plasma F2-isoprostanes previously observed in young adults [239]. In fact, specific antioxidant deficiencies (i.e., GSH, vitamin C) have been linked with oxidative stress development and impairment of physical function, which can be inverted by targeted antioxidant supplementation without significant side-effects reported [173, 174]. In light of the above, our results provide further insights into the significance of dietary cysteine intake in maintaining an optimal redox environment in erythrocytes as well as in skeletal muscle. Based on the partial correlation analysis, it is evident that dietary cysteine consumption per se is a significant determinant factor in relation to redox status and oxidative stress, irrespective of the individual's physical fitness and PA profile.

Redox biomarkers were also correlated with lower-limb strength performance. Specifically, knee extensors eccentric peak torque was positively correlated with muscle GR enzyme activity while 1RM in back squat exercise was positively correlated with erythrocyte GSH/GSSG ratio and negatively correlated with muscle GSSG and erythrocyte PC levels. A recent study revealed that decreased levels of cysteine intake and erythrocyte GSH are associated with reduced maximal isometric handgrip strength [239]. Our findings further corroborate that the aforementioned disturbances in redox homeostasis may be linked to impairments in skeletal muscle performance, especially in lower-limb muscle groups. It has become apparent that redox regulation is a crucial modulator of several physiological functions including muscle contractile activity and adaptation to exercise [277, 278]. An optimal redox equilibrium is required for normal muscle function as an exceedingly oxidized or reduced environment may result in lower muscle performance [96, 279]. Since low cysteine and GSH levels are associated with elevated oxidative stress and impaired muscle function, it has been hypothesized that a potential mechanism may involve a RONS-induced oxidative modification of specific cysteine and methionine residues in the actin-myosin junction, resulting in contractile inhibition and as such a decline of muscle function [280]. Furthermore, alterations in Ca2+ sensitivity have been also

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implicated in skeletal muscle function and fatigue development [281, 282]. In isolated muscle fibers exposed to H2O2, GSH and myoglobin addition partially prevented muscle force reduction via a rise in Ca2+ sensitivity, which was accompanied by the S-glutathionylation of cysteine residues on the troponin I (fast) protein [282, 283]. In humans, supplementation with N-acetylcysteine attenuated fatigue development during repeated bouts of exhaustive exercise [203, 284] and ameliorated performance decline two days after a muscle-damaging protocol of the knee extensors [91]. Of note, only mild side-effects (mainly gastrointestinal) were reported during these studies. However, it was documented that prolonged antioxidant supplementation hampers late strength recovery following exercise by interfering with key anabolic pathways (i.e., Akt-mTOR axis) [91, 234]. It is evident that in skeletal muscle, redox signalling and mild levels of oxidative stress are indispensable modulators of the inflammatory/regenerative phase following injury, through the induction of muscle stem cells proliferation and differentiation [1, 246]. It seems that, under physiological conditions, non-targeted use of antioxidants blunts redox signals and muscle's ability to regenerate. Specifically, it was shown that N-acetylcysteine treatment impairs myofiber growth and fusion at the late restorative phase (30 days) following muscle injury in mice [5], while Nrf2 over-activation (through antioxidant supplementation) hampers myoblasts differentiation in vitro [6]. Nevertheless, despite the established link between cysteine oxidation and muscle function, our analyses revealed that lower-limb muscle strength is also independently associated with glutathione metabolism and oxidative stress. However, from a physiological point of view, it is plausible to note that muscle function and strength can be redox-dependent and not vice versa [285].

To our knowledge, this is the first study to provide direct evidence regarding the association between redox status and objectively assessed (via accelerometers) PA profile. The use of accelerometry to objectively quantify PA profile has been documented to be a reproducible and valid methodological approach across the life-span [207, 286]. Our results indicate that PA, independent of its intensity, is associated with increased GPx enzyme activity in skeletal muscle, while light PA is inversely correlated with muscle GSSG concentration. Of note, we did not observe any association between GSH and PA variables. By incorporating partial correlation analysis, our results show that PA levels independently influence muscle antioxidant capacity, regardless of nutritional and physical fitness status. Regarding the relationship between PA/exercise and redox status, several reports have indicated that regular PA/exercise can exert antioxidant effects via an adaptive response characterized by the upregulation of antioxidant status (i.e., Trolox-equivalent antioxidant capacity) and key antioxidant enzymes in skeletal

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muscle (i.e., superoxide dismutase) [269, 287]. In healthy prepubertal children, higher fitness levels (assessed by questionnaires) positively correlated with selected erythrocyte redox status biomarkers (GSH, GSSG, GSH/GSSG ratio) [288]. Furthermore, a recent cross-sectional study investigated the influence of self-reported PA on redox status and nitric oxide bioavailability in non-overweight and overweight/obese prepubertal children. It was shown that engagement in regular PA increased nitric oxide bioavailability in non-overweight children, while systemic antioxidant capacity and insulin sensitivity were enhanced in overweight/obese children [242]. In the elderly, data suggests that moderate-to-vigorous PA and/or endurance training induces an adaptive increase in systemic antioxidant enzyme activity and decreases lipid peroxidation in both women and men [198, 243]. Additionally, research from our group has shown that men with elevated chronic systemic inflammation and increased oxidative stress, perform less steps and spent less time in moderate-to-vigorous PA per day compared to their healthy counterparts [207]. Collectively, our data suggest that increased PA positively impacts redox status, and especially muscle antioxidant enzyme activity in young physically active men. It appears that the maintenance of high PA levels may positively regulate redox status by reducing GSH oxidation, with significant implications for health and disease development.

3.5. Study limitations

At this point, it should be noted that the design and results of this observational investigation cannot establish a cause-and-effect relationship between redox status, cysteine intake, muscle strength and PA levels and the results can only be applied in young, healthy, physically active men. Moreover, alternatives to causality for the associations observed with dietary cysteine intake may include the interference of tissue inflammation/dysfunction markers not measured in the present study.

3.6. Conclusion

In summary, our findings support the conclusion that decreased dietary cysteine intake is associated with impaired GSH metabolism and increased levels of protein oxidation in erythrocytes. Moreover, redox status disturbances are linked to decreased lower-limb muscle strength performance, while a higher PA level, independent of its intensity, is associated with increased muscle antioxidant capacity, which may be implicated in the treatment of several pathologies and chronic diseases characterized by dysfunctional antioxidant status. Further

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research, with appropriately designed large-scale clinical trials, applying sensitive statistical analyses and varying relevant factors such as age, sex and physical condition is warranted to confirm the consistency of the associations observed in the present study and elucidate the mechanisms of redox regulation by dietary cysteine and PA/exercise.

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3.7. Supplementary information

Table 3.7.1. Significant partial and bivariate correlations between dietary cysteine intake, physical fitness and physical activity with redox biomarkers

Cysteine vs redox biomarkers	**Adjusted for confounders**	**Not-adjusted for confounders**
Erythrocytes		
Cysteine (g/day) and GSH (μmol/g Hb)	**r = 0.697; p < 0.001**	**r = 0.610; p < 0.001**
Cysteine (g/day) and GSH/GSSG (ratio)	**r = 0.530; p = 0.001**	**r = 0.456; p = 0.002**
Cysteine (g/day) and PC (nmol/g Hb)	**r = -0.325; p = 0.046**	NS
Skeletal muscle		
Cysteine (g/day) and GSH (nmol/mg protein)	**r = 0.654; p < 0.001**	**r = 0.630; p < 0.001**
Cysteine (g/day) and GR activity (U/mg protein)	**r = 0.352; p = 0.030**	NS
1RM squat vs redox biomarkers		
Erythrocytes		
1RM squat (kg) and GSSG (μmol/g Hb)	**r = -0.441; p = 0.006**	**r = -0.444; p = 0.002**
1RM squat (kg) and GSH/GSSG (ratio)	**r = 0.401; p = 0.014**	**r = 0.470; p = 0.001**
1RM squat (kg) and PC (nmol/g Hb)	**r = -0.413; p = 0.011**	**r = -0.374; p = 0.011**
Eccentric peak torque vs redox biomarkers		
Erythrocytes		
Eccentric peak torque (Nm) and PC (nmol/g Hb)	NS	**r = -0.373; p = 0.012**
Skeletal muscle		
Eccentric peak torque (Nm) and GR activity (U/mg protein)	**r = 0.355; p = 0.031**	**r = 0.544; p < 0.001**
VO_{2max} vs redox biomarkers		
Erythrocytes		
VO_{2max} (ml/kg/min) and PC (nmol/g Hb)	NS	**r = 0.441; p = 0.002**

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Steps vs redox biomarkers		
Erythrocytes		
Steps (n/day) and PC (nmol/g Hb)	NS	**r = -0.300; p = 0.045**
Skeletal muscle		
Steps (n/day) and GPx activity (U/mg protein)	**r = 0.520; p < 0.001**	**r = 0.552; p < 0.001**
Light PA vs redox biomarkers		
Erythrocytes		
Light PA (min/day) and PC (nmol/g Hb)	NS	**r = -0.309; p = 0.039**
Skeletal muscle		
Light PA (min/day) and GSSG (nmol/mg protein)	**r = -0.353; p = 0.022**	**r = -0.346; p = 0.020**
Light PA (min/day) and GPx activity (U/mg protein)	**r = -0.406; p = 0.008**	**r = 0.441; p = 0.002**
Light PA (min/day) and GR activity (U/mg protein)	NS	**r = 0.327; p = 0.028**
Moderate PA vs redox biomarkers		
Skeletal muscle		
Light PA (min/day) and GPx activity (U/mg protein)	**r = 0.417; p = 0.006**	**r = 0.449; p = 0.002**
Moderate-to-vigorous PA vs redox biomarkers		
Skeletal muscle		
Moderate-to-vigorous PA (min/day) and GPx activity (U/mg protein)	**r = 0.417; p = 0.006**	**r = 0.449; p = 0.002**
Vigorous PA vs redox biomarkers		
Erythrocytes		
Vigorous PA (min/day) and GSSG (μmol/g Hb)	NS	**r = 0.341; p = 0.022**
Skeletal muscle		
Vigorous PA (min/day) and GPx activity (U/mg protein)	**r = 0.352; p = 0.022**	**r = 0.355; p = 0.017**
Very vigorous PA vs redox biomarkers		
Skeletal muscle		
Very vigorous PA (min/day) and GPx activity (U/mg protein)	**r = 0.326; p = 0.035**	**r = 0.367; p = 0.013**

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Data are presented as correlation coefficients and p values (*n*=45). Correlations between dietary cysteine intake, physical fitness and PA with redox biomarkers are presented both with and without the adjustments for confounders. *GSH* reduced glutathione, *GSSG* oxidized glutathione, *GPx* glutathione peroxidase, *GR* glutathione reductase, *PA* physical activity, *PC* protein carbonyls, VO_{2max} maximal oxygen consumption

Table 3.7.2. Partial and bivariate correlations between corrected dietary cysteine intakes (cysteine/energy, cysteine/protein and cysteine/SAA) and legume intake with redox biomarkers

Cysteine/energy vs redox biomarkers	**Adjusted for confounders**	**Not-adjusted for confounders**
Erythrocytes		
Cysteine/energy (g/kcal/day) and GSH (μmol/g Hb)	**r = 0.618; p < 0.001**	**r = 0.594; p < 0.001**
Cysteine/energy (g/kcal/day) and GSSG (μmol/g Hb)	r = 0.022; p = 0.899	r = -0.074; p = 0.629
Cysteine/energy (g/kcal/day) and GSH/GSSG (ratio)	**r = 0.439; p = 0.008**	**r = 0.458; p = 0.002**
Cysteine/energy (g/kcal/day) and PC (nmol/g Hb)	r = -0.258; p = 0.135	**r = -0.334; p = 0.025**
Skeletal muscle		
Cysteine/energy (g/kcal/day) and GSH (nmol/mg protein)	**r = 0.668; p < 0.001**	**r = 0.678; p < 0.001**
Cysteine/energy (g/kcal/day) and GSSG (nmol/mg protein)	r = 0.274; p = 0.112	r = 0.279; p = 0.063
Cysteine/energy (g/kcal/day) and GSH/GSSG (ratio)	r = 0.015; p = 0.933	r = 0.021; p = 0.893
Cysteine/energy (g/kcal/day) and GPx activity (U/mg protein)	r = -0.010; p = 0.953	r = 0.102; p = 0.506
Cysteine/energy (g/kcal/day) and GR activity (U/mg protein)	r = 0.209; p = 0.228	r = 0.249; p = 0.099
Cysteine/protein vs redox biomarkers		
Erythrocytes		
Cysteine/protein (g/g/day) and GSH (μmol/g Hb)	**r = 0.654; p < 0.001**	**r = 0.616; p < 0.001**
Cysteine/protein (g/g/day) and GSSG (μmol/g Hb)	r = -0.069; p = 0.692	r = -0.087; p = 0.569
Cysteine/protein (g/g/day) and GSH/GSSG (ratio)	**r = 0.513; p = 0.002**	**r = 0.463; p = 0.001**

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Cysteine/protein (g/g/day) and PC (nmol/g Hb)	r = -0.230; p = 0.183	**r = -0.298; p = 0.047**
Skeletal muscle		
Cysteine/protein (g/g/day) and GSH (nmol/mg protein)	**r = 0.735; p < 0.001**	**r = 0.730; p < 0.001**
Cysteine/protein (g/g/day) and GSSG (nmol/mg protein)	r = 0.183; p = 0.294	r = 0.164; p = 0.282
Cysteine/protein (g/g/day) and GSH/GSSG (ratio)	r = 0.118; p = 0.501	r = 0.136; p = 0.371
Cysteine/protein (g/g/day) and GPx activity (U/mg protein)	r = -0.014; p = 0.938	r = 0.127; p = 0.406
Cysteine/protein (g/g/day) and GR activity (U/mg protein)	r = 0.188; p = 0.281	r = 0.231; p = 0.126
Cysteine/SAA vs redox biomarkers		
Erythrocytes		
Cysteine/SAA (g/g/day) and GSH (μmol/g Hb)	**r = 0.537; p = 0.001**	**r = 0.396; p = 0.007**
Cysteine/SAA (g/g/day) and GSSG (μmol/g Hb)	r = -0.106; p = 0.544	r = 0.175; p = 0.251
Cysteine/SAA (g/g/day) and GSH/GSSG (ratio)	r = 0.332; p = 0.051	**r = 0.307; p = 0.040**
Cysteine/SAA (g/g/day) and PC (nmol/g Hb)	r = -0.218; p = 0.208	r = -0.109; p = 0.477
Skeletal muscle		
Cysteine/SAA (g/g/day) and GSH (nmol/mg protein)	**r = 0.522; p = 0.001**	**r = 0.415; p = 0.005**
Cysteine/SAA (g/g/day) and GSSG (nmol/mg protein)	r = 0.158; p = 0.364	r = 0.039; p = 0.799
Cysteine/SAA (g/g/day) and GSH/GSSG (ratio)	r = 0.047; p = 0.791	r = 0.106; p = 0.487
Cysteine/SAA (g/g/day) and GPx activity (U/mg protein)	r = 0.108; p = 0.539	r = 0.176; p = 0.246
Cysteine/SAA (g/g/day) and GR activity (U/mg protein)	r = 0.249; p = 0.149	r = 0.121; p = 0.429
Legumes vs redox biomarkers		
Erythrocytes		
Legumes (g/day) and GSH (μmol/g Hb)	r = -0.144; p = 0.408	r = -0.234; p = 0.121
Legumes (g/day) and GSSG (μmol/g Hb)	r = -0.075; p = 0.667	r = -0.009; p = 0.955
Legumes (g/day) and GSH/GSSG (ratio)	r = -0.125; p = 0.474	r = -0.177; p = 0.246
Legumes (g/day) and PC (nmol/g Hb)	r = 0.082; p = 0.639	r = 0.126; p = 0.409
Skeletal muscle		

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Legumes (g/day) and GSH (nmol/mg protein)	r = -0.227; p = 0.189	r = -0.257; p = 0.088
Legumes (g/day) and GSSG (nmol/mg protein)	r = -0.077; p = 0.659	r = -0.127; p = 0.404
Legumes (g/day) and GSH/GSSG (ratio)	r = 0.058; p = 0.742	r = 0.055; p = 0.718
Legumes (g/day) and GPx activity (U/mg protein)	r = 0.131; p = 0.452	r = -0.008; p = 0.958
Legumes (g/day) and GR activity (U/mg protein)	r = 0.006; p = 0.971	r = 0.001; p = 0.997

Data are presented as correlation coefficients and p values (*n*=45). Correlations between corrected dietary cysteine intakes (cysteine/energy, cysteine/protein and cysteine/SAA) with redox biomarkers are presented both with and without the adjustments for confounders. Confounding variables included physical fitness and physical activity profile. SAA represents the total cysteine and methionine dietary intake. *GSH* reduced glutathione, *GSSG* oxidized glutathione, *GPx* glutathione peroxidase, *GR* glutathione reductase, *PC* protein carbonyls, SAA sulfur amino acids

Table 3.7.3. The SPSS code for non-parametric (Spearman) partial correlation analysis

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/MATRIX OUT(*).
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PARTIAL CORR Variable1 Variable2 BY Variable3
/significance = twotail
/MISSING = LISTWISE
/MATRIX IN(*).
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Reference

Schemper, M. (1991), Non-Parametric Partial Association Revisited. Journal of the Royal Statistical Society: Series D (The Statistician), 40: 73-76. https://doi.org/10.2307/2348226

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Chapter 4

Unpublished data:

Results related to glutathione metabolism, antioxidant status, muscle damage/regeneration, satellite cells, myogenic programming and muscle function

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4.1. Participants' physical profile, dietary intake and muscle fiber characteristics

Table 4.1.1. summarizes participants' physical characteristics, before each trial, for the total sample and the low/high pax7 groups. Participants' physical characteristics were comparable among groups before each trail with the exception of 1RM squat strength, which was higher in $pax7^{High}$ compared to $pax7^{Low}$ group in both conditions (PLA: p=0.011; NAC: p=0.002).

Table 4.1.2. depicts participants' dietary nutrient intake profile, during each trial, for the total sample and the low/high pax7 groups. No changes were noted regarding participants dietary nutrient intake during trials.

Table 4.1.3. presents participants' muscle fiber characteristics at basal state before each trial.

In total sample, muscle fiber CSA, myonuclear content and myonuclear domain were greater in type II compared to type I fibers in both trials (CSA: PLA, p<0.001; NAC, p<0.001 / myonuclear content: PLA, p=0.006; NAC, p=0.006 / myonuclear domain: PLA, p=0.047; NAC, p=0.011).

In low/high pax7 groups, type II muscle fiber CSA was greater compared with type I in both groups and trials (PLA: $pax7^{Low}$, p<0.001; $pax7^{High}$, p=0.001 / NAC: $pax7^{Low}$, p<0.001; $pax7^{High}$, p=0.001). Type I and II muscle fiber CSA was greater in $pax7^{High}$ compared to $pax7^{Low}$ group in both trials (PLA: type I, p=0.049; type II, p=0.013 / NAC: type I, p=0.022; type II, p=0.020). Interestingly, myonuclear content was greater in type II fibers compared to type I only in $pax7^{Low}$ group in both trials (PLA: p=0.017; NAC: p=0.002). Myonuclear content in type I fibers was greater in $pax7^{High}$ group compared to $pax7^{Low}$ group in both trials (PLA: p=0.042; NAC: p=0.022). Myonuclear domain was greater in type II fibers compared to type I fibers only in $pax7^{Low}$ group in both trials (PLA: p=0.031; NAC: p=0.044).

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Table 4.1.1. Participants' physical characteristics before each trial

	PLA			NAC		
Variable	**Total sample**	**Pax7^{Low} group**	**Pax7^{High} group**	**Total sample**	**Pax7^{Low} group**	**Pax7^{High} group**
Age (yrs)	22.5 ± 2.2	22.9 ± 2.6	22.2 ± 2.2	22.6 ± 2.3	23.0 ± 2.6	22.3 ± 2.2
Body mass (kg)	76.8 ± 9.9	74.8 ± 9.2	79.7 ± 10.5	77.6 ± 11.1	76.6 ± 11.4	79.0 ± 11.1
Height (m)	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
BMI (kg/m^2)	24.1 ± 2.4	23.4 ± 2.1	25.2 ± 2.5	24.4 ± 2.6	23.9 ± 2.6	24.9 ± 2.6
RMR (kcal/day)	1840.3 ± 251.3	1813.6 ± 252.3	1877.7 ± 258.3	1855.8 ± 266.3	1834.5 ± 299.5	1885.4 ± 223.4
Body fat (%)	21.7 ± 6.8	21.6 ± 6.3	21.8 ± 7.7	20.8 ± 7.1	20.7 ± 6.8	20.8 ± 7.9
Lean mass (kg)	56.7 ± 6.0	55.6 ± 6.1	58.2 ± 5.8	57.4 ± 6.2	56.1 ± 6.0	59.2 ± 6.4
1RM squat (kg)	111.9 ± 17.1	104.6 ± 10.3	122.0 ± 20.1*	112.3 ± 18.6	103.2 ± 12.5	125.0 ± 18.7*
VO_{2max} (ml/kg/min)	49.6 ± 5.0	50.5 ± 4.1	48.4 ± 6.0	50.0 ± 4.6	50.6 ± 3.9	49.1 ± 5.6
Resting HR (b/min)	51.4 ± 5.2	50.6 ± 4.7	52.6 ± 5.8	50.7 ± 4.6	50.4 ± 4.0	51.2 ± 5.4
Maximum HR (b/min)	200.0 ± 6.4	200.2 ± 5.5	199.7 ± 7.8	200.2 ± 7.0	201.3 ± 5.2	198.7 ± 9.0

Values are presented as mean ± SD. *Significant within-trial difference between groups. PLA, placebo; NAC, N-acetylcysteine; BMI, body mass index; RMR, resting metabolic rate; 1RM, one repetition maximum; VO_{2max}, maximal oxygen consumption; HR, heart rate. Total sample N=24; Pax7^{Low} group, N=14; Pax7^{High} group, N=10.

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Table 4.1.2. Participants' dietary profile during each trial

	PLA			NAC		
Variable	**Total sample**	**Pax7^{Low} group**	**Pax7^{High} group**	**Total sample**	**Pax7^{Low} group**	**Pax7^{High} group**
Energy (kcal/day)	2300.0 ± 565.7	2281.3 ± 473.5	2326.1 ± 702.0	2312.7 ± 636.5	2207.6 ± 586.3	2459.9 ± 705.4
Protein (g/kg/day)	1.4 ± 0.4	1.4 ± 0.3	1.4 ± 0.4	1.3 ± 0.4	1.2 ± 0.4	1.4 ± 0.4
Protein (% of total energy/day)	19.3 ± 3.4	18.1 ± 3.3	19.0 ± 3.7	17.9 ± 3.7	16.7 ± 2.8	18.9 ± 4.5
Carbohydrates (g/day)	243.7 ± 75.2	254.1 ± 72.4	229.0 ± 80.4	246.9 ± 80.0	238.1 ± 72.9	259.2 ± 91.5
Carbohydrates (% of total energy/day)	41.9 ± 5.5	44.1 ± 5.4	40.8 ± 4.3	42.6 ± 6.6	43.6 ± 6.9	41.9 ± 6.2
Fat (g/day)	98.8 ± 28.2	95.9 ± 21.7	102.9 ± 36.4	101.9 ± 32.4	99.1 ± 31.7	105.9 ± 34.7
Fat (% of total energy/day)	38.8 ± 4.9	37.8 ± 4.5	40.2 ± 5.3	39.5 ± 5.9	39.7 ± 6.3	39.2 ± 5.6
Zinc (mg/day)	13.7 ± 4.3	14.2 ± 3.8	13.0 ± 5.1	14.0 ± 6.3	14.2 ± 7.2	13.8 ± 5.3
Selenium (μg/day)	68.3 ± 21.1	67.6 ± 16.6	69.3 ± 27.2	65.5 ± 18.7	60.5 ± 14.9	72.4 ± 21.8
Vitamin A (μg RAE[1]/day)	780.8 ± 454.9	742.4 ± 345.5	834.6 ± 592.4	840.8 ± 428.9	880.6 ± 453.4	785.2 ± 408.9
Vitamin C (mg/day)	92.9 ± 51.3	90.7 ± 44.2	96.0 ± 62.3	118.3 ± 67.7	109.0 ± 58.6	131.3 ± 80.1
Vitamin E (mg a-TE[2]/day)	8.5 ± 3.6	8.5 ± 2.4	8.6 ± 5.0	9.6 ± 4.2	9.3 ± 4.2	10.1 ± 4.4

Values are presented as mean ± SD. PLA, placebo; NAC, N-acetylcysteine. [1] In retinol activity equivalents [2] In a-tocopherol equivalents. Total sample N=24; Pax7^{Low} group, N=14; Pax7^{High} group, N=10.

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Table 4.1.3. Participants' baseline muscle fiber characteristics before each trial

	PLA			NAC		
Variable	**Total sample**	**Pax7^{Low} group**	**Pax7^{High} group**	**Total sample**	**Pax7^{Low} group**	**Pax7^{High} group**
Fiber type distribution (%)						
Type I	51.2 ± 9.8	51.2 ± 9.6	51.3 ± 10.7	55.4 ± 10.9	55.1 ± 11.5	55.9 ± 10.6
Type II	48.8 ± 9.8	48.8 ± 9.6	48.7 ± 10.7	44.6 ± 10.9	44.9 ± 11.5	44.1 ± 10.6
Muscle fiber CSA (μm^2)						
Type I	6021 ± 1344	5586 ± 728	6630 ± 1774$^{\#}$	6025 ± 1373	5498 ± 681	6763 ± 1765$^{\#}$
Type II	7731 ± 982*	7325 ± 829*	8300 ± 924$^{*,\#}$	7696 ± 1281*	7198 ± 1168*	8393 ± 1138$^{*,\#}$
Myonuclear content (myonuclei/fiber)						
Type I	2.6 ± 0.5	2.5 ± 0.4	2.9 ± 0.6$^{\#}$	2.7 ± 0.6	2.5 ± 0.5	3.0 ± 0.6$^{\#}$
Type II	3.0 ± 0.7*	2.8 ± 0.6*	3.3 ± 0.6	3.0 ± 0.5*	2.9 ± 0.6*	3.2 ± 0.3
Myonuclear domain (μm^2)						
Type I	2391 ± 819	2361 ± 654	2432 ± 1045	2318 ± 637	2337 ± 574	2292 ± 749
Type II	2683 ± 604*	2722 ± 511*	2628 ± 740	2606 ± 507*	2571 ± 491*	2654 ± 551

Values are presented as mean ± SD. *Significant difference within group for fiber type. $^{\#}$Significant difference for Pax7 groups within trial. PLA, placebo; NAC, N-acetylcysteine; CSA, cross sectional area. Pax7^{Low} group, n=14; Pax7^{High} group, n=10.

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4.2. Exercise-induced muscle damage (EIMD) protocol

Total sample (**Figure 4.2.1**): Eccentric mean torque decreased at set 2 only in PLA (p=0.042) and in both trials throughout the protocol thereafter (p<0.05).

Low vs. High Pax7 groups (**Figure 4.2.2**): In both groups and trials, eccentric mean torque decreased from set 3 and throughout the protocol (p<0.05) with no between group nor trial differences.

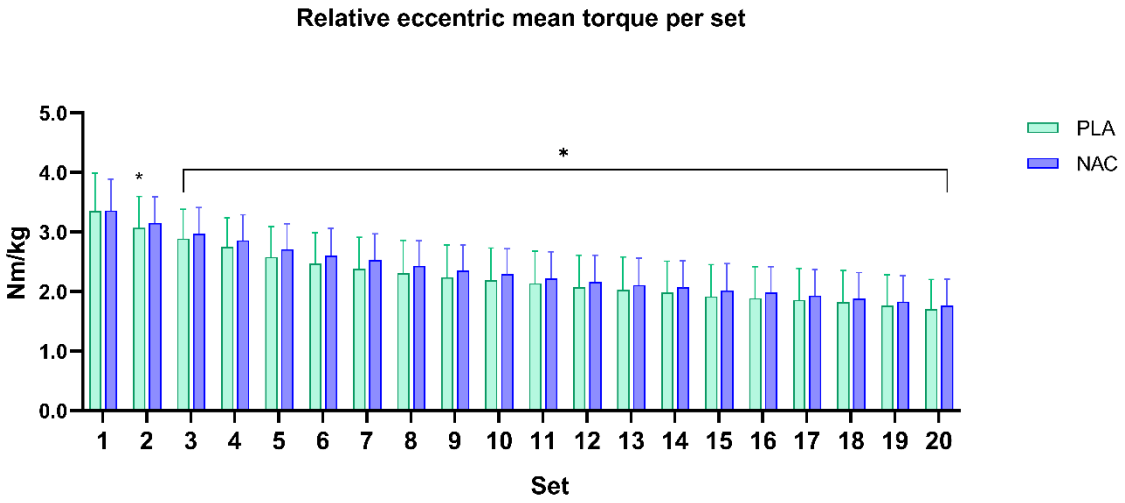

Figure 4.2.1. Changes in eccentric mean torque per set in total sample. *Difference with set 1 (basal), p<0.05. Total sample n=24.

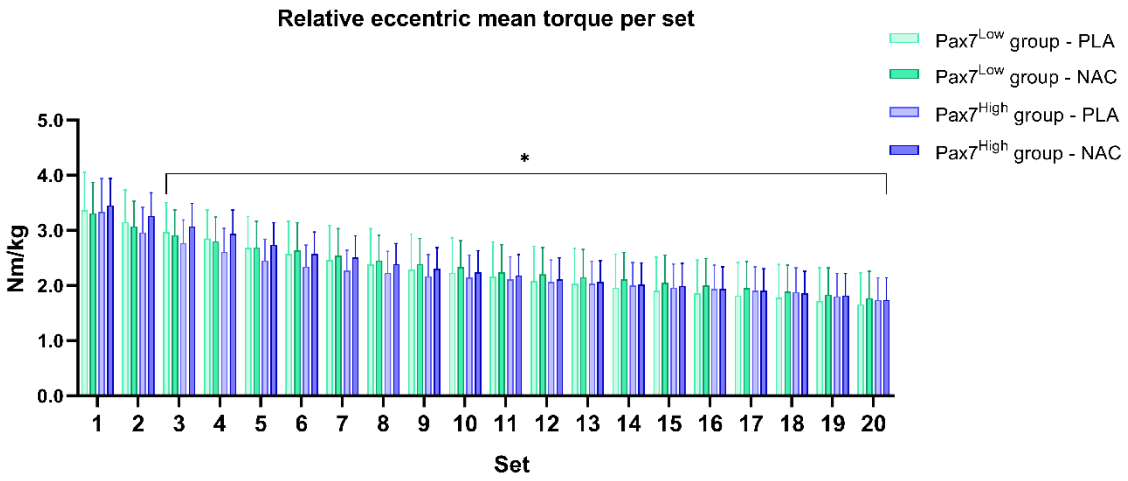

Figure 4.2.2. Changes in eccentric mean torque per set in low and high Pax7 groups. *Difference with set 1 (basal), p<0.05. $Pax7^{Low}$ group, n=14; $Pax7^{High}$ group, N=10.

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4.3. Glutathione metabolism and antioxidant status

4.3.1 Reduced glutathione (GSH)

Total sample (**Figure 4.3.1.1**): GSH increased in NAC at post-load (p=0.015). GSH decreased at 2-days post-injury in both trials (PLA: p<0.001; NAC: p=0.002) compared to basal. At 8-days post-injury, GSH levels in NAC were higher compared to basal (p<0.001). GSH levels were higher in NAC compared to PLA at post-load (p=0.044), 2-days (p=0.005) and 8-days (p<0.001) post-injury.

Low vs. High Pax7 groups (**Figure 4.3.1.2**): In pax7^{Low} group, GSH increased in NAC at post-load (+10.7%p=0.005). GSH decreased at 2-days post-injury only in PLA (-32.6%, p<0.001) while in NAC, GSH levels recovered and exhibited an increase at 8-days post-injury (+14%, p=0.013) compared to basal. In pax7^{High} group, GSH decreased at 2-days post-injury in both trials (PLA: -29%, p<0.001; NAC: -23.7%, p=0.003). At 8-days post injury, NAC restored GSH levels to a higher extent compared to basal (+13.1%, p=0.024). No changes were noted at post-load. At 2-days post-injury, GSH was higher in NAC compared to PLA in pax7^{Low} group (p=0.002). At 8-days post-injury, GSH levels were higher in NAC compared to PLA in both groups (pax7^{High} group: p<0.001; pax7^{Low} group: p=0.034).

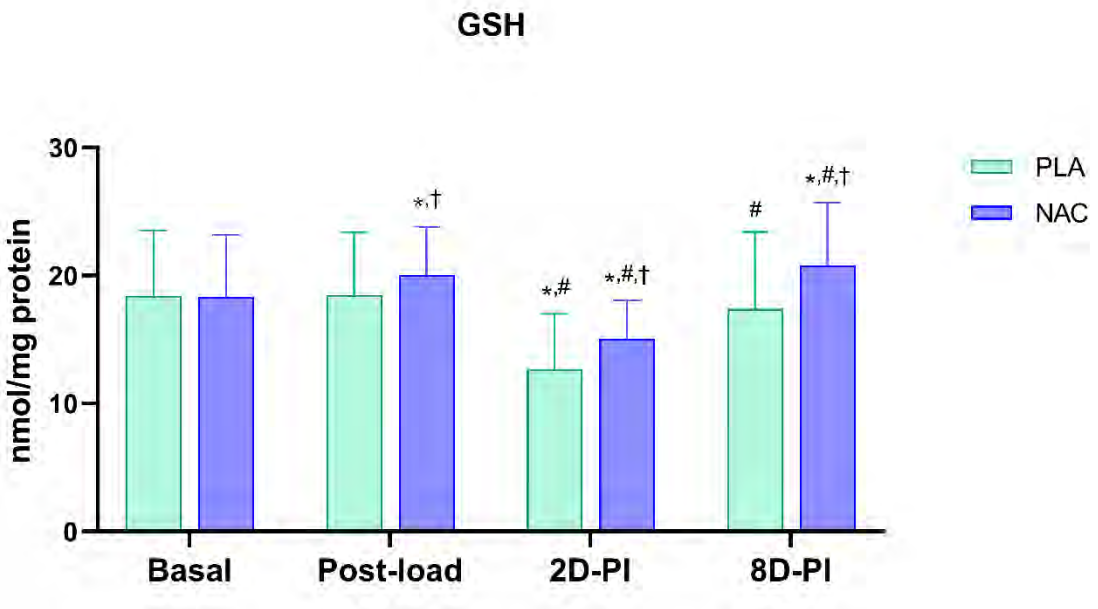

Figure 4.3.1.1. Changes in muscle GSH in total sample. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between trials, p<0.05. Total sample n=24.

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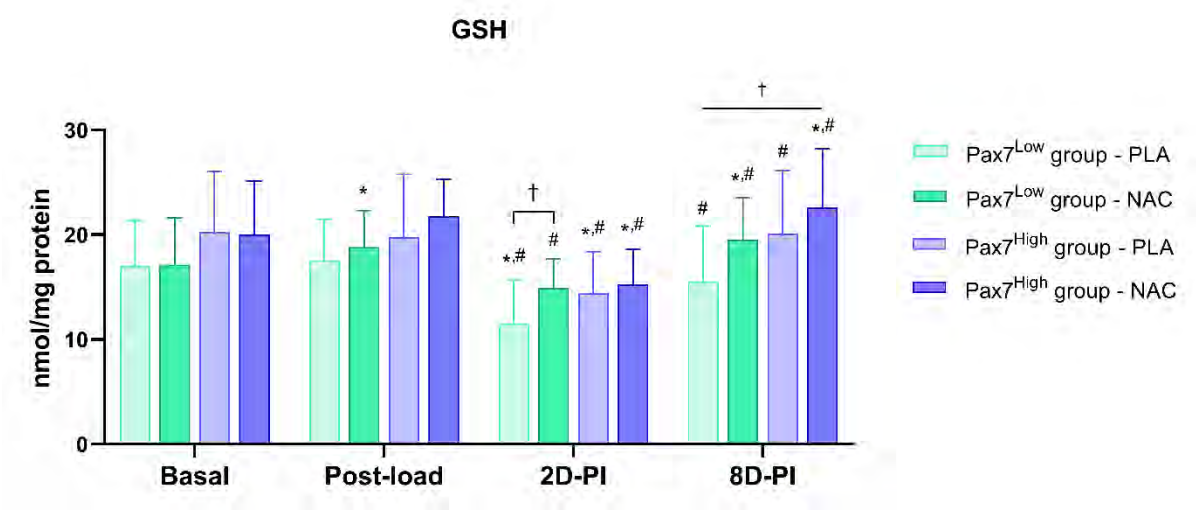

Figure 4.3.1.2. Changes in muscle GSH in low and high Pax7 groups. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between conditions, within group, p<0.05. Pax7^{Low} group, n=14; Pax7^{High} group, N=10.

4.3.2 Oxidized glutathione (GSSG)

Total sample (**Figure 4.3.2.1**): GSSG decreased in NAC at post-load (p=0.045). GSSG increased at 2-days post-injury only in PLA compared to basal (p=0.001). NAC resulted in lower GSSG levels at 8-days post-injury compared to basal (p=0.044). At 2-days post-injury, GSSG levels were lower in NAC compared to PLA (p<0.001). No changes were noted at post-load.

Low vs. High Pax7 groups (**Figure 4.3.2.2**): In pax7^{Low} group, GSSG decreased at post-load (-15.9%, p<0.001). GSSG increased only at 2-days post-injury in PLA (+68%, p=0.003) while in NAC, GSSG levels were lower at 8-days post-injury compared to basal (-22.1%, p=0.032). In pax7^{High} group, GSSG increased at 2-days post-injury only in PLA (+38.8%, p=0.026) with no time-dependent changes reported in NAC. At 2-days post-injury, GSSG levels were lower in NAC compared to PLA in both groups (pax7^{Low} group: p=0.001; pax7^{High} group: p=0.017). No changes were reported at post-load.

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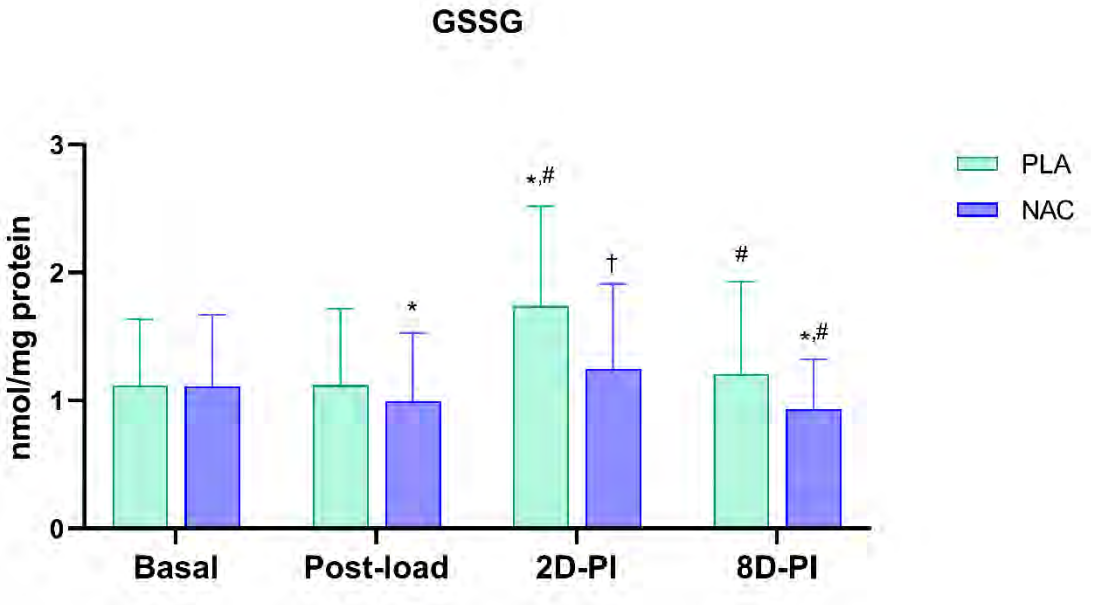

Figure 4.3.2.1. Changes in muscle GSSG in total sample. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between trials, p<0.05. Total sample n=24.

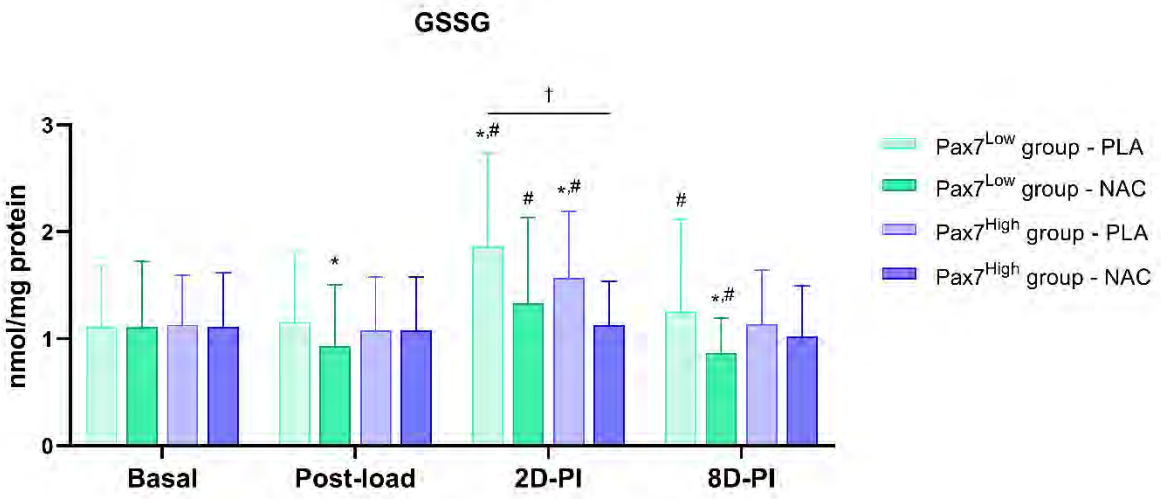

Figure 4.3.2.2. Changes in muscle GSSG in low and high Pax7 groups. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between conditions, within group, p<0.05. $Pax7^{Low}$ group, n=14; $Pax7^{High}$ group, N=10.

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4.3.3 GSH/GSSG ratio

Total sample (**Figure 4.3.3.1**): GSH/GSSG ratio decreased only at 2-days post-injury in both trials compared to basal (PLA: $p<0.001$; NAC: $p=0.045$) while at 8-days post-injury, NAC elicited an increase in GSH/GSSG ratio compared to basal ($p<0.001$). At post-load, NAC increased GSH/GSSG ratio ($p=0.048$). At 2- and 8-days post-injury, GSH/GSSG ratio was higher in NAC compared to PLA (2-days: $p<0.001$; 8-days: $p=0.003$). GSH/GSSG ratio tended to be higher in NAC compared to PLA at post-load ($p=0.052$).

Low vs. High Pax7 groups (**Figure 4.3.3.2**): In pax7^{Low} group, GSH/GSSG ratio decreased at 2-days post-injury only in PLA compared to basal (-54%, $p<0.001$), while NAC elicited an increase in GSH/GSSG ratio at 8-days post-injury compared to basal (+39.7%, $p=0.001$). NAC also increased GSH/GSSG ratio at post-load (+41.2%, $p=0.027$). In pax7^{High} group, GSH/GSSG ratio decreased at 2-days post-injury only in PLA compared to basal (-48.6%, $p=0.001$) and it was restored thereafter. GSH/GSSG ratio was higher in NAC compared to PLA in pax7^{Low} group at post-load ($p=0.032$) as well as 2-days ($p<0.001$) and 8-days ($p=0.006$) post-injury. GSH/GSSG ratio was also higher in NAC compared to PLA in pax7^{High} group at 2-days ($p=0.006$) post-injury.

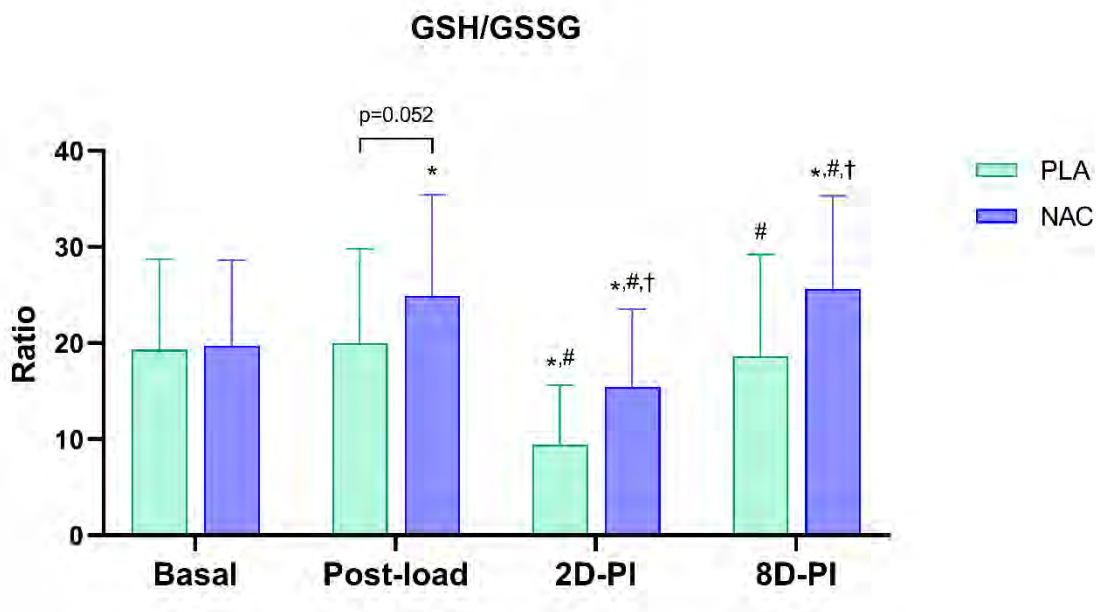

Figure 4.3.3.1. Changes in muscle GSH/GSSG ratio in total sample. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$; †Difference between trials, $p<0.05$. Total sample n=24.

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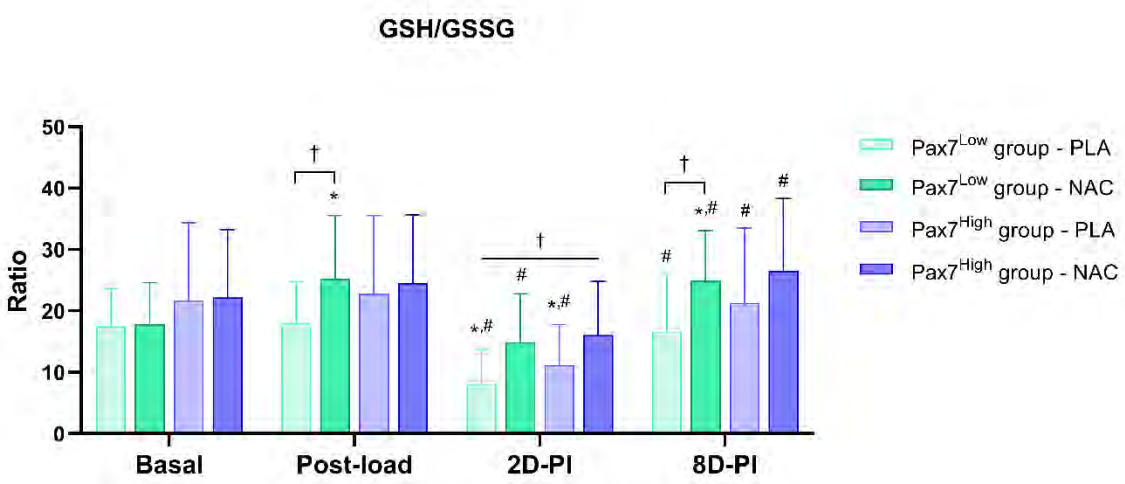

Figure 4.3.3.2. Changes in muscle GSH/GSSG ratio in low and high Pax7 groups. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between conditions, within group, p<0.05. Pax7^{Low} group, n=14; Pax7^{High} group, N=10.

4.3.4 Glutathione peroxidase (GPx) activity

Total sample (**Figure 4.3.4.1**): GPx activity increased in PLA only at 2-days post-injury (p<0.001), while in NAC it increased at post-load (p=0.002) as well as 2- (p<0.001) and 8-days (p=0.001) post-injury compared to basal. NAC resulted in higher GPx activity levels compared to PLA at post-load (p=0.001) as well as 2- (p<0.001) and 8-days (p<0.001) post-injury.

Low vs. High Pax7 groups (**Figure 4.3.4.2**): In pax7^{Low} group, GPx activity increased in PLA only at 2-days post-injury compared to basal (+15.6%, p<0.001), while in NAC it increased at post-load (+13.3%, p=0.009) as well as 2-days (+35.6%, p<0.001) and 8-days (+13.2%, p=0.027) post-injury compared to basal. In pax7^{High} group, GPx activity increased in PLA only at 2-days post-injury compared to basal (+27.3%, p<0.001), while in NAC it increased at 2-days (+48.6%, p<0.001) and 8-days (+16.3%, p=0.029) post-injury compared to basal. GPx activity levels were higher in NAC compared to PLA in both groups at 2-days (pax7^{Low} group: p<0.001; pax7^{High} group: p=0.009) and 8-days (pax7^{Low} group: p<0.001; pax7^{High} group: p=0.010) post-injury.

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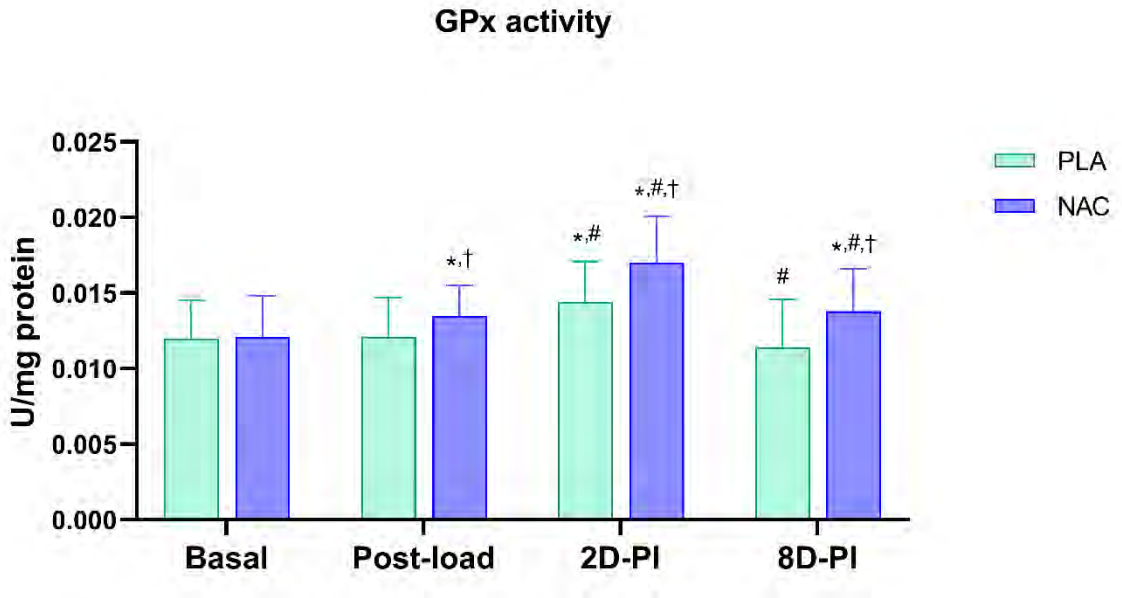

Figure 4.3.4.1. Changes in muscle GPx activity in total sample. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between trials, p<0.05. Total sample n=24.

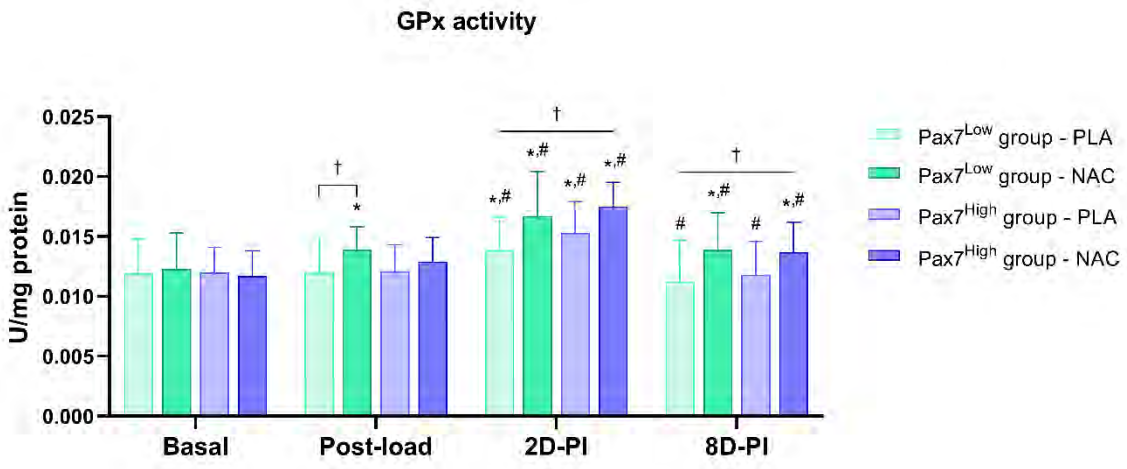

Figure 4.3.4.2. Changes in muscle GPx activity in low and high Pax7 groups. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between conditions, within group, p<0.05. Pax7^{Low} group, n=14; Pax7^{High} group, N=10.

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4.3.5 Glutathione reductase (GR) activity

Total sample (**Figure 4.3.5.1**): GR activity increased in PLA only at 2-days post-injury (p=0.012), while in NAC it increased at post-load (p=0.036) as well as 2- (p<0.001) and 8-days (p=0.023) post-injury compared to basal. NAC resulted in higher GR activity levels compared to PLA at post-load (p=0.011) as well as 2- (p=0.009) and 8-days (p<0.001) post-injury.

Low vs. High Pax7 groups (**Figure 4.3.5.2**): In pax7^{Low} group, GR activity increased in NAC at post-load (+11.8%, p=0.047) as well as 2-days (+58.2%, p=0.001) post-injury compared to basal. No changes were detected in PLA trial. In pax7^{High} group, GR activity increased in both trials only at 2-days post-injury compared to basal (PLA: +40.2%, p=0.010; NAC: +50.9%, p=0.011). GR activity was higher in NAC compared to PLA in pax7^{Low} group at 2-days post-injury (p=0.010) and at 8-days post-injury in both groups (pax7^{Low} group: p=0.002; pax7^{High} group: p<0.001).

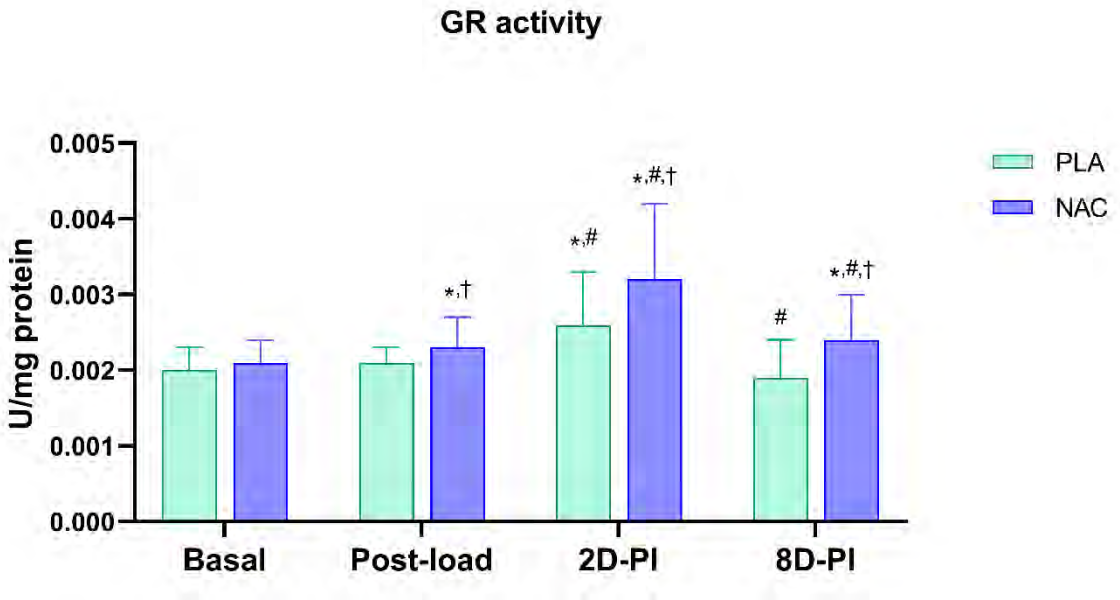

Figure 4.3.5.1. Changes in muscle GR activity in total sample. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between trials, p<0.05. Total sample n=24.

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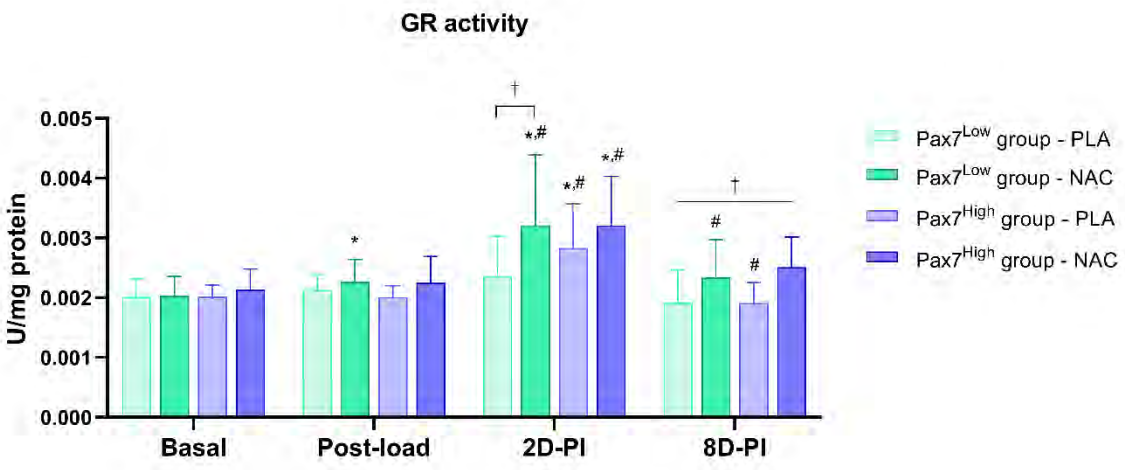

Figure 4.3.5.2. Changes in muscle GR activity in low and high Pax7 groups. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between conditions, within group, p<0.05. Pax7^{Low} group, n=14; Pax7^{High} group, N=10.

4.3.6 Nuclear Nrf2 fluorescent intensity

Total sample (**Figure 4.3.6.1**): Nuclear Nrf2 increased in PLA only at 2-days post-injury compared to basal (p<0.001), while in NAC, Nrf2 increased at post-load (p=0.015) as well as 2-days (p<0.001) and 8-days (p=0.001) post-injury compared to basal. Nuclear Nrf2 levels were higher in NAC compared to PLA at 2-days (p=0.024) and 8-days post-injury (p=0.030).

Low vs. High Pax7 groups (**Figure 4.3.6.2**): In pax7^{Low} group, nuclear Nrf2 increased at post-load in NAC (+16%, p=0.027) and at 2-days post-injury in both trials (PLA: +32%, p=0.004; NAC: +58%, p=0.001) compared to basal. In pax7^{High} group, nuclear Nrf2 increased in both trials at 2-days post-injury (PLA: +38%, p=0.003; NAC: +63%, p=0.004) and at 8-days post-injury only in NAC (+62%, p=0.002) compared to basal. At 2-days post-injury, nuclear Nrf2 levels tended to be higher in pax7^{Low} group in NAC compared to PLA (p=0.051). At 8-days post-injury, NAC elicited a greater increase in nuclear Nrf2 levels compared to PLA in pax7^{High} group (p=0.015).

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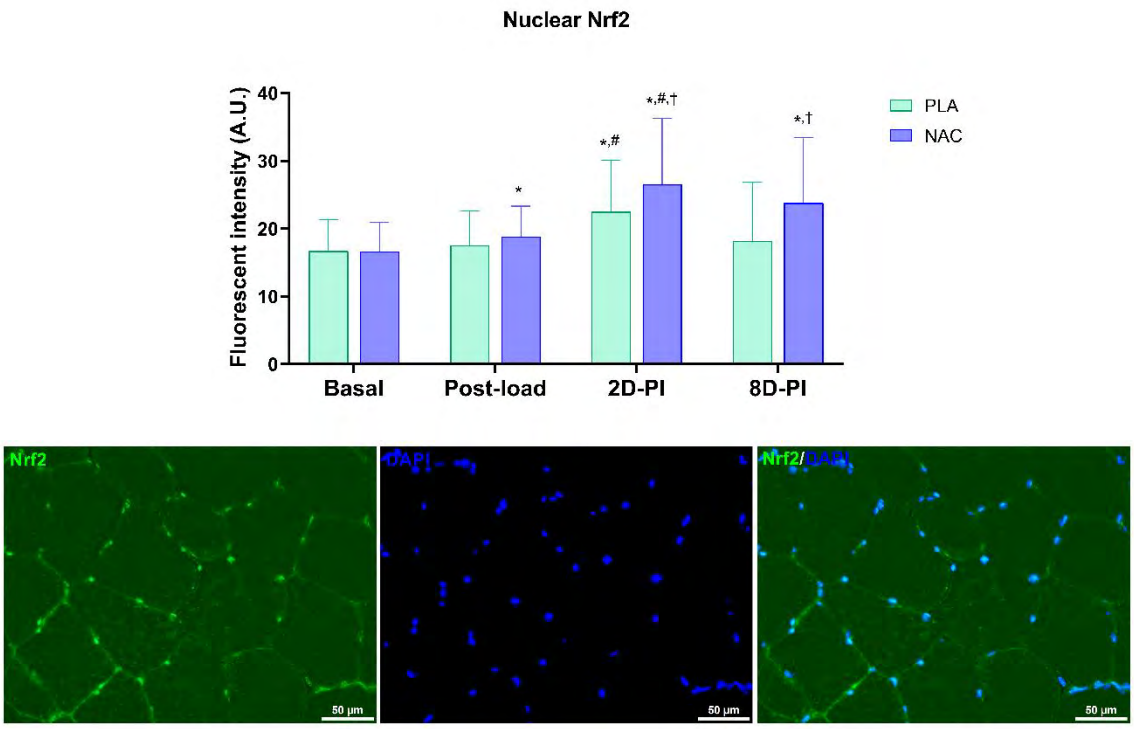

Figure 4.3.6.1. Changes in nuclear Nrf2 fluorescent intensity in total sample. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between trials, p<0.05. Lower panels: Representative images of Nrf2, and DAPI immunofluorescent staining of muscle cross sections. Total sample n=24.

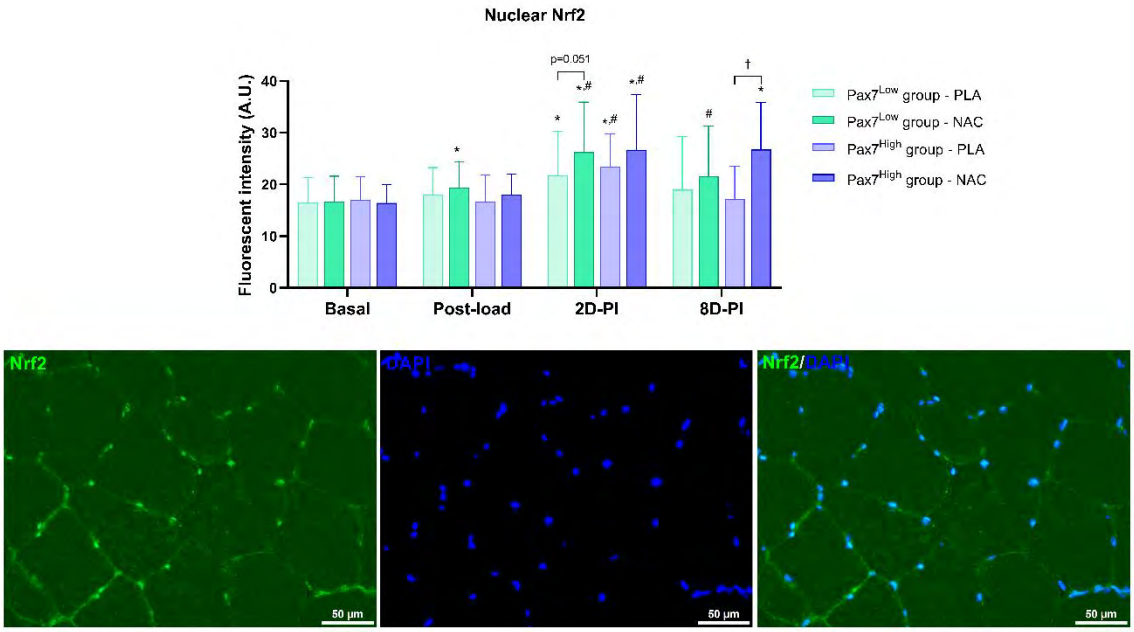

Figure 4.3.6.2. Changes in nuclear Nrf2 fluorescent intensity in low and high Pax7 groups. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between conditions, within group. Lower panels: Representative images of Nrf2, and DAPI immunofluorescent staining of muscle cross sections. $Pax7^{Low}$ group, n=14; $Pax7^{High}$ group, N=10.

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4.4. Muscle damage and regeneration

4.4.1 Skeletal muscle ultrastructural morphology

Qualitative analysis of skeletal muscle ultrastructural morphology revealed that at basal state, muscle fiber z-lines appear in their normal morphological status. Moreover, intermyofibrillar mitochondria are also evident between the myofibrils. Following EIMD, moderate z-line streaming is evident as early as 2D-PI and severe z-line streaming is noted at 8D-PI (**Figure 4.4.1.1**). Following injury, skeletal muscle stem cells (satellite cells) are activated an assist in muscle repair. **Figure 4.4.1.2** depicts a satellite cell located between the sarcolemma and basement membrane of a muscle fiber at 8D-PI.

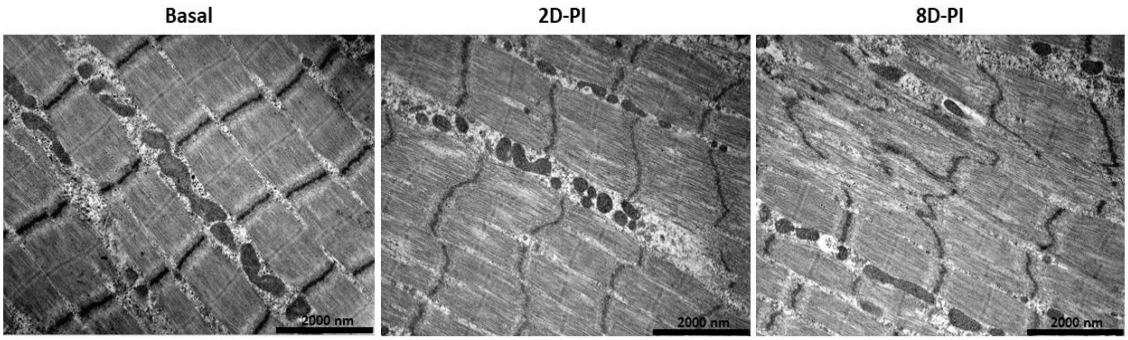

Figure 4.4.1.1. Representative images of transmission electron microscopy (TEM) analyses of skeletal muscle ultrathin sections.

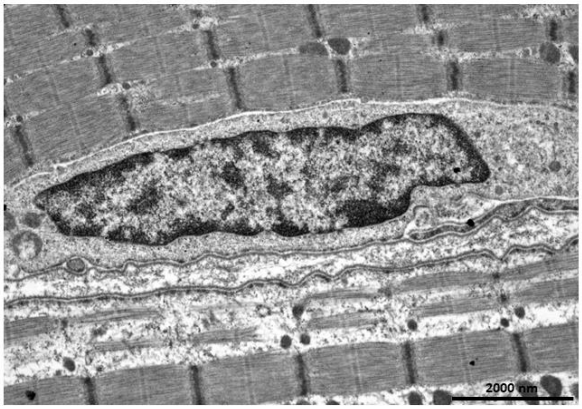

Figure 4.4.1.2. Representative images of transmission electron microscopy (TEM) analyses depicting a skeletal muscle stem cell (satellite cell) located between the sarcolemma and basement membrane of a muscle.

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4.4.2 Creatine kinase (CK) activity

Total sample (**Figure 4.4.2.1**): CK increased at 2-days (PLA: $p<0.001$; NAC: $p<0.001$) and 8-days (PLA: $p=0.004$; NAC: $p<0.001$) in both trials, with NAC elicited a lower CK elevation at 2-days post-injury ($p<0.001$). No changes were noted at post-load in both trials.

Low vs. High Pax7 groups (**Figure 4.4.2.2**): In pax7^{Low} group, CK increased only at 2-days in PLA (21-fold; $p<0.001$) and throughout recovery in NAC (2-days: 16.5-fold; $p<0.001$; 8-days: 3.4-fold; $p=0.018$). In pax7^{High} group, CK increased in both trials at 2-days (PLA: 17.6-fold; $p<0.001$; NAC: 11.5-fold; $p<0.001$) and 8-days (PLA: 7.9-fold; $p=0.047$; NAC: 5.5-fold; $p=0.001$) post-injury. NAC exhibited a lower raise in CK activity at 2-days in both groups compared to PLA (pax7^{Low}: $p=0.003$; pax7^{High}: $p=0.028$). No changes were noted at post-load in both groups and trials.

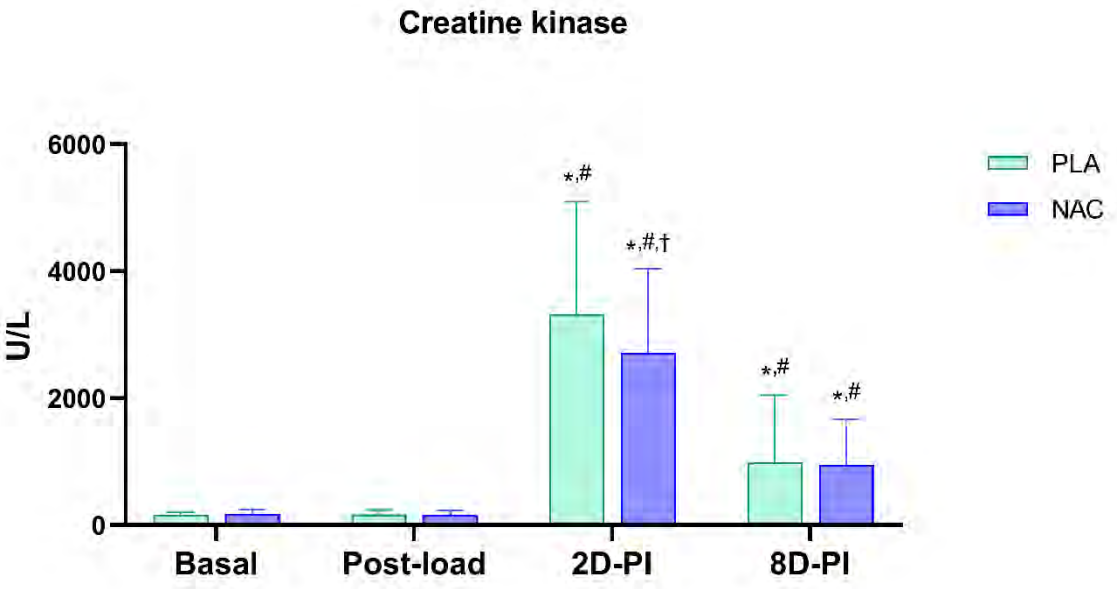

Figure 4.4.2.1. Changes in creatine kinase activity in total sample. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$; †Difference between trials, $p<0.05$. Total sample n=24.

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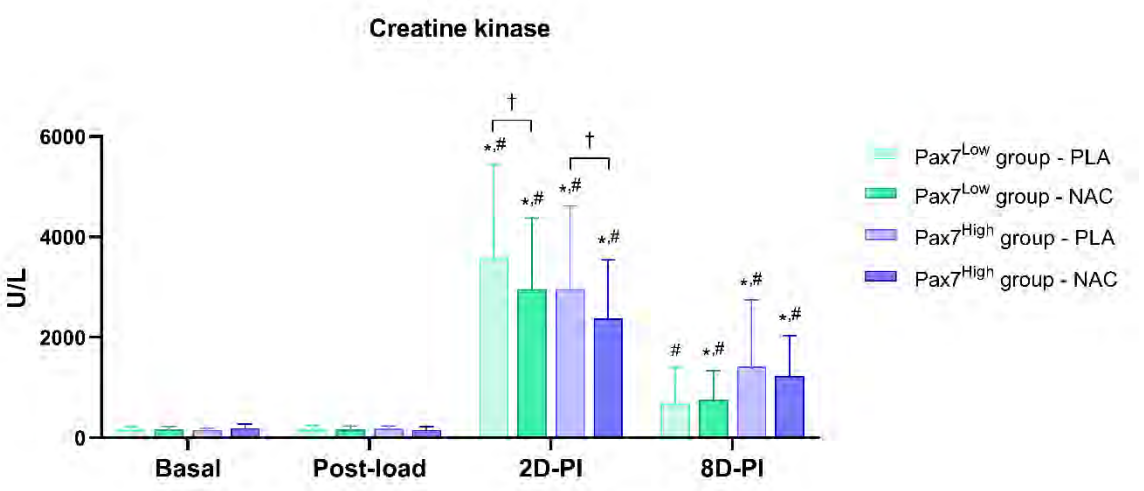

Figure 4.4.2.2. Changes in creatine kinase activity in low and high Pax7 groups. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between trials, within group, p<0.05. Pax7^{Low} group, n=14; Pax7^{High} group, N=10.

4.4.3 Delayed onset of muscle soreness (DOMS)

Total sample (**Figure 4.4.3.1**): DOMS increased at 2- (p<0.001) and 8-days (p<0.001) in PLA and only at 2-days in NAC (p<0.001) compared to basal, with NAC eliciting a lower increase in DOMS at 2- (p<0.001) and 8-days (p=0.001) post-injury. No changes were detected at post-load in both trials.

Low vs. High Pax7 groups (**Figure 4.4.3.2**): In both groups, DOMS increased in PLA at 2-days (pax7^{Low}: 7.4-fold, p<0.001; pax7^{High}: 6.5-fold, p<0.001) and 8-days (pax7^{Low}: 1.6-fold, p<0.001; pax7^{High}: 1.1-fold, p=0.006), while in NAC, DOMS increased only at 2-days in both groups (pax7^{Low}: 6.1-fold, p<0.001; pax7^{High}: 5.2-fold, p<0.001) compared to basal. NAC elicited a lower increase in DOMS in both groups at 2-days (pax7^{Low}: p=0.004; pax7^{High}: p=0.012) and at 8-days only in pax7^{Low} group (p=0.001). At 2-days post-injury, pax7^{High} group exhibited a lower increase in DOMS compared to pax7^{Low} group in PLA (p=0.028). No changes were detected at post-load in both trials.

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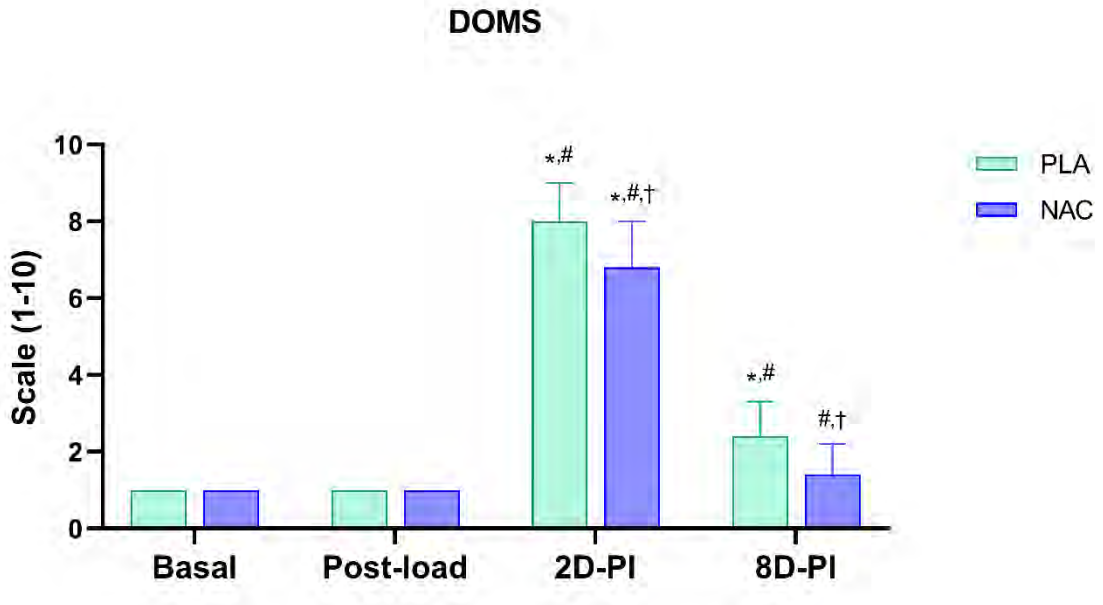

Figure 4.4.3.1. Changes in DOMS in total sample. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between trials, p<0.05. Total sample n=24.

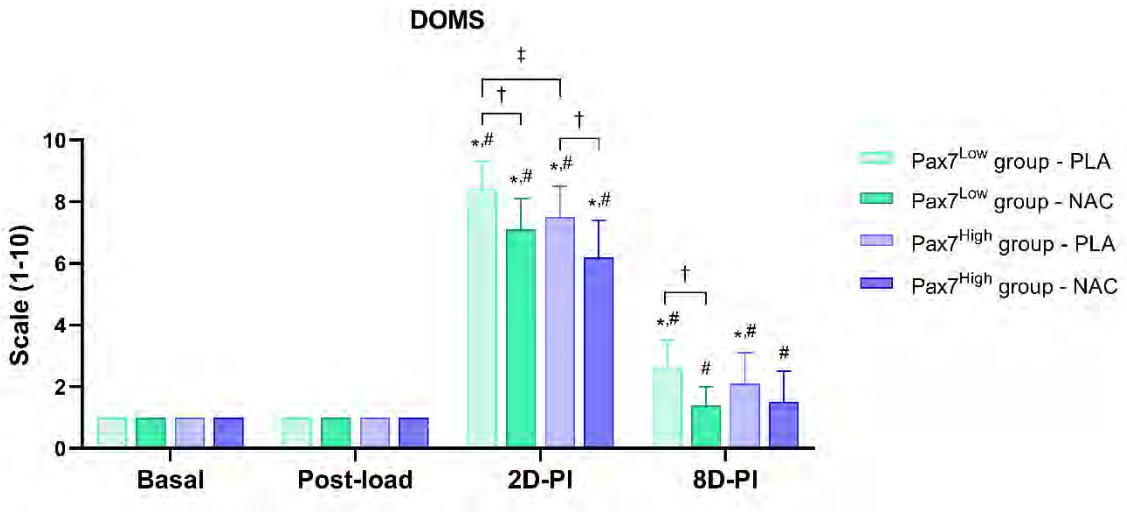

Figure 4.4.3.2. Changes in DOMS in low and high Pax7 groups. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between trials, within group, p<0.05. ‡Difference between groups, within trial, p<0.05. $Pax7^{Low}$ group, n=14; $Pax7^{High}$ group, N=10.

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4.4.4 Muscle cross sectional area (CSA)

Total sample (**Figure 4.4.4.1**): Muscle CSA decreased in both trials at 2-days (PLA: $p<0.001$; NAC: $p=0.008$) and 8-days (PLA: $p=0.010$; NAC: $p=0.003$) post-injury compared to basal. No between groups and trial differences were detected. No differences were noted at post-load

Low vs. High Pax7 groups (**Figure 4.4.4.2**): In pax7^{Low} group, muscle CSA decreased at 2-days in PLA (-12%, $p=0.013$) and at 8-days in both trials (PLA: -13.4%, $p=0.010$; NAC: -10.3%, $p=0.026$) compared to basal. In pax7^{High} group, muscle CSA decreased in both trials only at 2-days post-injury compared to basal (PLA: -12.5%, $p=0.011$; NAC: -9.8%, $p=0.043$). Muscle CSA was greater in pax7^{High} group compared to pax7^{Low} group in both trials at basal (PLA: $p=0.019$; NAC: $p=0.012$) and post-load state (PLA: $p=0.007$; NAC: $p=0.003$). At 8-days, pax7^{Low} group exhibited a more pronounced decrease in muscle CSA compared to pax7^{High} group in NAC ($p=0.030$).

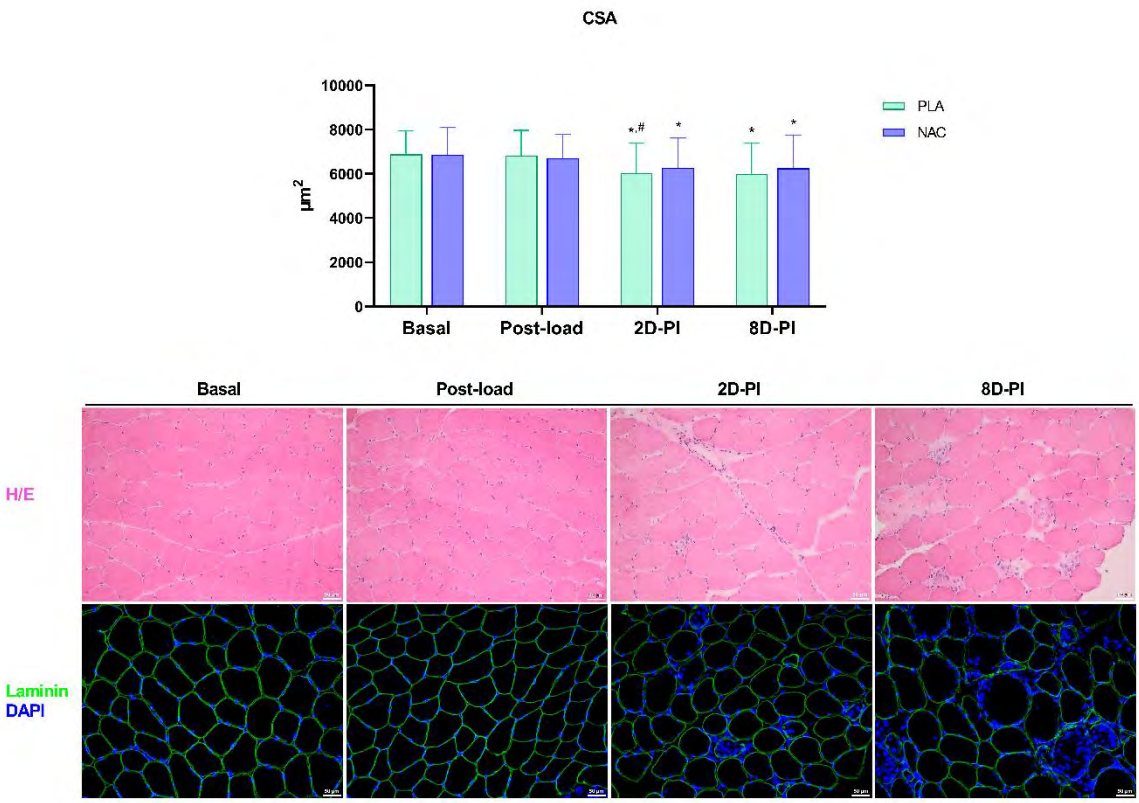

Figure 4.4.4.1. Changes in muscle CSA in total sample. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$. Lower panels: Representative images of H/E and laminin/DAPI immunofluorescent staining of muscle cross sections at basal, post-load, 2- and 8D-PI. At 2- and 8D-PI samples show progressively increased levels of myofiber damage. Total sample n=24.

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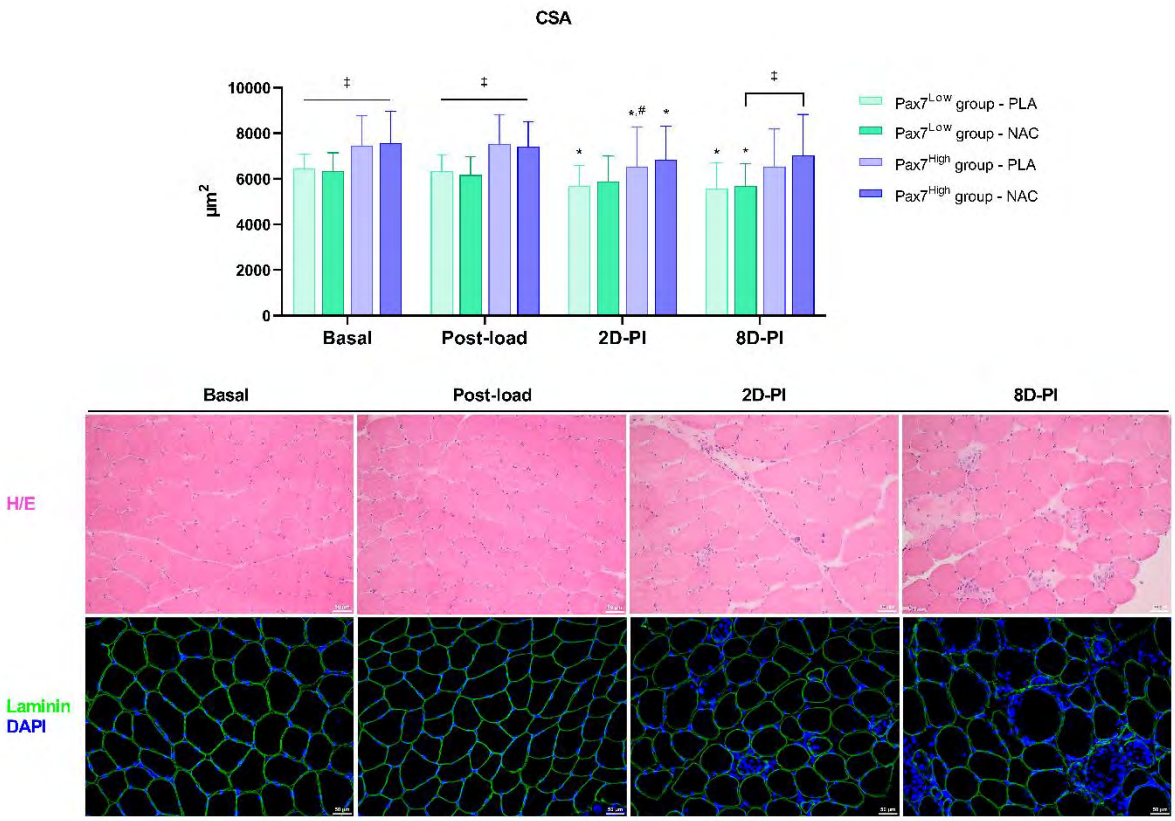

Figure 4.4.4.2. Changes in muscle CSA in low and high Pax7 groups. *Difference with basal, p<0.05; # Difference with previous time-point, p<0.05; ‡Difference between groups, within trial, p<0.05. Lower panels: Representative images of H/E and laminin/DAPI immunofluorescent staining of muscle cross sections at basal, post-load, 2- and 8D-PI. At 2- and 8D-PI samples show progressively increased levels of myofiber damage. $Pax7^{Low}$ group, n=14; $Pax7^{High}$ group, N=10.

4.4.5 Damaged fibers

Total sample (**Figure 4.4.5.1**): Damaged fibers proportion increased in both trials at 8-days (PLA: p<0.001; NAC: p<0.001) post-injury compared to basal with no between trial differences in any time-point. No changes were detected at post-load.

Low vs. High Pax7 groups (**Figure 4.4.5.2**): In both groups, damaged fibers proportion increased in both trials only at 8-days post-injury compared to basal ($pax7^{Low}$ group: PLA, p=0.006; NAC, p=0.013 / $pax7^{High}$ group: PLA, p=0.001; NAC, p<0.001). At 8-days, damaged fibers proportion was greater in $pax7^{High}$ group compared to $pax7^{Low}$ group in NAC (p=0.021).

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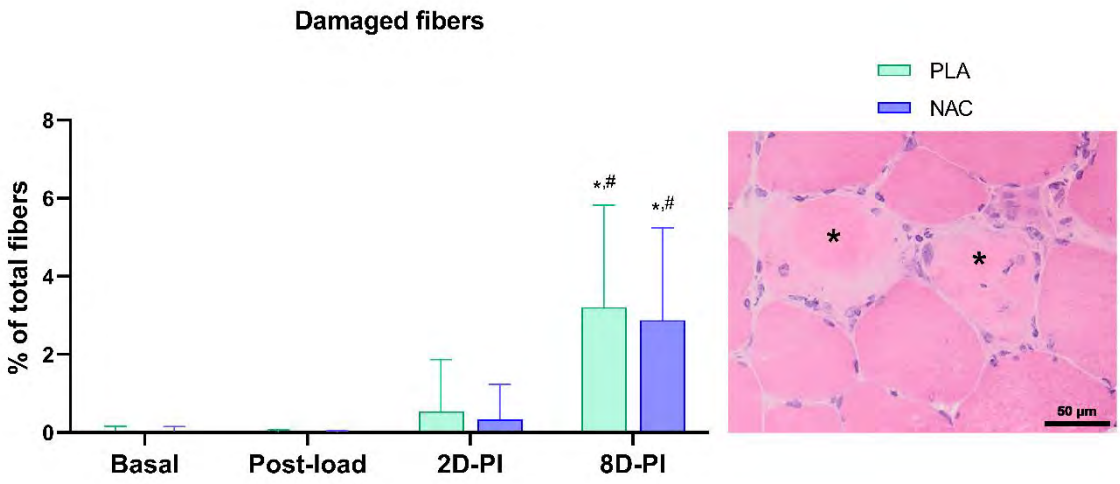

Figure 4.4.5.1. Changes in damaged fibers in total sample. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05. Right panel: Representative image of H/E staining for damaged fibers evaluation. Damaged muscle fibers show signs of sarcolemma disorganization (black asterisks). Total sample n=24.

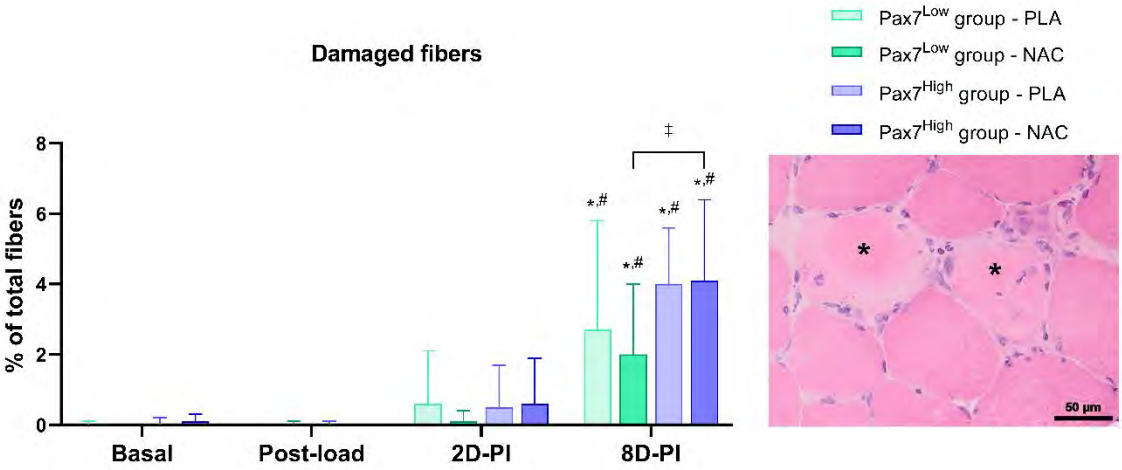

Figure 4.4.5.2. Changes in damaged fibers in low and high Pax7 groups. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; ‡Difference between groups, within trial, p<0.05. Right panel: Representative image of H/E staining for damaged fibers evaluation. Damaged muscle fibers show signs of sarcolemma disorganization (black asterisks). $Pax7^{Low}$ group, n=14; $Pax7^{High}$ group, N=10.

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4.4.6 Infiltrated fibers

Total sample (**Figure 4.4.6.1**): Infiltrated fibers proportion increased in both trials at 8-days (PLA: p=0.001; NAC: p<0.000) post-injury compared to basal with no between trial differences in any time-point. No changes were detected at post-load.

Low vs. High Pax7 groups (**Figure 4.4.6.2**): In both groups, infiltrated fibers proportion increased in both trials only at 8-days post-injury compared to basal (pax7^{Low} group: PLA, p=0.028; NAC, p=0.004 / pax7^{High} group: PLA, p=0.033; NAC, p<0.001). At 8-days, infiltrated fibers proportion was greater in pax7^{High} group compared to pax7^{Low} group in NAC (p=0.032).

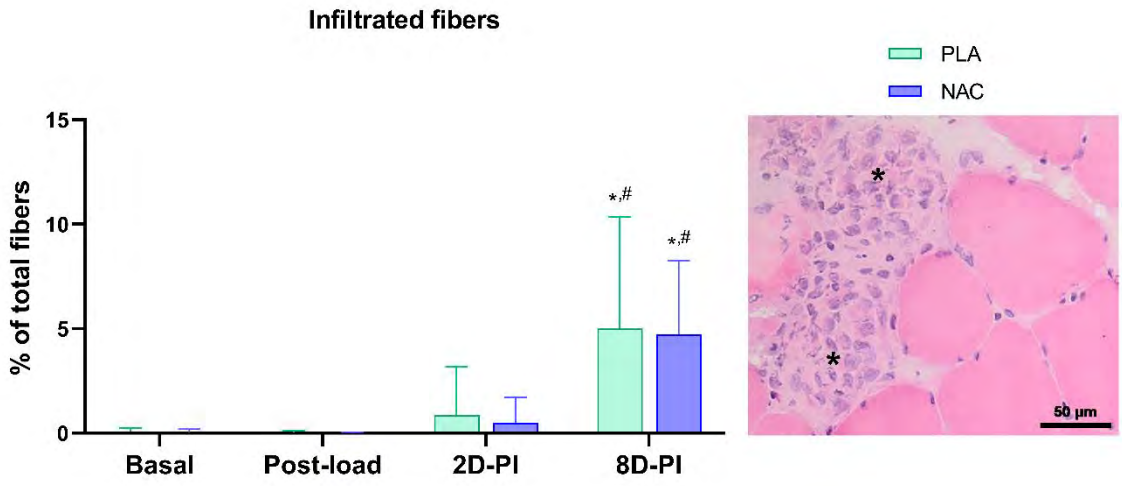

Figure 4.4.6.1. Changes in infiltrated fibers in total sample. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05. Right panel: Representative image of H/E staining for infiltrated fibers evaluation (black asterisks). Total sample n=24.

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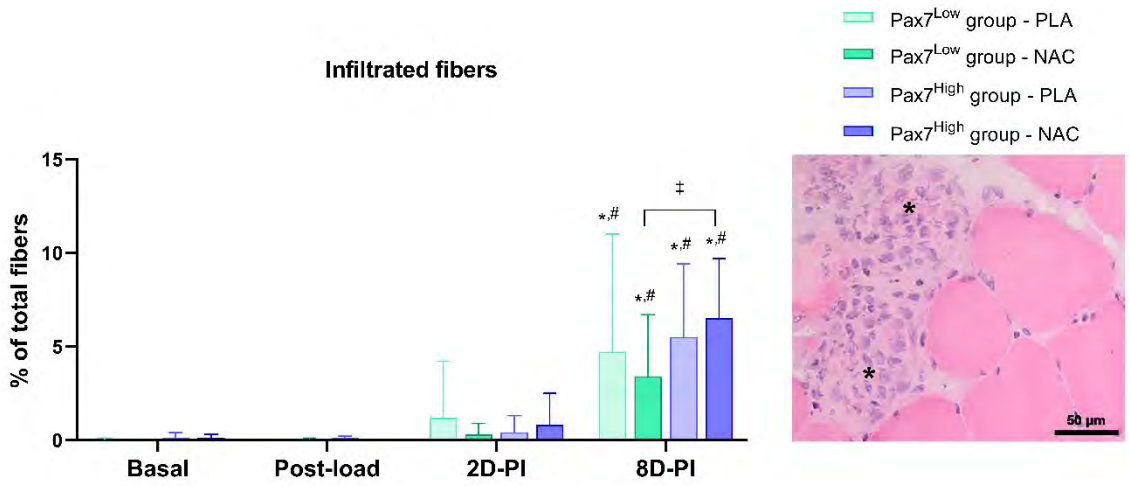

Figure 4.4.6.2. Changes in infiltrated fibers in low and high Pax7 groups. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; ‡Difference between groups, within trial, p<0.05. Right panel: Representative image of H/E staining for infiltrated fibers evaluation (black asterisks). Pax7^{Low} group, n=14; Pax7^{High} group, N=10.

4.4.7 Centrally nucleated fibers

Total sample (**Figure 4.4.7.1**): Centrally nucleated fibers proportion increased at 2-days (p=0.034) and 8-days (p<0.001) in PLA and only at 8-days in NAC (p<0.001). No between groups and trial differences were detected. No differences were noted at post-load.

Low vs. High Pax7 groups (**Figure 4.4.7.2**): In both groups, centrally nucleated fibers proportion increased in both trials only at 8-days post-injury compared to basal (pax7^{Low} group: PLA, p<0.001; NAC, p<0.001 / pax7^{High} group: PLA, p<0.001; NAC, p=0.033). At 8-days, centrally nucleated fibers proportion was greater in pax7^{Low} group compared to pax7^{High} group in NAC (p=0.028).

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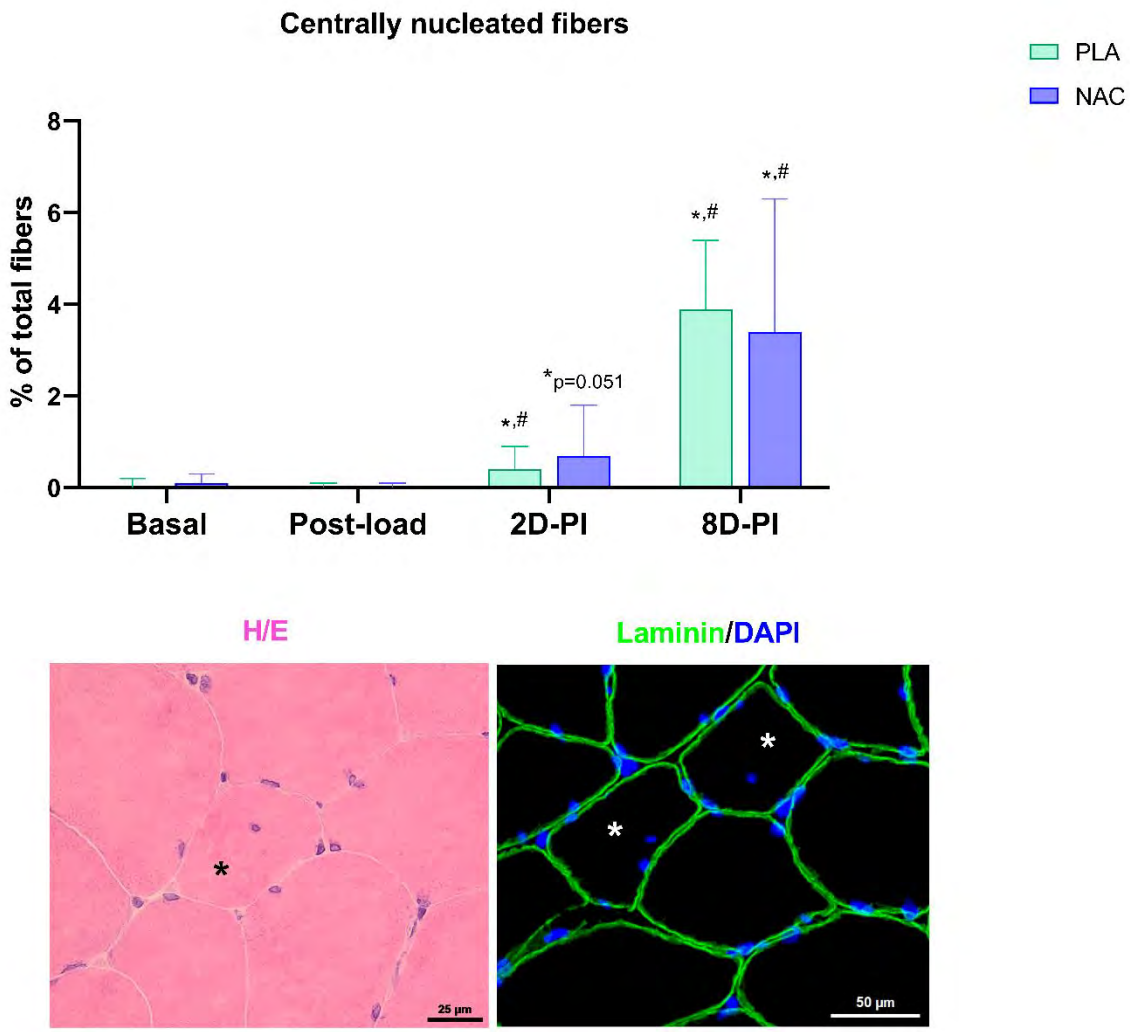

Figure 4.4.7.1. Changes in centrally nucleated fibers in total sample. *Difference with basal, $p<0.05$; # Difference with previous time-point, $p<0.05$. Right panel: Representative image of H/E and laminin/DAPI immunofluorescent staining for centrally nucleated fibers evaluation (black and white asterisks). Total sample n=24.

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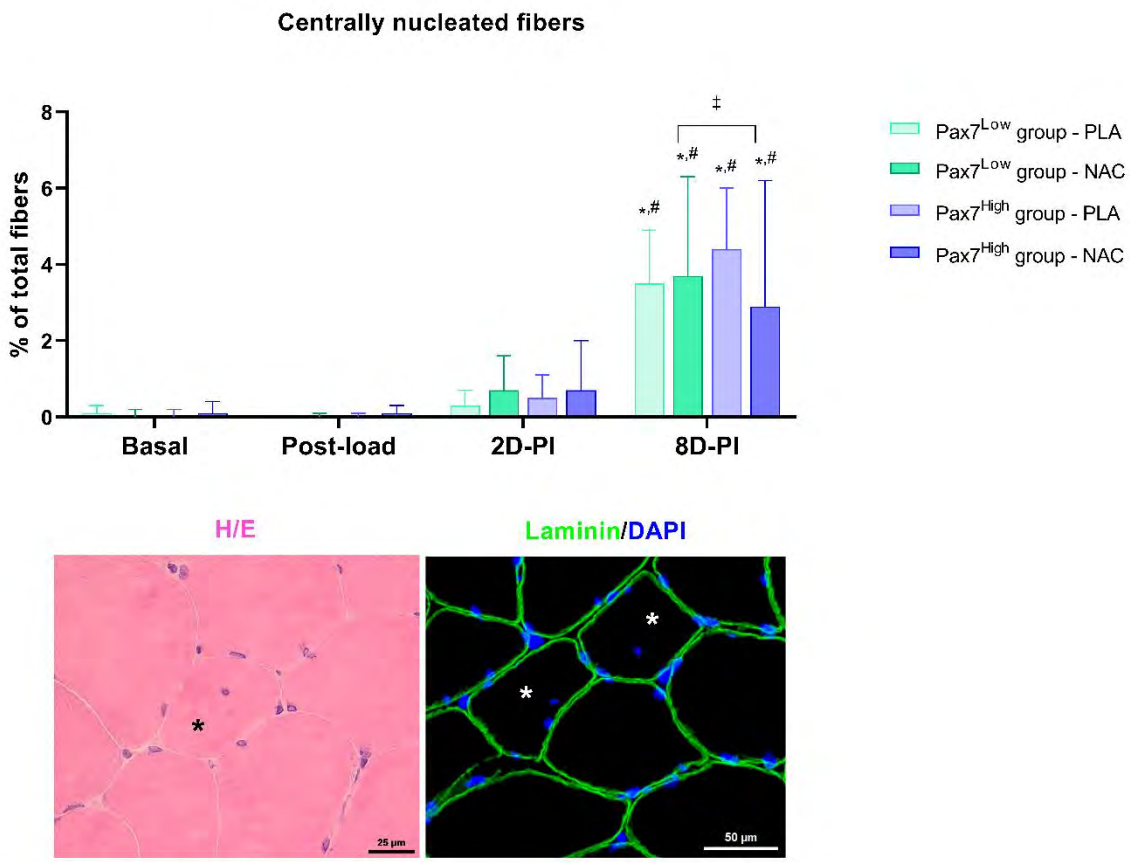

Figure 4.4.7.2. Changes in centrally nucleated fibers in low and high Pax7 groups. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$; ‡Difference between groups, within trial, $p<0.05$. Right panel: Representative image of H/E and laminin/DAPI immunofluorescent staining for centrally nucleated fibers evaluation (black and white asterisks). $Pax7^{Low}$ group, n=14; $Pax7^{High}$ group, N=10.

4.4.8 MHCe/n+ fibers

<u>Total sample (**Figure 4.4.8.1**):</u> MHCe/n+ fibers increased at 8-days post-injury compared to basal, only in PLA ($p<0.001$). No between groups and trial differences were noted. No differences were detected at post-load.

<u>Low vs. High Pax7 groups (**Figure 4.4.8.2**):</u> MHCe/n+ fibers increased at 8-days post-injury compared to basal, only in $pax7^{High}$ group in PLA ($p<0.001$). At 8-days, $pax7^{High}$ group exhibited a more pronounced increase in MHCe/n+ fibers compared to $pax7^{Low}$ group in PLA ($p=0.003$). No changes were detected at post-load.

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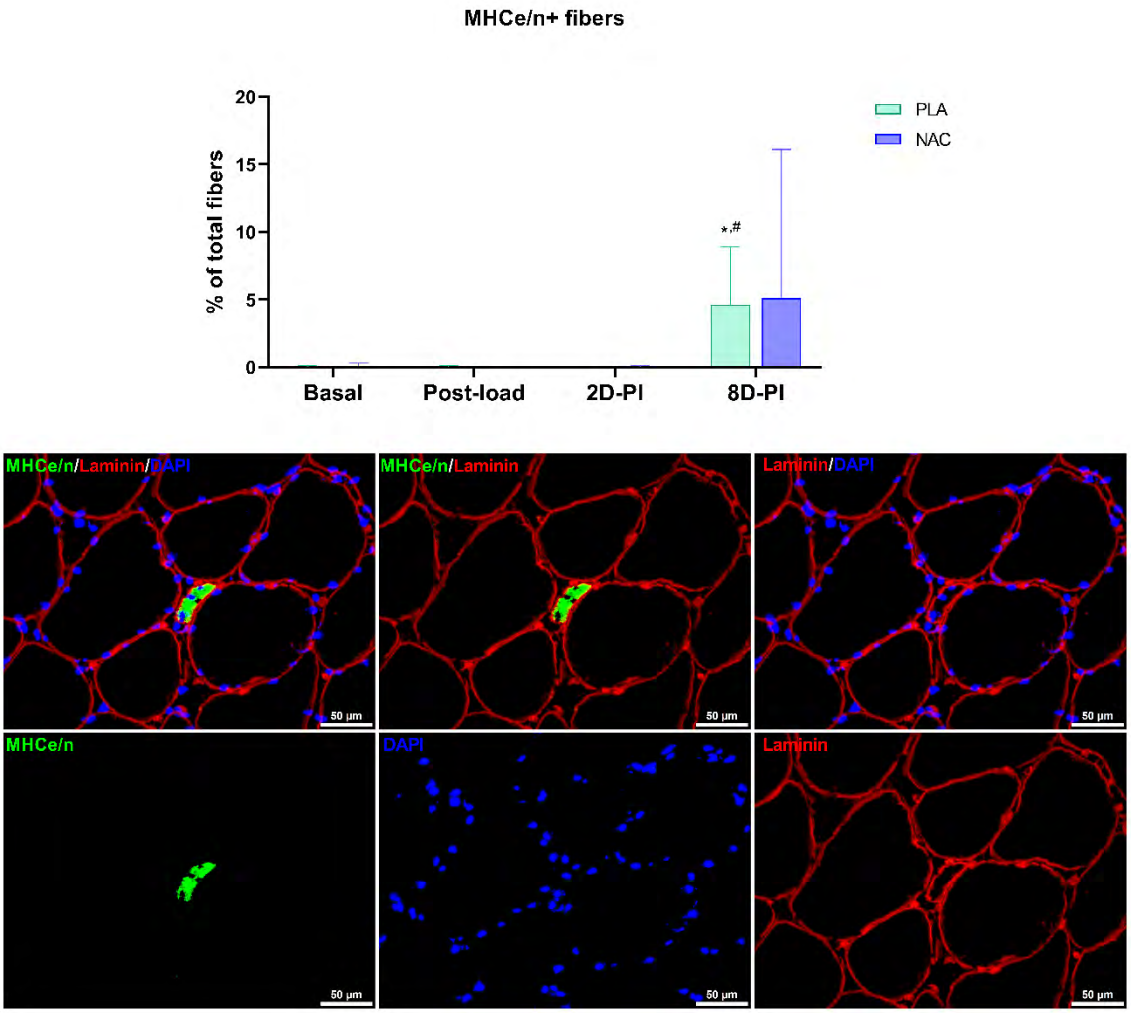

Figure 4.4.8.1. Changes in MHCe/n+ fibers in total sample. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$. Lower panels: Representative images of MHCe/n, laminin and DAPI staining of muscle cross sections. Total sample n=24.

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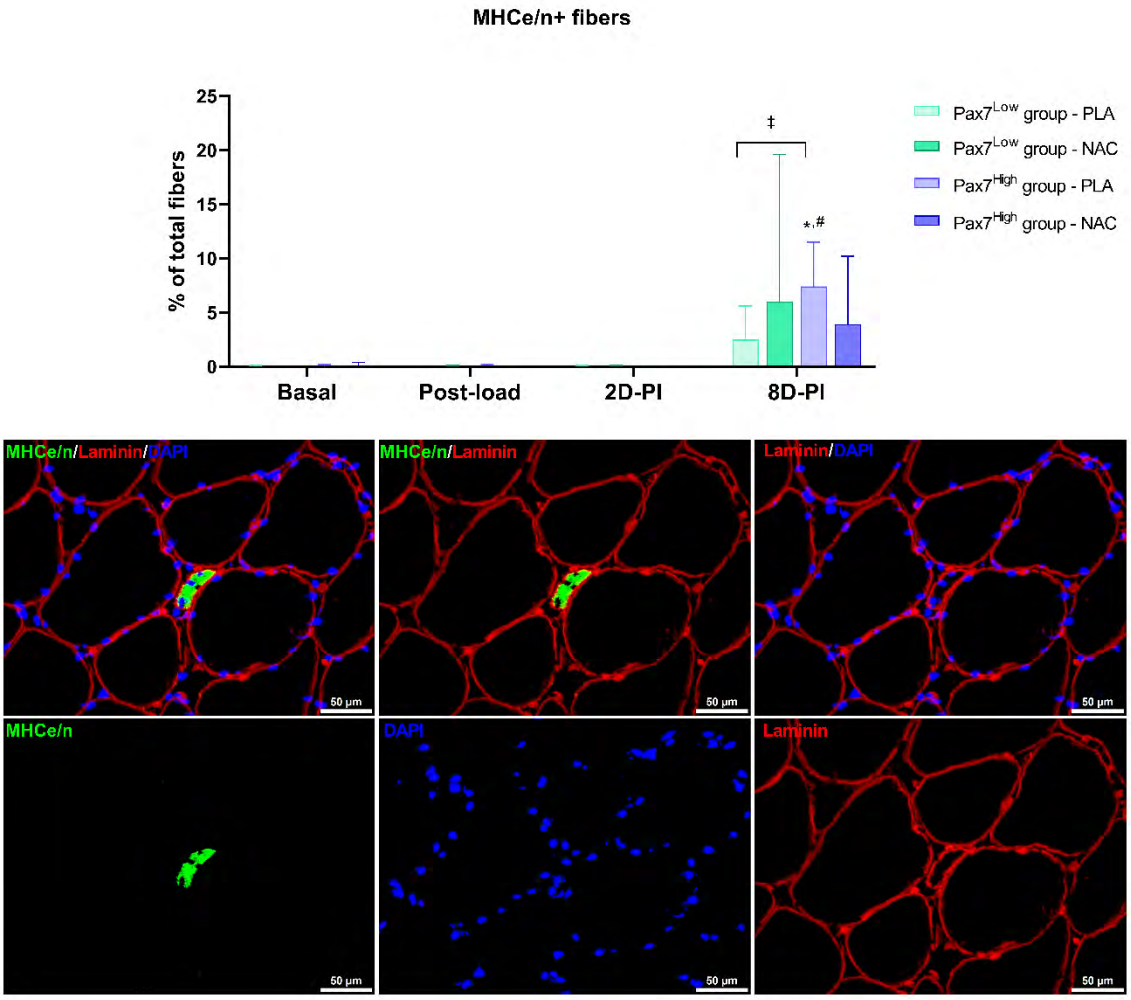

Figure 4.4.8.2. Changes in MHCe/n+ fibers in low and high Pax7 groups. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$; ‡Difference between groups, within trial, $p<0.05$. Lower panels: Representative images of MHCe/n, laminin and DAPI staining of muscle cross sections. $Pax7^{Low}$ group, n=14; $Pax7^{High}$ group, N=10.

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4.5. Satellite cells

4.5.1 Pax7+ cells per fiber vs. GSH/GSSG ratio at basal state (**Figure 4.5.1.1**)

A correlation and linear regression analysis revealed that basal pax7+ cells content per fiber was significantly correlated with basal levels of GSH/GSSG ratio (r=0.419; p=0.042).

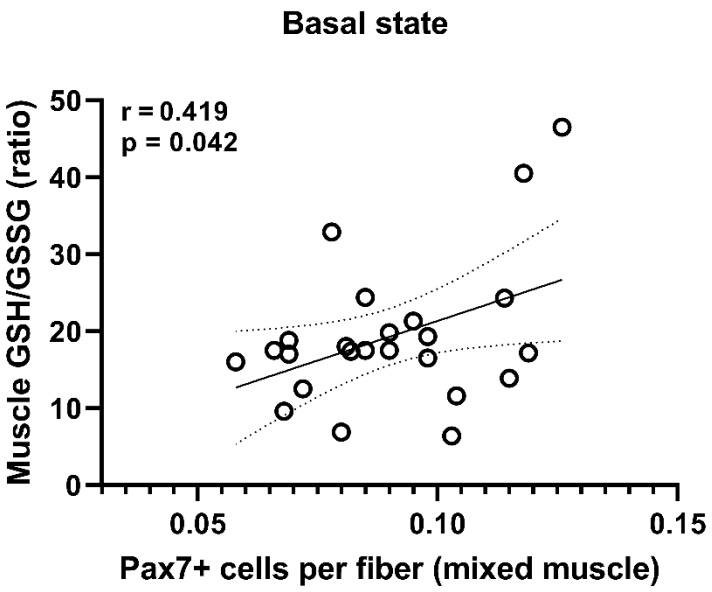

Figure 4.5.1.1 Correlation and linear regression analysis of pax7+ cells per fiber and GSH/GSSG ratio at basal state. Data are presented as individual values with 95% confidence bands of the best-fit line. Total sample n=24.

4.5.2 Pax7+ cells

Total sample (**Figure 4.5.2.1**): Pax7+cells increased at 2- and 8-days post-injury in both conditions (all p<0.001) compared to basal, with no between trial differences. No changes were detected at post-load.

High vs. Low Pax7 groups (**Figure 4.5.2.2**): At basal and post-load state, pax7+ cells were higher in pax7^{High} group compared to pax7^{Low} group in both trials (all p<0.001). In pax7^{Low} group, pax7+ cells increased in both trials at 2-days (PLA: +41.6%, p=0.001; NAC: +61.4%, p<0.001) and 8-days (PLA: +61.2%, p<0.001; NAC: +96.1%, p<0.001) post-injury compared to basal. In pax7^{High} group, pax7+cells increased at 2-days post-injury only in PLA (+38.2%, p<0.001) and at 8-days post-injury in both trials (PLA: +45.5%, p<0.001; NAC: +45.2%,

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p=0.011) compared to basal. Pax7+ cells were higher in pax7^{High} group compared to pax7^{Low} group in PLA at 2-days (p<0.001) and 8-days (p=0.015) post-injury.

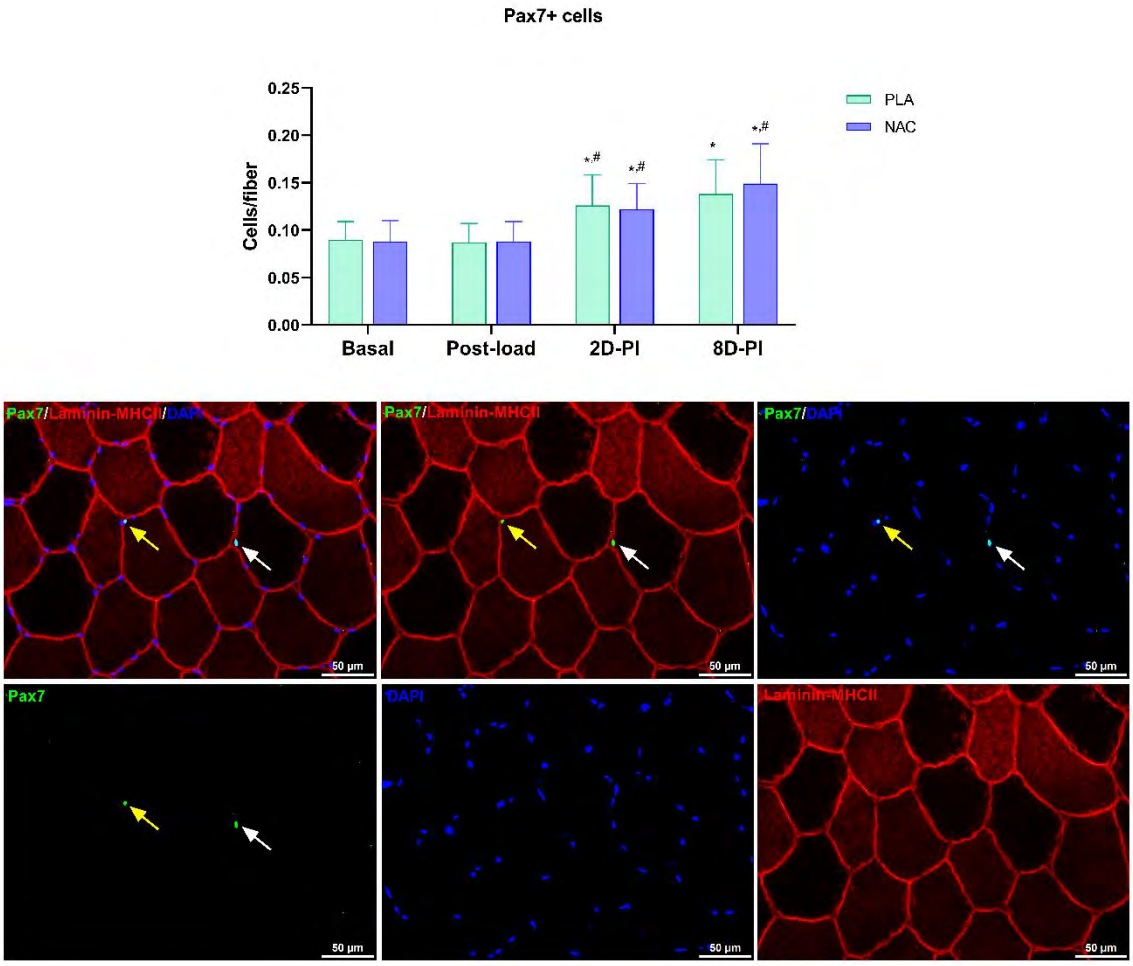

Figure 4.5.2.1. Changes in Pax7+ cells in total sample. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05. Lower panels: Representative images of Pax7, laminin-MHCII and DAPI staining of muscle cross sections. White arrow pinpoints a type I-associated satellite cell and yellow arrow a type II-associated satellite cell. Total sample n=24.

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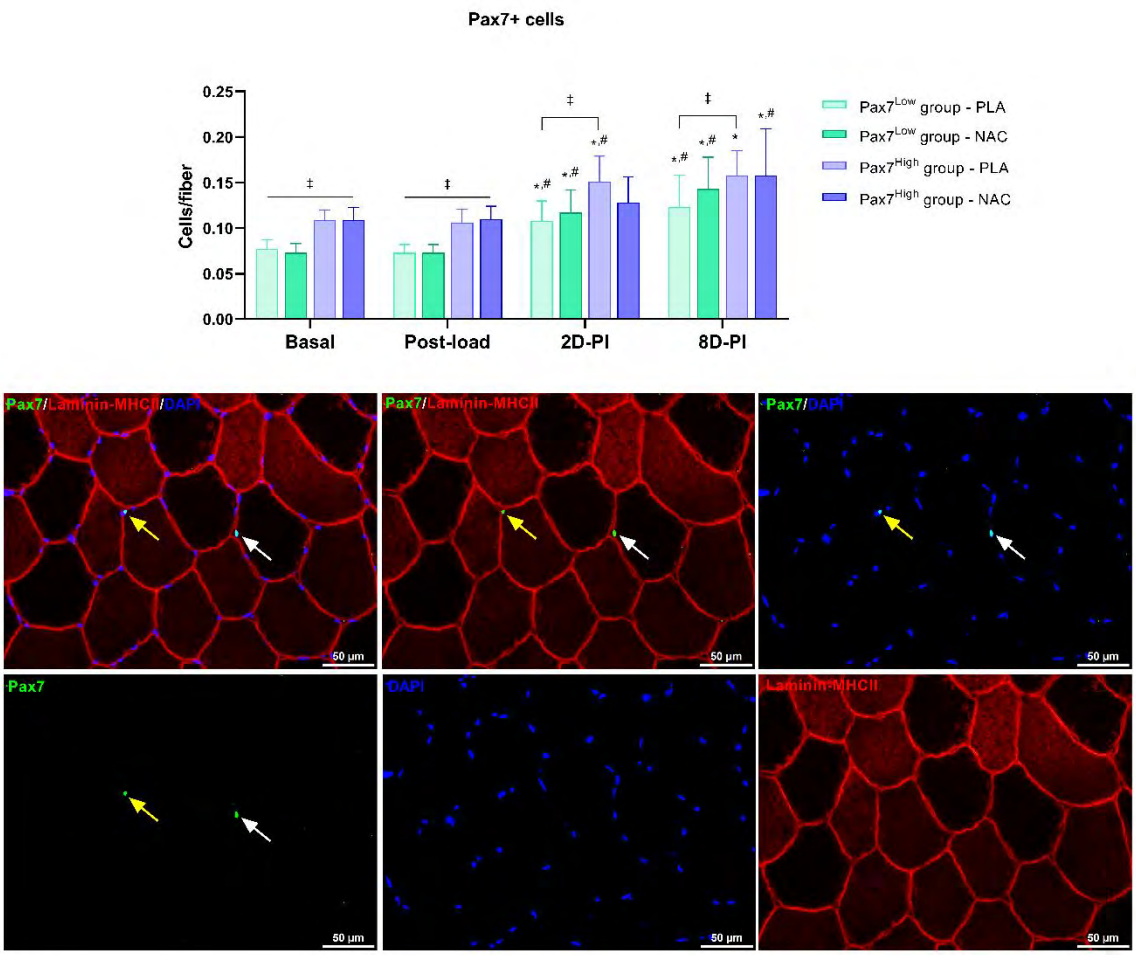

Figure 4.5.2.2. Changes in Pax7+ cells in low and high Pax7 groups. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$; ‡Difference between groups, within trial, $p<0.05$. Lower panels: Representative images of Pax7, laminin-MHCII and DAPI staining of muscle cross sections. White arrow pinpoints a type I-associated satellite cell and yellow arrow a type II-associated satellite cell. $Pax7^{Low}$ group, n=14; $Pax7^{High}$ group, N=10.

4.5.3 MyoD+ cells

Total sample (**Figure 4.5.3.1**): MyoD+ cells increased at 2-days post-injury compared to basal in both trials (all $p<0.001$) with no between trial differences. No changes were detected at post-load.

Low vs. High Pax7 groups (**Figure 4.5.3.2**): At basal and post-load state, MyoD+ cells proportion was higher in $pax7^{High}$ group compared to $pax7^{Low}$ group in both trials (basal: PLA, $p<0.001$; NAC, $p=0.004$ / post-load: PLA, $p<0.001$; NAC, $p=0.005$). In $pax7^{Low}$ group, MyoD+ cells increased at 2-days post-injury compared to basal in both trials (PLA: +284%,

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p<0.001; NAC: +352%, p<0.001) and remained elevated at 8-days post-injury only in NAC (+92.5%, p=0.036). In pax7^{High} group, MyoD+ cells increased only at 2-days post-injury compared to basal in both trials (PLA: +309%, p<0.001; +278%NAC: p<0.001). MyoD+ cells proportion was higher in pax7^{High} group compared to pax7^{Low} group in PLA at 2-days post-injury (p=0.012).

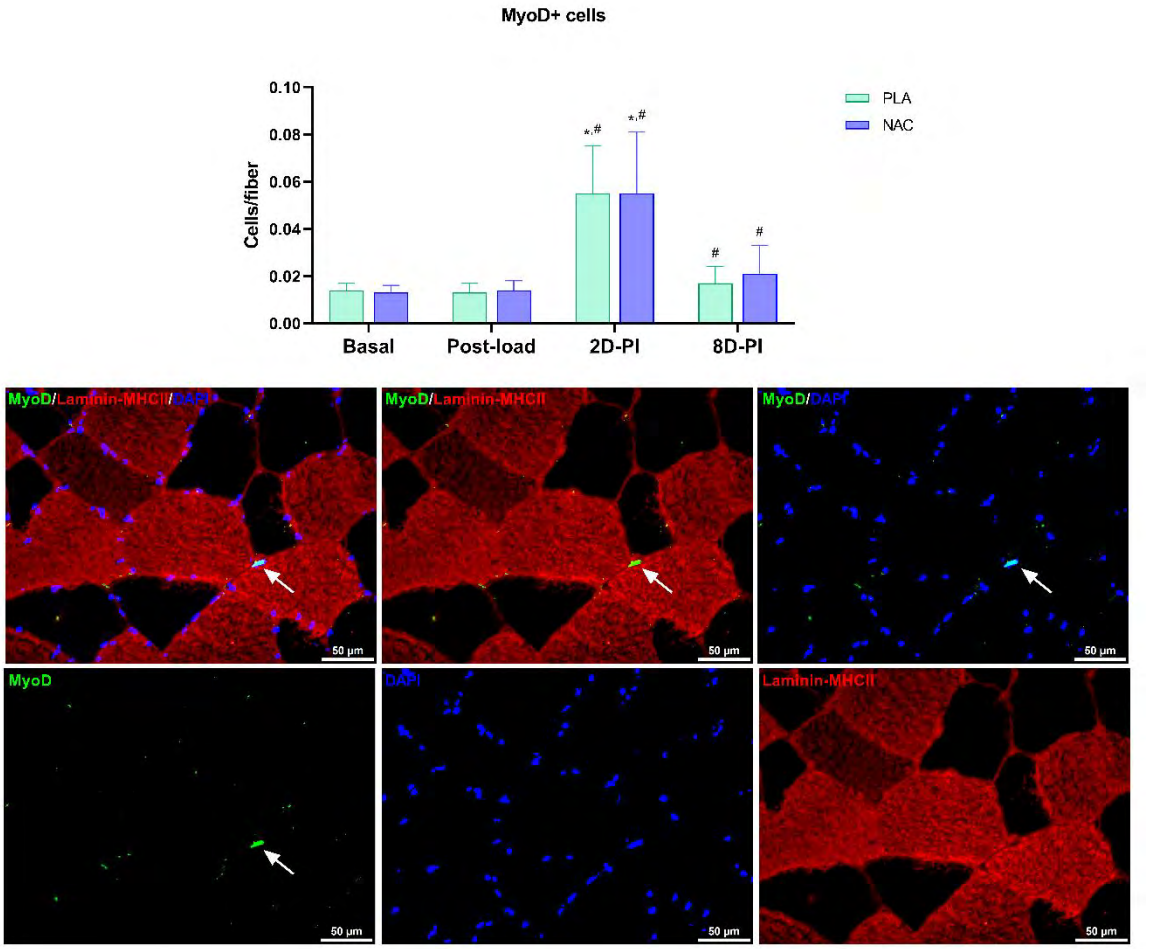

Figure 4.5.3.1. Changes in MyoD+ cells in total sample. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05. Lower panels: Representative images of MyoD, laminin-MHCII and DAPI staining of muscle cross sections. White arrow pinpoints a MyoD+ satellite cell. Total sample n=24.

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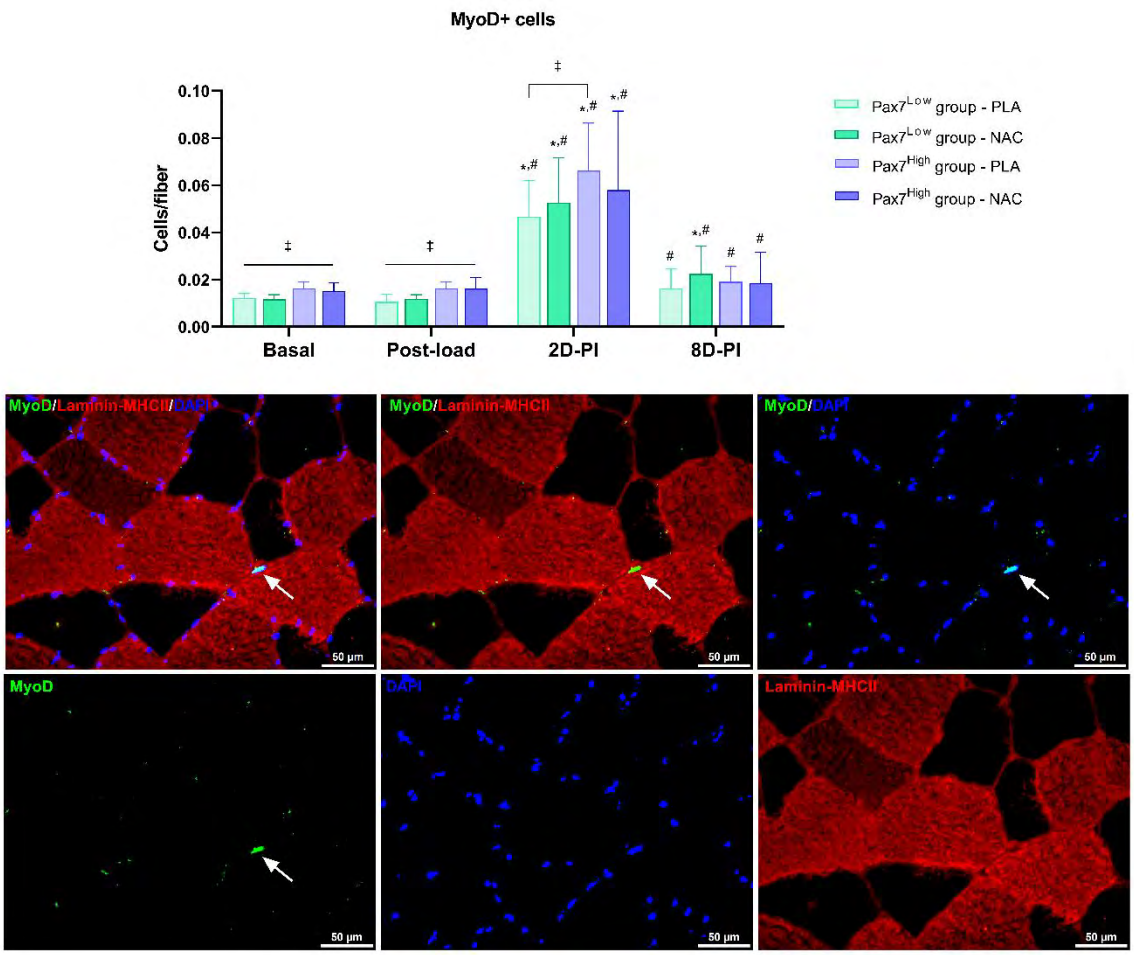

Figure 4.5.3.2. Changes in MyoD+ cells in low and high Pax7 groups. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$; ‡Difference between groups, within trial, $p<0.05$. Lower panels: Representative images of MyoD, laminin-MHCII and DAPI staining of muscle cross sections. White arrow pinpoints a MyoD+ satellite cell. Pax7^{Low} group, n=14; Pax7^{High} group, N=10.

4.5.4 Myogenin+ cells

Total sample (**Figure 4.5.4.1**): Myogenin+ cells increased at 8-days post-injury compared to basal only in PLA ($p=0.003$) with no between trial differences. No changes were detected at post-load and 2-days post-injury.

Low vs. High Pax7 groups (**Figure 4.5.4.2**): Myogenin+ cells increased at 8-days post-injury compared to basal only in pax7^{High} group in PLA (67-fold, $p=0.004$) with no between group or trial differences. No changes were detected at post-load and 2-days post-injury.

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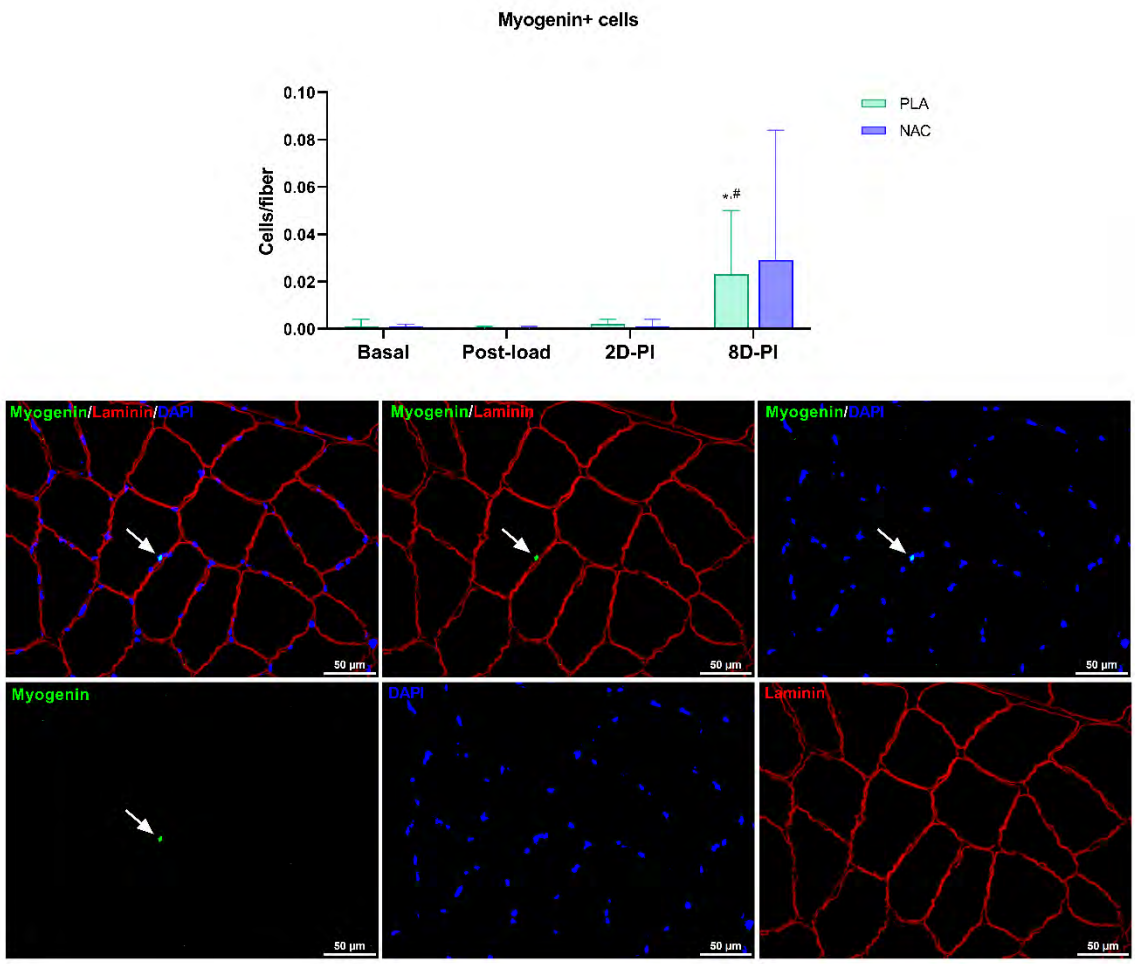

Figure 4.5.4.1. Changes in Myogenin+ cells in total sample. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$. Lower panels: Representative images of Myogenin, laminin-MHCII and DAPI staining of muscle cross sections. White arrow pinpoints a Myogenin+ satellite cell. Total sample n=24.

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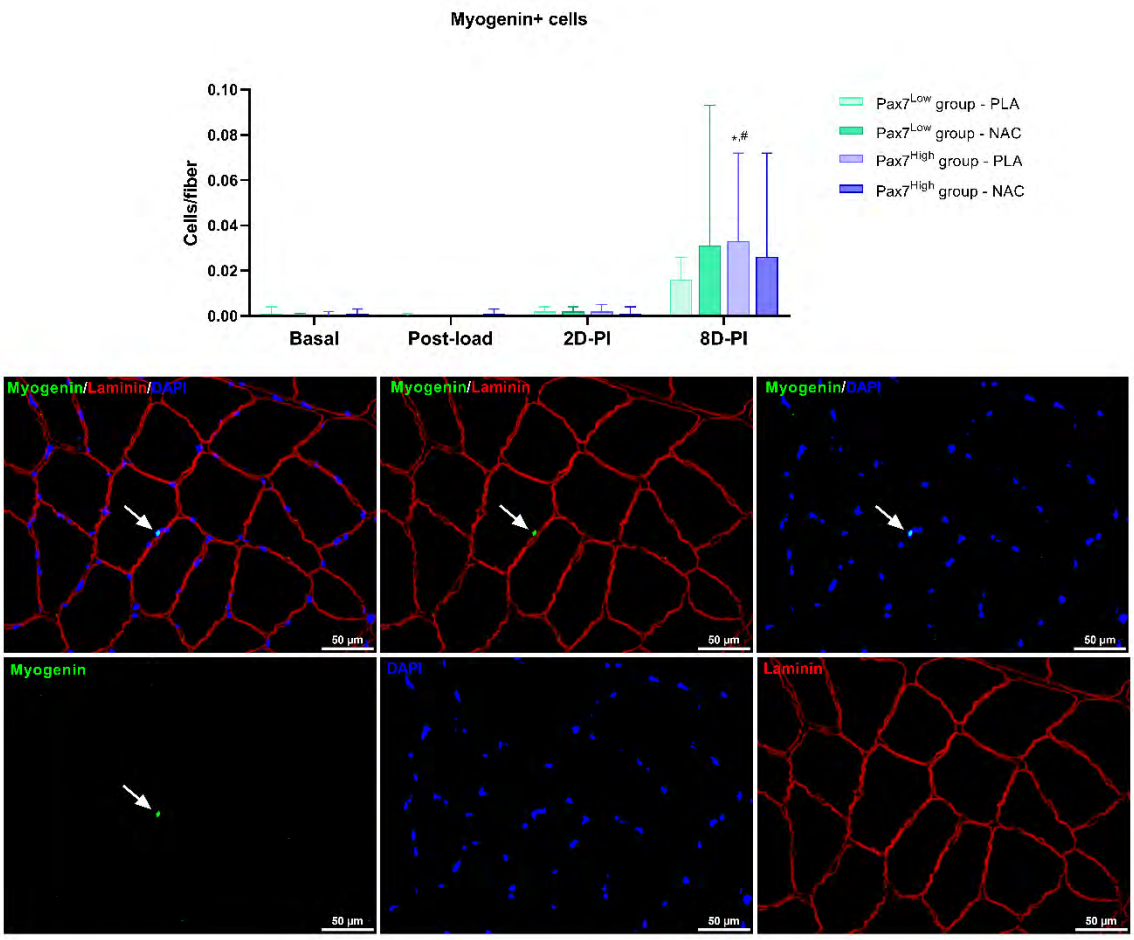

Figure 4.5.4.2. Changes in Myogenin+ cells in low and high Pax7 groups. *Difference with basal; #Difference with previous time-point. Lower panels: Representative images of Myogenin, laminin-MHCII and DAPI staining of muscle cross sections. White arrow pinpoints a Myogenin+ satellite cell. $Pax7^{Low}$ group, n=14; $Pax7^{High}$ group, N=10.

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4.6. Myogenic programming

4.6.1 MYF5 mRNA expression

Total sample (**Figure 4.6.1.1**): MYF5 mRNA expression increased at 2- (p=0.003) and 8-days (p=0.001) post-injury compared to basal only in PLA with no between trial differences. No changes were detected at post-load.

Low vs. High Pax7 groups (**Figure 4.6.1.2**): MYF5 mRNA expression increased at 2-days post-injury compared to basal only in pax7^{High} group in PLA (2.3-fold, p=0.019) and at 8-days post-injury only in pax7^{Low} group in PLA (2.2-fold, p=0.005) compared to basal with no between group or trial differences. No changes were detected at post-load and 2-days post-injury.

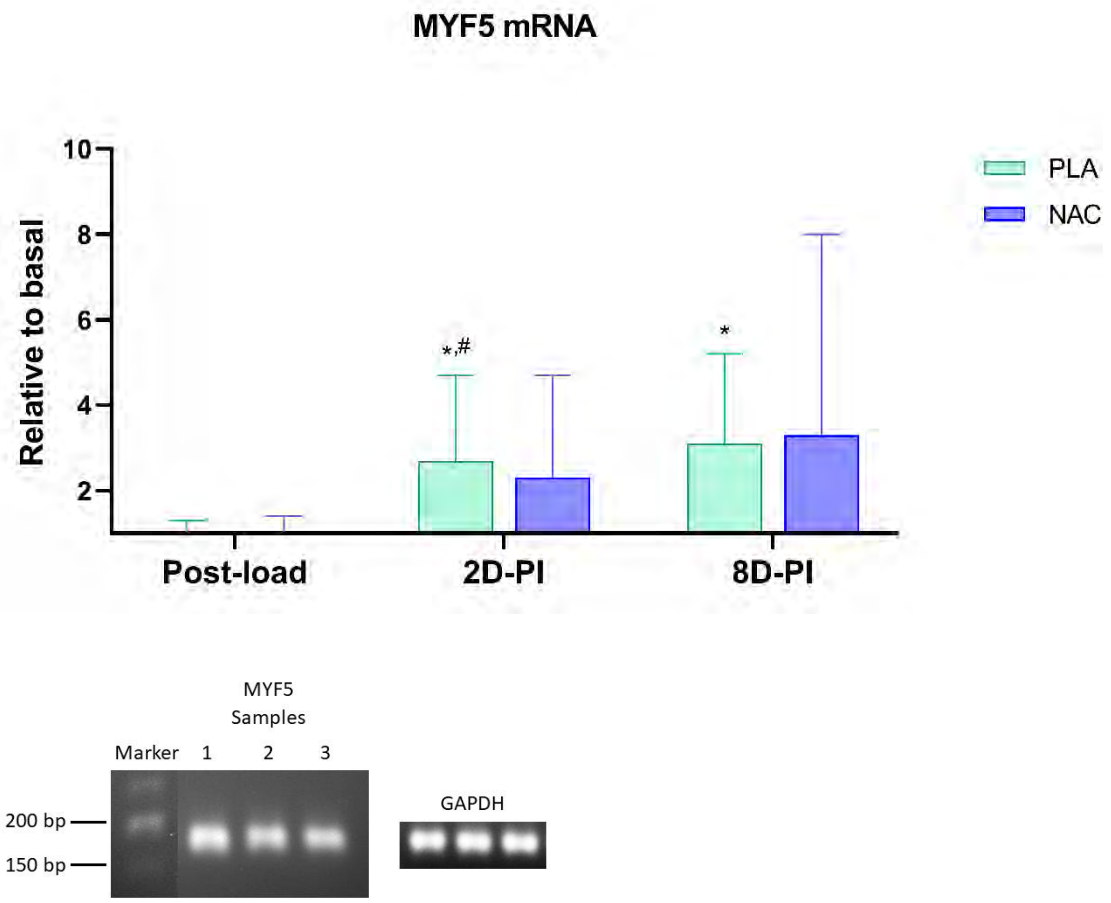

Figure 4.6.1.1. Changes in muscle MYF5 mRNA expression in total sample. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05. Lower panels: Representative images of agarose gel electrophoresis of PCR amplified products. Total sample n=24.

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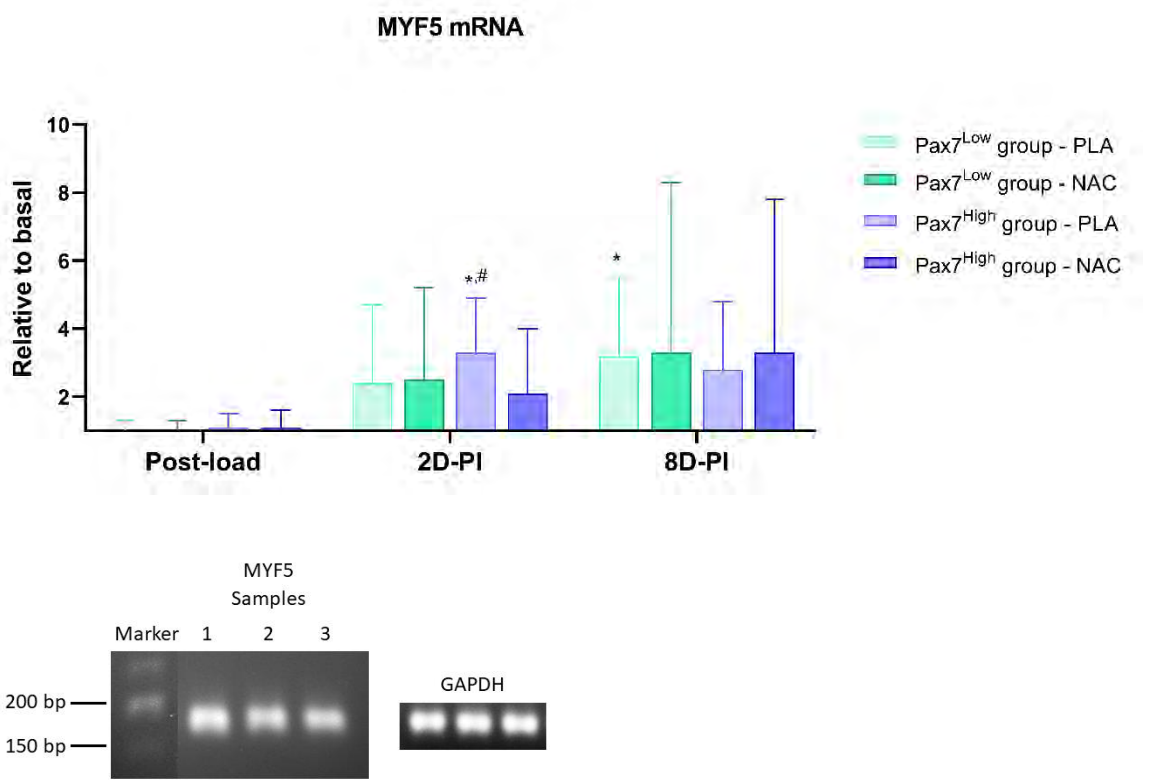

Figure 4.6.1.2. Changes in muscle MYF5 mRNA expression in low and high Pax7 groups. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05. Lower panels: Representative images of agarose gel electrophoresis of PCR amplified products. Pax7^{Low} group, n=14; Pax7^{High} group, N=10.

4.6.2 MYOD mRNA expression

Total sample (**Figure 4.6.2.1**): MYOD mRNA expression increased in both trials at 2-days (PLA: p=0.003; NAC: p=0.008) and 8-days (PLA: p=0.003; NAC: p=0.027) post-injury compared to basal with no between trials differences. No changes were noted at post-load.

Low vs. High Pax7 groups (**Figure 4.6.2.2**): In pax7^{Low} group, MYOD mRNA expression tended to increase at 2-days post-injury compared to basal in NAC (2.7-fold, p=0.070). In pax7^{High} group, MYOD mRNA expression increased at 8-days post-injury in PLA (0.9-fold%, p=0.021). MYOD mRNA expression tended to increase at 2-days post-injury compared to basal in PLA (2.3-fold, p=0.050) and at 8-days post-injury compared to basal in NAC (1.2-fold, p=0.053). No between group or trial differences were detected. No changes were noted at post-load.

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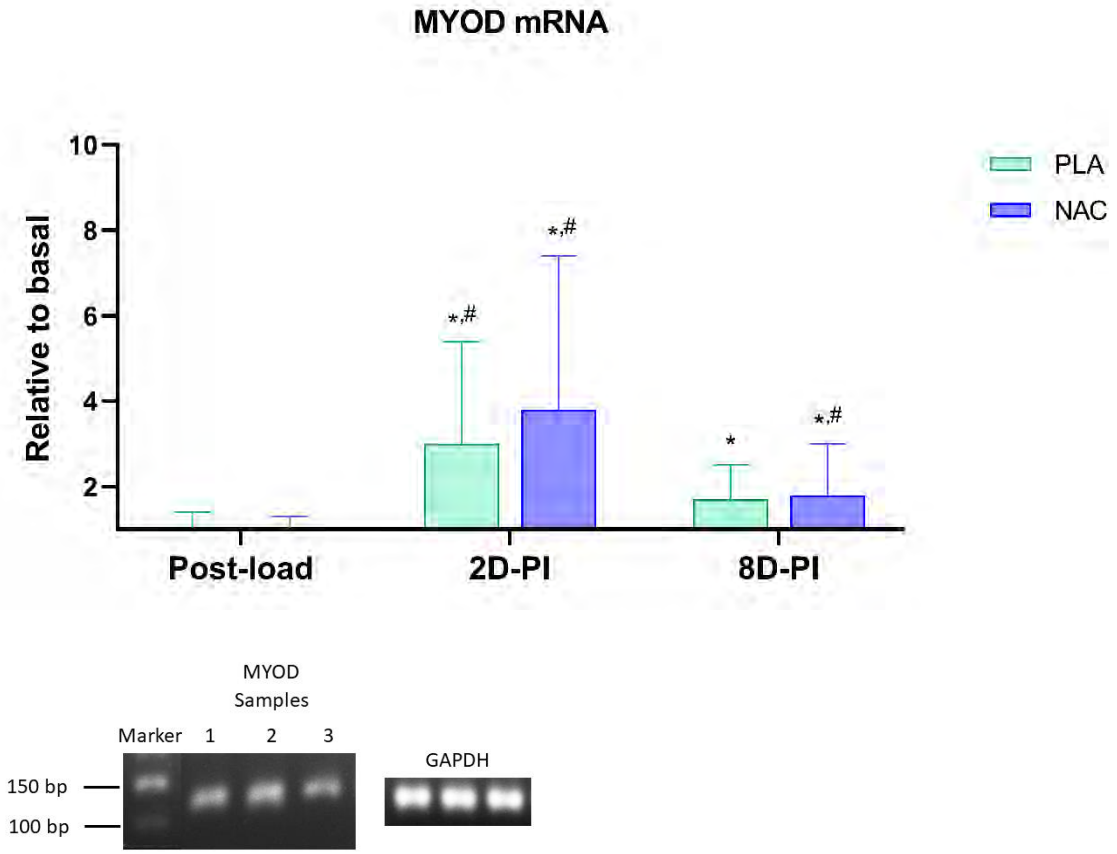

Figure 4.6.2.1. Changes in muscle MYOD mRNA expression in total sample. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$. Lower panels: Representative images of agarose gel electrophoresis of PCR amplified products. Total sample n=24.

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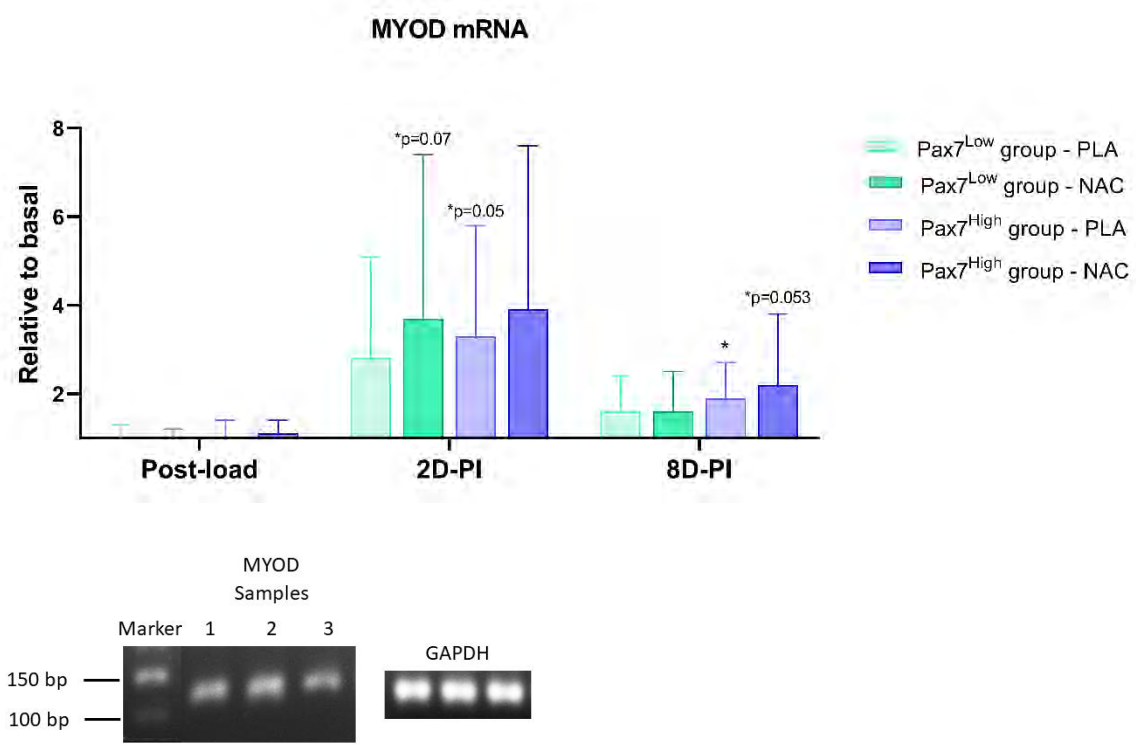

Figure 4.6.2.2. Changes in muscle MYOD mRNA expression in low and high Pax7 groups. Lower panels: Representative images of agarose gel electrophoresis of PCR amplified products. Pax7^{Low} group, n=14; Pax7^{High} group, N=10.

4.6.3 MYOGENIN mRNA expression

<u>Total sample (**Figure 4.6.3.1**):</u> MYOGENIN mRNA expression increased in both trials at 2-days (PLA: p=0.004; NAC: p=0.048) and at 8-days post-injury only in PLA (p<0.001) compared to basal, with no between trial differences. No changes were detected at post-load and 2-days post-injury.

<u>Low vs. High Pax7 groups (**Figure 4.6.3.2**):</u> In pax7^{Low} group, MYOGENIN mRNA expression increased only in PLA at 2-days (3.5-fold, p=0.036) and 8-days (0.8-fold, p=0.035) post-injury compared to basal. In pax7^{High} group, MYOGENIN mRNA expression increased at 8-days post-injury compared to basal only in PLA (1.5-fold, p=0.001). No between group or trial differences were detected. No changes were noted at post-load.

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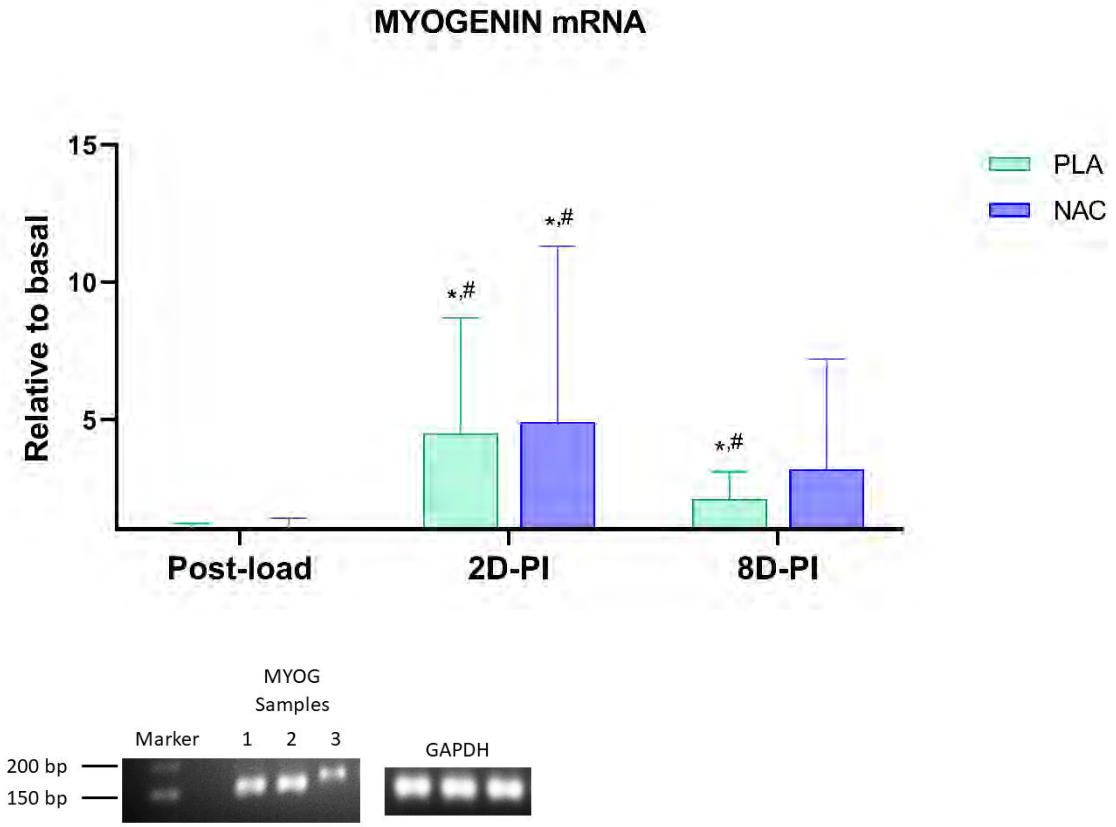

Figure 4.6.3.1. Changes in muscle MYOGENIN mRNA expression in total sample. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$. Lower panels: Representative images of agarose gel electrophoresis of PCR amplified products. Total sample n=24.

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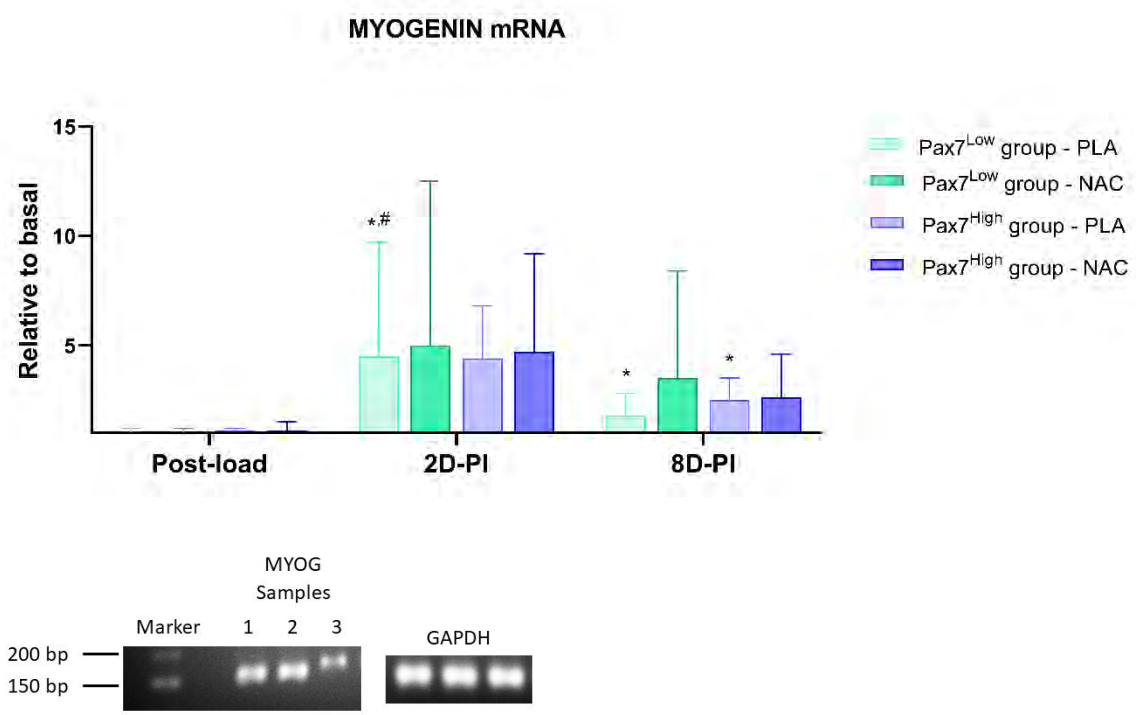

Figure 4.6.3.2. Changes in muscle MYOGENIN mRNA expression in low and high Pax7 groups. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$. Lower panels: Representative images of agarose gel electrophoresis of PCR amplified products. $Pax7^{Low}$ group, n=14; $Pax7^{High}$ group, N=10.

4.6.4 MRF4 mRNA expression

Total sample (**Figure 4.6.4.1**): MRF4 mRNA expression increased in both trials at 2-days (PLA: p=0.015; NAC: p=0.049) and 8-days (PLA: p=0.011; NAC: p<0.001) post-injury compared to basal with no between trials differences. No changes were noted at post-load.

Low vs. HighPax7 groups (**Figure 4.6.4.2**): In $pax7^{Low}$ group, MRF4 mRNA expression increased only in NAC at 2-days (1.1-fold, p=0.020) and 8-days (1.2-fold, p=0.010) post-injury compared to basal. In $pax7^{High}$ group, MRF4 mRNA expression increased at 8-days post-injury compared to basal in both trials (PLA: 2.3-fold, p=0.039; NAC: 1.3-fold, p=0.030). No between group or trial differences were detected. No changes were noted at post-load.

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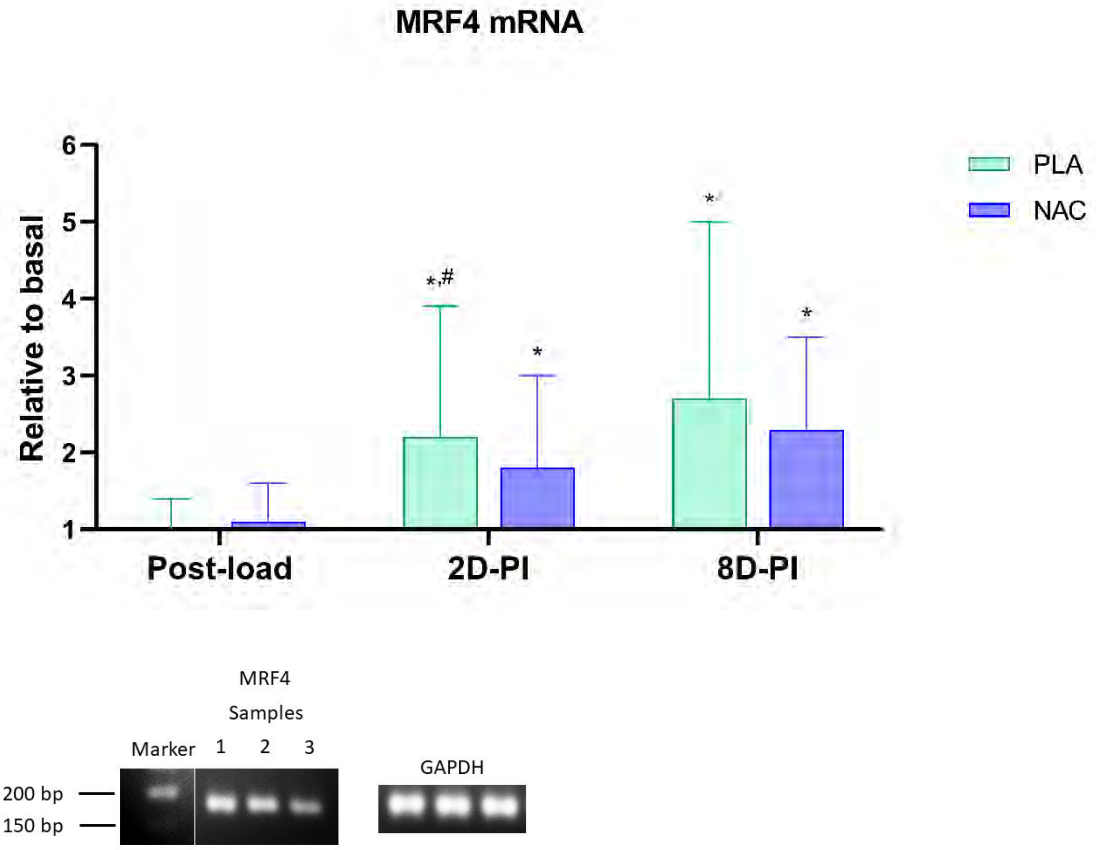

Figure 4.6.4.1. Changes in muscle MRF4 mRNA expression in total sample. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$. Lower panels: Representative images of agarose gel electrophoresis of PCR amplified products. Total sample n=24.

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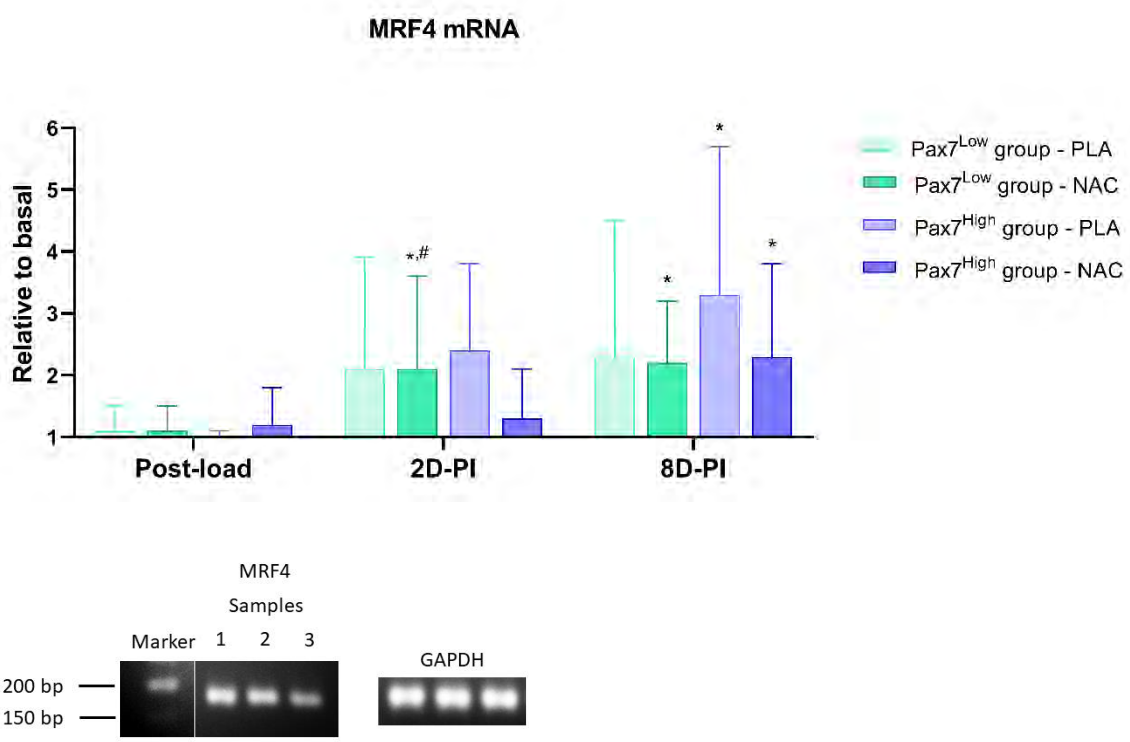

Figure 4.6.4.2. Changes in muscle MRF4 mRNA expression in low and high Pax7 groups. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$. Lower panels: Representative images of agarose gel electrophoresis of PCR amplified products. $Pax7^{Low}$ group, n=14; $Pax7^{High}$ group, N=10.

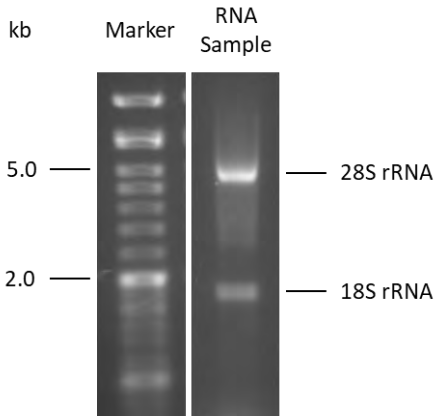

Figure 4.6.4.3. Representative image obtained from agarose gel electrophoresis of total RNA. Intact RNA, for downstream q RT-PCR, was verified by the appearance of sharp 28S and 18S ribosomal RNA (rRNA) bands.

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4.7. Muscle function

4.7.1 Knee extensors eccentric peak torque

Total sample (**Figure 4.7.1.1**): Eccentric peak torque decreased only at 2-days post-injury in both trials (all $p<0.001$) compared to basal, with NAC eliciting a lower decline compared to PLA ($p=0.020$). No changes were noted at post-load.

Low vs. High Pax7 groups (**Figure 4.7.1.2**): In both groups, eccentric peak torque decreased only at 2-days post-injury in both trials ($pax7^{Low}$ group: PLA, -42%, $p<0.001$; NAC, -27%, $p<0.001$ / $pax7^{High}$ group: PLA, -30%, $p=0.004$; NAC, -29%, $p=0.001$) compared to basal. At 2-days post-injury NAC ameliorated eccentric peak torque decline in $pax7^{Low}$ group ($p=0.003$). At 8-days, eccentric peak torque level tended to be higher in $pax7^{Low}$ group in NAC compared to PLA ($p=0.054$). No differences were observed between groups nor at post-load.

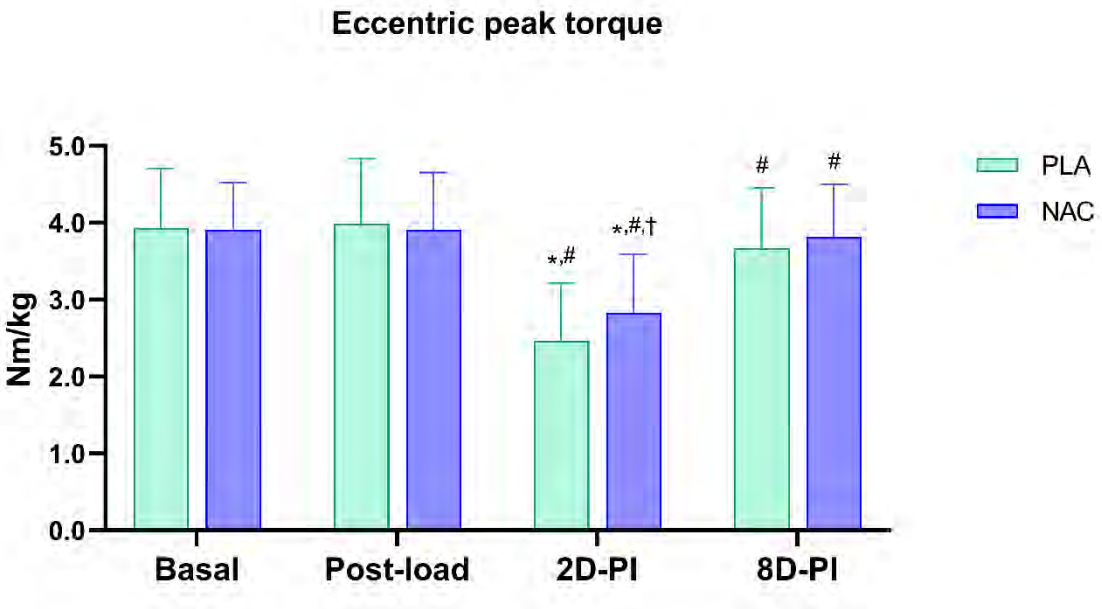

Figure 4.7.1.1. Changes in eccentric peak torque in total sample. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$; †Difference between trials, $p<0.05$. Total sample n=24.

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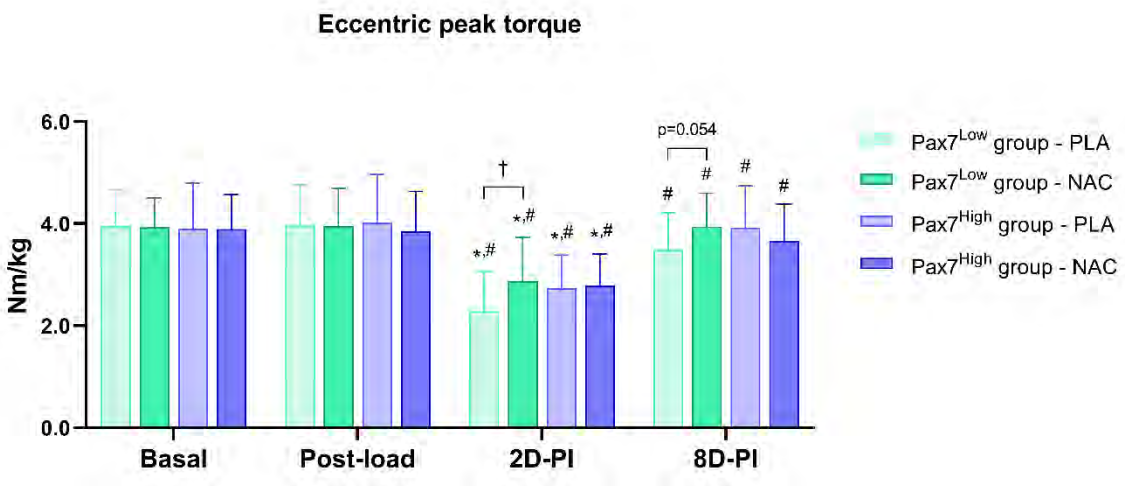

Figure 4.7.1.2. Changes in eccentric peak torque in low and high Pax7 groups. *Difference with basal, p<0.05; #Difference with previous time-point, p<0.05; †Difference between conditions, within group, p<0.05. Pax7^{Low} group, n=14; Pax7^{High} group, N=10.

4.7.2 Knee extensors isometric peak torque

Total sample (**Figure 4.7.2.1**): Isometric peak torque decreased in both trials at 2-days (PLA: p<0.001; NAC: p<0.001) and 8-days (PLA: p=0.010; NAC: p=0.036) post-injury compared to basal, with no between trial differences. No changes were noted at post-load.

Low vs. High Pax7 groups (**Figure 4.7.2.2**): In pax7^{Low} group, isometric peak torque decreased in both trials only at 2-days post-injury compared to basal (PLA: -33%, p<0.001; NAC: -29%, p<0.001). Isometric peak torque tended to decreased in PLA at 8-days post-injury compared to basal (-14%, p=0.060). In pax7^{High} group, isometric peak torque decreased in both trials at 2-days (PLA: -25%, p=0.020; NAC: -30%, p<0.001) and at 8-days post-injury only in NAC (-16%, p=0.021). At 8-days post-injury isometric peak torque levels in pax7^{Low} group were higher in NAC compared to PLA (p=0.008). No differences were observed at post-load.

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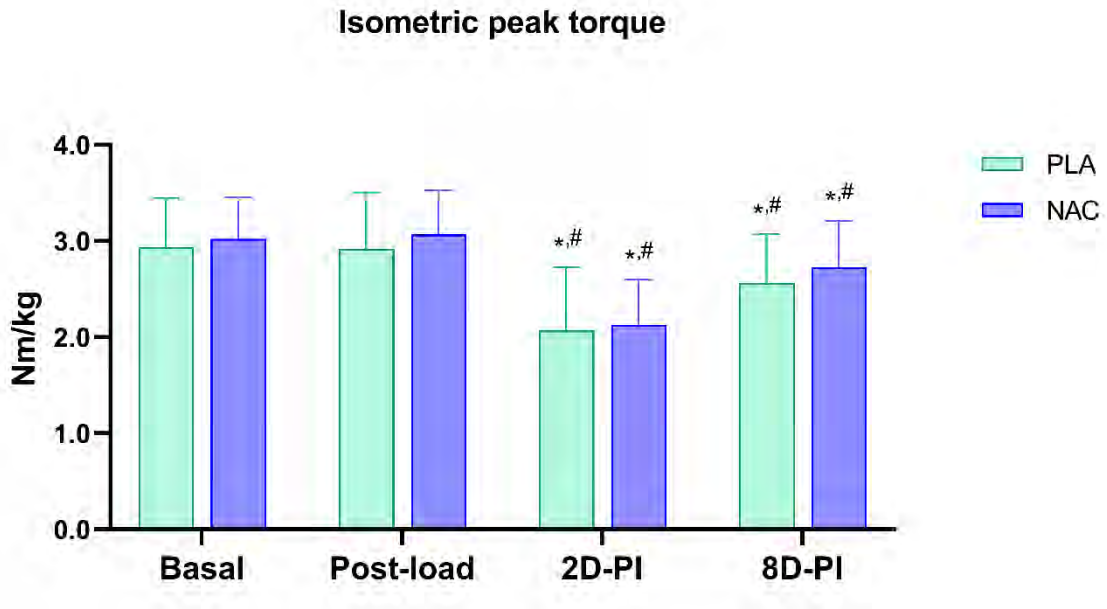

Figure 4.7.2.1. Changes in isometric peak torque in total sample. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$. Total sample n=24.

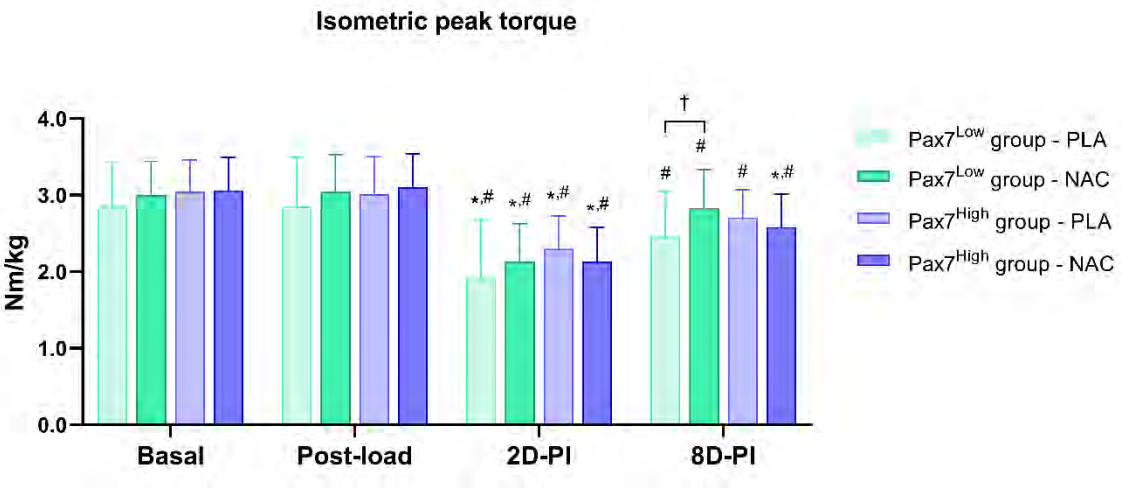

Figure 4.7.2.2. Changes in isometric peak torque in low and high Pax7 groups. *Difference with basal, $p<0.05$; #Difference with previous time-point, $p<0.05$; †Difference between conditions, within group; $Pax7^{Low}$ group, n=14; $Pax7^{High}$ group, N=10.

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General Conclusions

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General conclusions

By summarizing the results of previous in vitro and in vivo studies, our literature review demonstrated the primary physiological and molecular processes linking glutathione metabolism and redox status with skeletal muscle satellite cell viability and response following EIMD [234]. Redox regulation in satellite cells is crucial not only for their viability at quiescent state but also for their successful activation and commitment through the myogenic program following myotrauma. A balance between ROS production and neutralization is of paramount importance regarding satellite cells activity, ensuring proper satellite cells function and tissue repair during regeneration [234]. Low ROS levels are essential for physiological cell signaling and function, while high levels of ROS lead to irreversible post-translational modifications, such as thiol oxidation of cysteine residues, and deactivation of key signaling molecules which may be detrimental for the successful satellite cells activation and subsequent muscle tissue healing during regeneration. Given the established link between redox status and satellite cells, our results provide evidence for a redox-dependent regulation of satellite cells mainly at basal state and partially following EIMD in human skeletal muscle.

At basal state, in the total sample, muscle fiber CSA, myonuclear content and myonuclear domain size were higher in type II compared to type I fibers in both trials with no changes observed in participants physical and dietary nutrient profile. $Pax7^{High}$ group was characterized by higher muscle fiber CSA and myonuclear content, especially in type I muscle fibers, compared to $Pax7^{Low}$ group, while no differences were observed in myonuclear domain size, indicative of a compensatory mechanism regulating a constant myonuclear density in rested skeletal muscle [289]. Accordingly, participants in the $Pax7^{High}$ group exhibited higher levels of lower limb muscle strength (1RM in back squat) compared to $Pax7^{Low}$ group. No differences were detected in participants' dietary profile in both groups and trials.

At post-load, NAC treatment decreased GSSG and increased GSH, GSH/GSSG ratio, GPx and GR enzyme activity as well as nuclear levels of Nrf2 in skeletal muscle, in the total group, a finding that is consistent with the literature [171]. Interestingly, in the low and high Pax7 groups, NAC decreased GSSG and increased GSH, GSH/GSSG ratio, GPx and GR enzyme activity as well as nuclear levels of Nrf2 in skeletal muscle, only in the low Pax7 group. Notably, Pax7 low group demonstrated lower GSH values compared to high group at basal, however without reaching statistical significance. Our results indicate that NAC treatment upregulates GSH metabolism only

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in individuals with lower and/or inadequate levels of GSH, an observation that is in line with recent studies on young individuals [240, 241].

Following injury, in the total sample, GSH and GSH/GSSG ratio decreased at 2-days post-injury in both trials while GSSG increased only in PLA. NAC treatment ameliorated GSH and GSH/GSSG ratio decline at 2-days and increased their levels at 8-days post-injury compared to PLA. GPx and GR enzyme activity increased at 2-days in PLA and throughout recovery from EIMD in NAC. GPx and GR enzyme activity was augmented by NAC administration compared to PLA throughout recovery. Similar redox responses, have been reported in the literature, suggesting a protective effect of NAC administration in the first days following EIMD in human skeletal muscle [77, 91, 193]. To determine the antioxidant response in skeletal muscle, we also measured nuclear levels of the Nuclear factor-erythroid factor 2-related factor 2 (Nrf2) using immunofluorescence. Nrf2 is a master regulator of cellular antioxidant response orchestrating the expression of several antioxidant and myogenic genes in skeletal muscle [290-292]. Nuclear levels of Nrf2 increased at 2-days in PLA while NAC sustained this increase for up to 8-days post-injury. In the low and high Pax7 groups, GSH and GSH/GSSG ratio decreased at 2-days post-injury in $Pax7^{High}$ group and in $Pax7^{Low}$ group only in PLA, with NAC mitigating the decrease of redox status at 2-days post-injury and increasing its level at 8-days post-injury only in $Pax7^{Low}$ group. GPx and GR enzyme activity increased at 2-days to a similar extent in both groups in PLA, while NAC further increased both enzymes activity at 2- and 8-days post-injury in both groups. Nuclear levels of Nrf2 similarly increased at 2-days post-injury in both groups and trials, while NAC tended to increase the rise in Nrf2 levels in the $Pax7^{Low}$ group. Notably, in the $Pax7^{High}$ group, a sustained activation of Nrf2 up to 8-days post-injury was observed in NAC. Collectively, our results corroborate previous data in the literature showing a beneficial effect of NAC on redox responses and antioxidant status following muscle injury. Furthermore, our data also indicate a selective effect of NAC treatment in individuals with a lower satellite cells content, probably by reversing a redox defect in GSH metabolism.

In regards to the development of muscle damage following injury, we assessed CK activity in serum, DOMS as well as histological markers in muscle cross sections. Our data suggest that EIMD is associated with a pronounced increase in all muscle damage markers, with minimal effects of NAC treatment, especially in the histological parameters that were evaluated in the present study. In the total sample, CK activity and DOMS increased up to 8 days post-injury with NAC eliciting a lower increase at 2 days post-injury, which is in line with previous research [91]. Damaged and infiltrated fibers proportion increased at 8 days post-injury in both trials, while muscle fiber CSA decreased at 2- and 8-days post-injury with no effect of NAC. In the low and high pax7 groups, CK

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activity and DOMS increased up to 8 days post-injury with NAC mitigating this increase at 2 days post-injury in both groups. Damaged and infiltrated fibers proportion increased in both groups at 8 days post-injury in both trials, with no effect of NAC. Of note, damaged and infiltrated fibers proportion was higher in the $Pax7^{High}$ group compared to $Pax7^{Low}$ group in NAC. Muscle fiber CSA decreased at 2-days in both trials (except for $Pax7^{Low}$ group in NAC) and at 8-days post-injury only in $Pax7^{Low}$ group. At 8-days, muscle fiber CSA was greater in $Pax7^{High}$ group compared to $Pax7^{Low}$ group in NAC. It seems that a higher satellite cells content may result in expedited recovery of muscle fiber CSA following injury probably because of an increased satellite cells quantity and mobilization.

Regarding muscle regeneration, we assessed the proportion of centrally nucleated fibers as well as the proportion of fibers showing immunoreactivity for embryonic and neonatal myosin heavy chain (MHCe/n+). In the total sample, centrally nucleated fibers increased at 2-days post-injury in PLA and at 8-days post-injury in both trials, while MHCe/n+ fibers increased at 8-days post injury only in PLA. In the low and high Pax7 groups, centrally nucleated fibers increased at 8-days post-injury similarly in both groups and trials, while MHCe/n+ fibers increased at 8-days post injury only in the $Pax7^{High}$ group. Our results are in line with previous studies showing an increase in the proportion of centrally nucleated and MHCe/n+ fibers following injury in human skeletal muscle [181, 293]. In our study, we did not detect any effect of NAC on regeneration variables, however it was observed that $Pax7^{High}$ group exhibited a higher proportion of MHCe/n+ fibers compared to $Pax7^{Low}$ group in PLA, indicative of increased regeneration potential in individuals with higher satellite cells content.

Concerning satellite cells, we observed a significant correlation between Pax7+ cells and muscle GSH/GSSG ratio at basal state, providing evidence that satellite cells content is redox-dependent in a dose-response manner. In regard to satellite cells and myogenic responses, EIMD induced an increase in satellite cells quantity, proliferation and differentiation as well as in mRNA levels of myogenic factors with no significant effect of NAC treatment neither in total sample nor in low and high groups. Specifically, in the total sample, Pax7+ cells increased at 2- and 8-days post-injury and MyoD+ cells increased only at 2-days post-injury in both groups. Myogenin+ cells increased only at 8-days post-injury in PLA. Regarding the mRNA expression of myogenic genes, MYF5 increased at 2- and 8-days post-injury only in PLA, MYOD and MRF4 increased in both trials at 2- and 8-days post-injury and MYOGENIN increased at 2-days post-injury in both trials and at 8-days post-injury only in PLA. Our results and time-course of responses are in accordance with previous studies investigating satellite cells and myogenic responses following EIMD in humans

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[66, 68, 180, 181, 197, 294]. In low and high Pax7 groups, it was observed that Pax7^{High} group had higher Pax7+ and MyoD+ cells content compared to Pax7^{Low} group at basal state. Following EIMD, Pax7+cells increased in both groups and trials at 2-days (except for Pax7^{High} group in NAC) and 8-days post-injury. MyoD+ cells increased in both groups and trials at 2-days and at 8-days post-injury only in Pax7^{Low} group. Myogenin+ cells increased at 8-days post-injury only in Pax7^{High} group in PLA. Regarding myogenic programing, MYF5 increased at 2-days in Pax7^{High} group and at 8-days in Pax7^{Low} group in PLA only. MYOGENIN increased at 2-days post-injury in Pax7^{Low} group in PLA only and at 8-days post-injury in both groups only in PLA. MRF4 increased at 2-days post-injury in Pax7^{Low} group in PLA only and at 8-days post-injury in both groups and trials (except for Pax7^{Low} group in PLA). Previous studies have shown beneficial effects of antioxidant compounds administration in skeletal muscle regeneration following injury in vivo [3, 4, 176], while others indicated an opposite negative effect in myogenic potential and tissue repair [5, 6]. In our study, satellite cells responses and molecular myogenic markers were largely unaffected by NAC administration, with the exception of an augmented increase in MyoD+ cells at 8-days in the Pax7^{Low} group. Based on our results, a higher satellite cells content at baseline results in a more pronounced satellite cells response (activation, differentiation) during regeneration following EIMD.

Muscle function was assessed by measuring knee extensors peak eccentric and isometric torque. In the total sample, eccentric peak torque decreased in both trials at 2-days post-injury and recovered at 8-days post-injury with NAC mitigating eccentric peak torque decline at 2-days. Isometric peak torque decreased in both trials throughout the experimental period with no effect of NAC. In both low and high Pax7 groups eccentric peak torque decreased at 2-days post-injury with NAC eliciting a lower decline in the Pax7^{Low} group. Isometric peak torque decreased similarly in both groups and trials at 2-days post-injury while at 8-days post-injury isometric peak torque was higher in Pax7^{Low} group and lower in Pax7^{High} group in NAC. Our results showed that following EIMD, muscle function is greatly impaired for up to 8-days post-injury, which is in line with previous data [91, 181]. NAC treatment successfully mitigates muscle strength decline and improves performance following injury. Moreover, it is evident for the first time, that NAC treatment following muscle injury mitigates strength decline in participants with lower satellite cells content.

We also investigated the relationship between glutathione metabolism and redox status with dietary nutrient intake, physical fitness and physical activity profile in young adults. We observed that individuals with higher GSH and redox status levels consume higher amounts of dietary cysteine in their diet. Additionally, lower-limb muscle strength is higher in those individuals with higher redox status and lower oxidative stress levels. Moreover, higher antioxidant enzyme activity is

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associated with higher physical activity levels, irrespective of its intensity. Therefore, higher dietary cysteine intake and increased redox status appear to be discriminant factors of optimal muscle function and strength in young individuals.

In conclusion, the results of the present thesis clearly indicate that individuals with higher satellite cells content at baseline, demonstrate a superior regenerative capacity following EIMD compared to individuals with lower satellite cells content in skeletal muscle. It appears that samples homogeneity is an important factor in the investigation of satellite cell responses following EIMD. NAC treatment is beneficial only in individuals with lower satellite cells content, by upregulating redox status, satellite cells proliferation and muscle function recovery. On the contrary, NAC treatment does not affect redox and muscle damage/regeneration responses, but blunts satellite cells differentiation and muscle function recovery in individuals with higher satellite cells content. Of note, individuals with lower satellite cells content, also exhibit lower (without reaching statistical significance) levels of GSH – GSH/GSSG in skeletal muscle compared to the individuals with higher satellite cells content, indicative of a redox-dependent mechanism regulating satellite cells physiology in human skeletal muscle. Personalized redox nutrition, based on specific antioxidant deficiencies/inadequacies, seems a promising avenue in the area of redox biology and muscle regeneration.

Future directions

Future studies should further investigate the role of redox metabolism on satellite cells responses following EIMD in both the early (2-8 days) and late (30 days) phases of regeneration. Moreover, there is need for well-controlled clinical trials to explore the response of satellite cells activity and myogenic potential in conjunction with specific antioxidant deficiencies (i.e., GSH, NAD) in order to identify responsive redox phenotypes during skeletal muscle regeneration. Sample clustering based on redox inadequacies (i.e., GSH clustering) and different doses of antioxidants (titration studies) may also provide valuable insights. Furthermore, future studies should also investigate the redox-dependent regulation of satellite cells responses in pathophysiological conditions such as in aged or obese individuals characterized by increased inflammatory status and impaired regenerative potential.

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Limitations

The results of the present research are limited to young healthy men, while future studies should also explore the redox-dependent regulation of satellite cells in other men and women populations. Moreover, different exercise protocols of damaging (i.e., eccentric load) vs. non-damaging (sports participation) exercise and NAC supplementation may have a different effect on satellite cells and myogenic responses during recovery. Lastly, it should also be stated that the physiological differences observed in various markers (i.e., mRNA expression) did not always result in statistical significance, probably due to the high standard deviation values and the presence of outliers in the sample.

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APPENDIX A

(Ethics approval)

Institutional Repository - Library & Information Centre - University of Thessaly
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University of Thessaly
Department of Physical Education and Sport Science

Internal Ethics Committee

Trikala: 6/6/2018
Protocol Number: 1387

Approval of research entitled: "The effect of redox potential on the regulation of satellite cells and skeletal muscle healing following exercise-induced muscle damage"

Scientist responsible – supervisor: Ioannis Fatouros, Associate Professor

Main researcher - student: Konstantinos Papanikolaou

Institution & Department: School of Physical Education and Sport Science, University of Thessaly

The proposed research relates to a: PhD Thesis

Contact phone: +306986952200, +306937165213
Contact email: kpapanikolaou@uth.gr

The Internal Ethics Committee (IEC) of the Department of PE and Sport Science (DPESS), University of Thessaly, examined the proposal in its 4-2/6-6-2018 meeting and approves the implementation of the proposed research.

The Chair of the IEC – DPESS

Athanasios Tsiokanos, PhD

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ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ
ΤΜΗΜΑ ΕΠΙΣΤΗΜΗΣ ΦΥΣΙΚΗΣ ΑΓΩΓΗΣ ΚΑΙ ΑΘΛΗΤΙΣΜΟΥ

Εσωτερική Επιτροπή Δεοντολογίας

Τρίκαλα: 6/6/2018
Αριθμ. Πρωτ.: 1387

Βεβαίωση έγκρισης της πρότασης για διεξαγωγή Έρευνας με τίτλο: «Η επίδραση του οξειδοαναγωγικού δυναμικού στον μηχανισμό ελέγχου των δορυφόρων κυττάρων και της επούλωσης του σκελετικού μυ μετά από ασκησιογενή μυϊκό τραυματισμό»

Επιστημονικώς υπεύθυνος / επιβλέπων: Φατούρος Ιωάννης
Ιδιότητα: Αναπληρωτής Καθηγητής, ΣΕΦΑΑ-Π.Θ.
Ίδρυμα: Πανεπιστήμιο Θεσσαλίας (Π.Θ.)
Τμήμα: Επιστήμης Φυσικής Αγωγής και Αθλητισμού

Κύριος ερευνητής / φοιτητής: Παπανικολάου Κωνσταντίνος
Πρόγραμμα Σπουδών: Διδακτορικό ΠΣ Π.Θ.
Ίδρυμα: Πανεπιστήμιο Θεσσαλίας (Π.Θ.)
Τμήμα: Επιστήμης Φυσικής Αγωγής και Αθλητισμού

Η προτεινόμενη έρευνα θα είναι : Διδακτορική διατριβή

Τηλ. επικοινωνίας: 6986952200, 6937165213
Email επικοινωνίας: kpapanikolaou@uth.gr

Η Εσωτερική Επιτροπή Δεοντολογίας του Τ.Ε.Φ.Α.Α., Πανεπιστημίου Θεσσαλίας μετά την υπ. Αριθμ. 4-2/6-6-2018 συνεδρίασή της εγκρίνει τη διεξαγωγή της προτεινόμενης έρευνας.

Ο Πρόεδρος της Εσωτερικής Επιτροπής Δεοντολογίας – ΤΕΦΑΑ

Τσιόκανος Αθανάσιος
Αναπληρωτής Καθηγητής

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APPENDIX B

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B1: Candidate's responsibilities throughout the study

During the PhD program the candidate had the following duties:

- Completion of the courses required for the accomplishment of 10 to 20 ECTS
- Submission of the required documents to receive ethics approval for the study
- Submission of applications for research grands/funds/studentships
- Implementation of the study
- Implementation of the biochemical and molecular analysis on biological samples
- Perform the data analysis as well as the relevant power and statistical analysis
- Preparation of scientific manuscripts and submission for publication
- Writing the doctoral thesis and public defense
- Participation in relevant seminars and conferences
- Presentation of scientific results at internal group meetings, workshops and conferences

B2: Skills acquired during the PhD program

Through the present study and being also actively involved in other research projects and clinical trials during the PhD program, the candidate has gained considerable experience and advanced knowledge in the fields of molecular biology, muscle physiology, histology, redox metabolism and nutrition, reflected by the following acquired skills:

- Design and implementation of clinical trials
- Administration of nutritional supplements in clinical trials
- Evaluation of physical performance and physical activity in general population using accelerometry
- Assessment of body composition using advanced techniques such as dual energy x-ray absorptiometry - DXA
- Coordination of muscle biopsy and blood sampling procedures in clinical trials
- Application of advanced molecular and histological methodologies, including quantitative real-time PCR (RT-PCR), gel electrophoresis, histology, immunohistochemistry (IHC), immunofluorescence (IF), transmission electron microscopy (TEM) and enzymatic biochemical reactions
- Academic writing including scientific original papers, reviews and research proposals

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B3: Academic activities during the PhD program

Articles in Peer-Reviewed Scientific Journals

Number of Publications = 25

Citations (Google Scholar) = 406

h-index (Google Scholar) = 11

1. Deli, C.K., Poulios, A., Georgakouli, K., **Papanikolaou, K.,** Papoutsis, A., Selemekou, M., Karathanos, V.T., Draganidis, D., Tsiokanos, A., Koutedakis, Y., Fatouros, I.G., & Jamurtas, A.Z. (2018). The effect of pre-exercise ingestion of corinthian currant on endurance performance and blood redox status. *Journal of sports sciences*, 36(19), 2172–2180. https://doi.org/10.1080/02640414.2018.1442781 Impact Factor: 3.943

2. Draganidis, D., Jamurtas, A.Z., Stampoulis, T., Laschou, V.C., Deli, C.K., Georgakouli, K., **Papanikolaou, K.,** Chatzinikolaou, A., Michalopoulou, M., Papadopoulos, C., Tsimeas, P., Chondrogianni, N., Koutedakis, Y., Karagounis, L.G., & Fatouros, I.G. (2018). Disparate Habitual Physical Activity and Dietary Intake Profiles of Elderly Men with Low and Elevated Systemic Inflammation. *Nutrients*, 10(5), 566. https://doi.org/10.3390/nu10050566 Impact Factor: 6.706

3. Batrakoulis, A., Jamurtas, A.Z., Georgakouli, K., Draganidis, D., Deli, C.K., **Papanikolaou, K.,** Avloniti, A., Chatzinikolaou, A., Leontsini, D., Tsimeas, P., Comoutos, N., Bouglas, V., Michalopoulou, M., & Fatouros, I.G. (2018). High intensity, circuit-type integrated neuromuscular training alters energy balance and reduces body mass and fat in obese women: A 10-month training-detraining randomized controlled trial. *PloS one*, 13(8), e0202390. https://doi.org/10.1371/journal.pone.0202390 Impact Factor: 3.752

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5. Jamurtas, A.Z., Fatouros, I.G., Deli, C.K., Georgakouli, K., Poulios, A., Draganidis, D., **Papanikolaou, K.,** Tsimeas, P., Chatzinikolaou, A., Avloniti, A., Tsiokanos, A., & Koutedakis, Y. (2018). The Effects of Acute Low-Volume Impact Factor: 4.017

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6. Poulios, A., Fatouros, I.G., Mohr, M., Draganidis, D. Deli, C.K., **Papanikolaou, K.,** Sovatzidis, A., Nakopoulou, T., Ermidis, G., Tzatzakis, T., Laschou, V.C., Georgakouli, K., Koulouris, A., Tsimeas, P., Chatzinikolaou, A., Karagounis, L.G., Batsilas, D., Krustrup, P., & Jamurtas, A.Z. (2018). Post-Game High Protein Intake May Improve Recovery of Football-Specific Performance during a Congested Game Fixture: Results from the PRO-FOOTBALL Study. *Nutrients*, 10(4), 494. https://doi.org/10.3390/nu10040494 Impact Factor: 6.706

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Conference Abstracts (Oral and Poster Presentations)

Number of Presentations = 15

1. Stantsiou E., Sotiras S., Chatzipavlidis E., Tsolakis C., **Papanikolaou K.**, Draganidis D., Avloniti A., Fatouros I., Chatzinikolaou A. Bone adaptations in fencer: Case study. In: Proceedings of the 26th International Congress on Physical Education & Sport Science; 2018 May 18-20: Komotini (Greece). 2018. p. 214.

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15. **Papanikolaou K.,** Poulios A., Draganidis D., Margaritelis N., Baloyiannis I., Papadopoulos C., Deli C., Syrou N., Georgakouli K., Tsimeas P., Rosvoglou A., Tzatzakis T., Makri S., Kouretas D., Nikolaidis M., Jamurtas A., Fatouros I. Erythrocyte and skeletal muscle redox status is associated with dietary cysteine intake, strength and physical activity levels in young men. In: Proceedings of the 30th International Congress on Physical Education & Sport Science; 2022 May 20-22: Komotini (Greece). 2022. p. 267

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Traineeship at the German Institute of Human Nutrition

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MEMBER OF THE
LEIBNIZ ASSOCIATION

ARTHUR-SCHEUNERT-ALLEE 114–116
D-14558 NUTHETAL

PHONE +49 (0)33200 88-2216
FAX +49 (0)33200 88-2555
E-MAIL tilman.grune@dife.de
INTERNET http://www.dife.de

DEPARTMENT MOLECULAR TOXICOLOGY
Prof. Dr. Tilman Grune

TRAINEESHIP EVALUATION

Name of the trainee: Konstantinos Papanikolaou

Name of the receiving organisation/enterprise:
German Institute of Human Nutrition Potsdam-Rehbruecke (DIfE)

Sector of the receiving organisation/enterprise: Department of Molecular Toxicology (MTOX)

Address of the receiving organisation/enterprise:
German Institute of Human Nutrition Potsdam-Rehbruecke (DIfE),
Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany
phone: +49 (0)33200 88-0; fax: +49 (0)33200 88-2444; **website:** www.dife.de

Start and end of the traineeship: from 29/01/2018 till 30/04/2018

Traineeship title: Quantitative confocal laser scanning microscopy in human biopsies for quantification of skeletal muscle satellite cells and oxidized proteins.

Detailed programme of the traineeship period including tasks carried out by the trainee:

- Immunohistological stainings for determination of protein oxidation and nitration markers (protein carbonyls and 3-nitrotyrosine) in tissue samples of human skeletal muscle.
- Immunohistological stainings for the determination of the type I (Myosin heavy chain I; MCHI) and II (Myosin heavy chain II; MCHII) muscle fiber distribution as well as single fiber localization (Laminin staining) in human and mouse skeletal muscle tissue samples.
- Immunohistological stainings for the determination of skeletal muscle satellite cell markers (Pax7, MyoD) in human and mouse skeletal muscle tissue samples.
- Quantitative confocal laser scanning microscopy for the quantification of skeletal muscle satellite cells markers (Pax7, MyoD), oxidized proteins (protein carbonyls) and protein nitration (3-nitrotyrosine).

HEAD OF THE BOARD OF ADVISORS: DR. CLAUDIA HEROK
BOARD OF DIRECTORS: PROF. DR TILMAN GRUNE • DR. BIRGIT SCHRÖDER-SMEIBIDL
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- Confocal microscopic image acquisition, data collection and statistical analyses, preparation of the figures and presentation of scientific results of the immunohistological analyses.

Knowledge, skills (intellectual and practical) and competences acquired (learning outcomes achieved):

- Practice and experience in confocal laser scanning microscopy application
- Sample preparation from human biopsies
- Quantification of microscopic data and data analysis
- Theoretical knowledge of immunohistochemical analytic methods including immunology, histology and basic chemistry knowledge.
- Acquisition of techniques of sample preparation for immunohistological analyses from human and mouse skeletal muscle tissue samples.
- Acquisition of basic and advanced techniques for confocal laser scanning microscopy and multichannel imaging analysis.
- Experiments planning and optimization of the immunohistological stainings.
- Quantification of confocal microscopic images using advanced quantitative and qualitative techniques with different software platforms.
- Data acquisition and preparation of scientific outcomes and results.
- Overall scientific knowledge, cooperation and interaction with experienced researchers in the field of biology, biochemistry and nutrition science.

Evaluation of the trainee:
We are pleased to provide this letter for Konstantinos Papanikolaou. He was very dedicated, thirsty for knowledge and highly motivated. With keen perception he was quickly able to work independently as well as to plan and perform his experiments accurately.
He carried out every aspect of his work to a very high standard and his pleasant manner made him popular with the rest of the work group. Respecting the scientific outcome, transfer of knowledge and methods, this traineeship can be considered as very successful.

Date: 02/05/2018

Prof. Dr. Tilman Grune

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Teaching courses in the undergraduate program

Department of Physical Education and Sport Science, University of Thessaly

- Winter Semester 2018-2019: "Laboratory Assessment of Sports Performance", course code: MK1109, 4 hours per week.

- Spring Semester 2018-2019: "Exercise Techniques for Strength Development", course code: MK0917, 8 hours per week.

- Winter Semester 2019-2020: "Ergometry - Ergospirometry" course code: MK1126, 4 hours per week.

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APPENDIX C

(Publications included in the Thesis)

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Free Radical Biology and Medicine 161 (2020) 125–138

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Review Article

Redox-dependent regulation of satellite cells following aseptic muscle trauma: Implications for sports performance and nutrition

Konstantinos Papanikolaou [a], Aristidis S. Veskoukis [b,c], Dimitrios Draganidis [a], Ioannis Baloyiannis [d], Chariklia K. Deli [a], Athanasios Poulios [a], Athanasios Z. Jamurtas [a], Ioannis G. Fatouros [a,*]

[a] Department of Physical Education and Sport Science, University of Thessaly, Karies, Trikala, 42132, Greece
[b] Department of Nutrition and Dietetics, University of Thessaly, Argonafton 1, 42132, Trikala, Greece
[c] Department of Biochemistry and Biotechnology, University of Thessaly, Viopolis, Mezourlo, 41500, Larissa, Greece
[d] Department of Surgery, University Hospital of Larissa, Mezourlo, 41110, Larissa, Greece

ARTICLE INFO

Keywords:
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Antioxidants
Redox status
Recovery
Sports performance
Muscle damage
Healing

ABSTRACT

Skeletal muscle satellite cells (SCs) are indispensable for tissue regeneration, remodeling and growth. Following myotrauma, SCs are activated, and assist in tissue repair. Exercise-induced muscle damage (EIMD) is characterized by a pronounced inflammatory response and the production of reactive oxygen species (ROS). Experimental evidence suggests that SCs kinetics (the propagation from a quiescent to an activated/proliferative state) following EIMD is redox-dependent and interconnected with changes in the SCs microenvironment (*niche*). Animal studies have shown that following aseptic myotrauma, antioxidant and/or anti-inflammatory supplementation leads to an improved recovery and skeletal muscle regeneration through enhanced SCs kinetics, suggesting a redox-dependent molecular mechanism. Although evidence suggests that antioxidant/anti-inflammatory compounds may prevent performance deterioration and enhance recovery, there is lack of information regarding the redox-dependent regulation of SCs responses following EIMD in humans. In this review, SCs kinetics following aseptic myotrauma, as well as the intrinsic redox-sensitive molecular mechanisms responsible for SCs responses are discussed. The role of redox status on SCs function should be further investigated in the future with human clinical trials in an attempt to elucidate the molecular pathways responsible for muscle recovery and provide information for potential nutritional strategies aiming at performance recovery.

1. Introduction

Skeletal muscle is a highly dynamic and adaptive tissue which in healthy adults comprises ~40–50% of human body mass and is implicated in multiple mechanical, physiological and metabolic processes including movement, metabolism, and homeostasis [1]. Skeletal muscle is composed of large, multinucleated cells called myofibers, which are subjected to microtrauma and their ability for repair is crucial for proper and life-long muscle function and adaptation [2]. Due to its unique plasticity, skeletal muscle has the ability to regenerate and remodel in response to injury [3].

Muscle microtrauma is present in numerous physiological conditions such as exercise-induced muscle damage (EIMD), which is frequently observed following physical activity and/or strenuous exercise, especially when eccentric contractions are incorporated [4]. Moreover, numerous catabolic and muscle wasting chronic conditions such as myopathies, aging, and cachexia result in a chronic state of muscle microtrauma [5]. Acute or chronic muscle injury results in the initiation of an inflammatory cascade and the mobilization and infiltration of immune cells to the injured muscle tissue. Immune cell subpopulations (neutrophils, macrophages) invade damaged muscle tissue to remove cellular debris, neutralize pathogenic organelles and promote healing [2].

Muscle microtrauma is characterized by elevated inflammation and reactive oxygen species (ROS) which may result in a secondary damage of previously uninjured myofibers [6]. It should be noted that the term ROS will be used throughout the manuscript hereafter since, as it has been insightfully proposed in previous studies, it encompasses the initial species generated by oxygen reduction (superoxide or hydrogen peroxide) as well as their secondary reactive products [7,8]. During

* Corresponding author. School of Physical Education, Sport Science & Dietetics, University of Thessaly, Karies, Trikala, 42100, Greece.
E-mail address: ifatouros@pe.uth.gr (I.G. Fatouros).

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exercise, the main sources of ROS generation include NADPH oxidase enzymes [associated with the sarcoplasmic reticulum (SR), transverse tubules and plasma membrane], phospholipase A_2, xanthine oxidase and mitochondrial metabolism whereas an immune cell oxidative burst is active during post-exercise recovery [9–11]. In response to altered levels of ROS, transient and both reversible and non-reversible oxidative modifications occur in the skeletal muscle, including S-nitrosylation, S-glutathionylation and disulfide formation [12]. Specifically, S-glutathionylation of the cysteine residues may result in protein deactivation and activity inhibition [12]. This post-translational redox modification may therefore play important role in the skeletal muscle signaling during exercise and recovery [13].

The aforementioned inflammatory cascade is followed by a healing phase during which skeletal muscle fibers are repaired or regenerated mainly due to the activity of resident skeletal muscle-specific stem cells. Pro-inflammatory (M1) and anti-inflammatory (M2) macrophages seem to orchestrate skeletal muscle's myogenic response after microtrauma [14]. Postnatal muscle tissue repair and remodeling following injury is attributed to a heterogenous family of mononucleated myogenic stem cells, called satellite cells (SCs) [15]. In response to injury, SCs are activated, enter the cell cycle, proliferate and give rise to daughter cells that can either return to quiescence (self-renewal) or proceed to terminal differentiation by formatting myoblasts and fuse with damaged myofibers to form new muscle fibers or assist in growing the old ones [16,17]. This process is accompanied by increased transcriptional and translational capacity at nuclei and ribosomes, respectively, of muscle cells resulting in the accumulation of contractile proteins (e.g. myosin heavy chain isoforms) and extracellular matrix elements (e.g., proteins, cell adhesion receptors) leading to muscle remodeling and growth [18].

The influence of ROS-induced cellular processes on myogenic programming is complex as ROS present in concentrations below a threshold (that is largely unknown though) are implicated in signal transduction, SCs viability and differentiation while they result in inhibition of myogenesis through apoptotic and necrotic pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) when their levels surpass this threshold [19]. Recently, *in vitro* and *in vivo* studies that used antioxidant compounds to quench excess ROS production in skeletal muscle during the repair process following aseptic myotrauma have provided evidence of accelerated muscle recovery process via upregulation of the myogenic potential, viability, maintenance and activity of the resident SCs [20–22]. However, to this date literature lacks evidence on a redox-dependent regulation of SCs following aseptic myotrauma and inflammation in the human skeletal muscle and to what extent, antioxidant supplementation can affect activity of SCs and muscle's regeneration.

The aim of this review is to present an integrated overview of (i) the mechanisms governing SCs biology following EIMD and its impact on muscle regeneration; (ii) the available evidence demonstrating a redox-dependent regulation of SCs activation and myogenic action; (iii) the implications of a redox-dependent regulation of SCs on recovery of skeletal muscle performance following EIMD; and (iv) the potential role of antioxidant supplementation on SCs response to injury.

2. Skeletal muscle satellite cells biology and tissue regeneration

Skeletal muscle-specific stem cells (satellite cells – SCs) are the machinery of tissue repair, remodeling and growth. SCs represent approximately ~30–35% of the sublaminal nuclei of myofibers in early postnatal mouse muscle [23,24]. SCs numbers also depend on muscle fiber type, with oxidative (slow-twist, type I) fibers containing more SCs than glycolytic ones (fast-twist, type II) fibers [25]. In addition, SCs can be identified by their location and the expression of several surface and transcriptional factors, with paired box transcription factor Pax7 considered to be the most reliable and reproducible for this cell type [26]. Pax7 has been correlated with SCs self-renewal through the maintenance of an undifferentiated state [27]. Skeletal muscle has a remarkable capacity to preserve muscle structure and integrity by regenerating mature myofibers following injury [26]. Studies that utilized SCs deletion models confirmed that muscle's regenerative capacity relies on their myogenic potential and activity [28]. Intriguingly, Pax7-null mice demonstrated a dramatic reduction of the SCs pool, a completely abolished regenerative capacity following skeletal muscle injury as well as increased fibrotic and fat tissue that resulted in functional impairment and premature postnatal death [29,30]. Recent advances in transplantational medicine have shown that the detrimental effects induced by the absence of SCs can be reversed via exogenous intramuscular injection of SCs in the injured muscle [31]. Apart from Pax7, Pax3 is also expressed in quiescent SCs, influencing their specification [26]. After birth, SCs express Pax3 and are present in the skeletal muscle in the absence of Pax7 (although in reduced numbers) [32]. Nevertheless, the absence of Pax7 leads to cell cycle impairments and apoptosis resulting in ablation of the resident SCs [33]. Moreover, Pax3 has been shown to play a critical role in orchestrating SCs commitment in the myogenic program by independently regulating MyoD expression in activated SCs during regeneration [34].

Under physiological conditions, SCs reside to the adjunct myofiber and are mitotically quiescent outside the cell cycle (G_0 phase) with a low metabolic rate and RNA content [35]. Microarray transcriptomic analysis has revealed that >500 genes and antioxidant molecules, such as thioredoxin-1 (TRX1) and glutathione peroxidase-3 (GPX3), are upregulated during quiescence suggesting a protective mechanism against oxidative damage [36]. Upon muscle injury, SCs are activated by factors released within their microenvironment (also called *niche*) and enter the cell cycle. These factors include, but are not limited to, immune cells subpopulations which in turn release cytokines, chemokines and growth factors, blood vessels-derived factors, signaling molecules and extracellular matrix cues [37].

The transition from quiescence to activation is accompanied by a shift from fatty acid oxidation to glycolytic metabolism [38]. Activated SCs express both Pax7 and the myogenic factor 5 (Myf5) [24]. In response to muscle injury, the influx of calcium into the cytosol triggers calcineurin and calmodulin kinase through calcium binding to calmodulin, to activate Myf5 expression [39]. SCs activation also involves an IGF-dependent mechanism which induces the upregulation of Myf5 following injury through the PI3K/Akt and ERK signaling pathways [39]. Activated ($Pax7^+/Myf5^+$) SCs undergo several cell divisions, proliferate and follow two distinct fates by either assisting in tissue repair or return to quiescence to replenish the *in vivo* SCs pool (self-renewal). Activated SCs proceed to proliferation, forming myoblasts, while $Pax7^+/Myf5^-$ SCs are able to self-renew through symmetric or asymmetric division upon entry into the cell cycle [26].

During the proliferation state, myoblast determination factor (MyoD) is a key regulator of myogenic advancement and subsequent SCs differentiation to myocytes through the induction of cell cycle inhibitors such as p21 and p57 [40,41]. Proliferative ($Pax7^+/MyoD^+$) SCs migrate from their dormant location to the injured area and increase their mitotic activity [42]. Interestingly, $MyoD^{-/-}$ myoblasts exhibit incomplete muscle regeneration as well as downregulated myogenin and myogenic regulatory 4 (MRF4) gene levels [43,44].

Following differentiation, SCs-derived myocytes downregulate the expression of Pax7 and MyoD and upregulate the expression of myogenin [42]. Differentiating ($Myogenin^+$) myocytes undergo an early (myocyte-to-myocyte attachment) and late cell-to-cell fusion (fusion of myocytes to form myotubes) [45]. Upon terminal differentiation, multinucleated myotubes ($MRF4^+$) undergo a maturation process to form functional myofibers, a process which involves the expression of embryonic and neonatal myosin heavy chain isoforms ($MHCe/n^+$) [46]. During this process the Akt/mTOR pathway is the key signaling pathway, activated by IGF-1 which binds to its transmembrane receptor and phosphorylates Akt to subsequently stimulate protein synthesis through the phosphorylation of $p70^{S6}$/ribosomal protein S6 cascade that results in the accumulation of several contractile proteins such as slow

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and fast MHC isoforms in the intra- and extracellular matrix (ECM) [47]. Moreover, Akt-induced down-regulation of catabolic pathways [e.g. the Forkhead box protein (FOXO), muscle ring finger-1 (MuRF-1) and atrogin-1 pathways] is also associated with an increased hypertrophic response following myotube formation during myogenesis [48].

The final stage of muscle healing process involves an angiogenesis cascade, ECM remodeling and reinnervation of the regenerated myofibers [49]. During this stage, angiogenesis is driven by the transforming growth factor-β (TGF-β) which is upregulated during regeneration, inducing the physiological fibrosis cascade [50]. ECM components such as laminin, fibronectin, proteoglycans, elastin and various isoforms of collagen accumulate in the regenerative area to provide tissue stabilization, while also serving as a docking station towards the formation of neuromuscular junctions (NMJs) [51]. Importantly, nerve activity directly regulates protein turnover and gene expression within regenerative myotubes, while indirectly affects the activation and proliferation of SCs [52,53]. The influence of nerve activity in the maturation and functional restoration of the regenerating innervated myofibers can be assessed by analyzing the expression of developmental MHC isoforms (i.e. $MHCe^{+}/n^{+}$) [49]. Collectively, complete muscle restoration requires the recovery of functional performance, which is only beneficial if the regenerated myofibers become efficiently reinnervated. (Fig. 1).

Intensive exercise, particularly eccentric contractions, result in aseptic exercise-induced microtrauma and inflammation characterized by ultrastructural damage of sarcomeres, basal lamina compartments and cell membranes, with a concomitant increase of ROS [54,55]. The mechanical explanation of sarcomere disruption leads to the dissociation of contractile proteins from the ECM as actin and myosin filaments overstretch along with an increase in titin stiffness [56,57]. These events attenuate the muscle's excitation-contraction potential and lead to loss of force-production capacity, muscle soreness and functional impairment [2]. Several human studies have examined the SCs response after aseptic myotrauma caused by eccentric exercise, mostly in mixed muscle (Table 1). Overall, SCs number increases from the first hours of injury with significant changes observed at 24 h post-injury, peak at 2–3 days, while their number remains elevated even at 8 days post-injury compared to baseline values, especially in type II fibers [58–68]. Noteworthy, human studies investigating SCs responses in conjunction with changes in the redox status inside the SCs microenvironment following aseptic microtrauma are currently lacking.

3. The inflammatory response underlies the evolution of SCs-mediated tissue healing

SCs are essential for muscle healing, though proper muscle recovery relies on the interaction with numerous cell types and secreted factors within the SCs niche. In response to muscle injury, cytokines, chemotactic and growth factors are rapidly released propagating an inflammatory cascade that is mainly characterized by infiltration of mast cells (MCs) and leukocyte subpopulations into the injured tissue [69]. In addition, the local MCs are also activated and release pro-inflammatory cytokines such as tumour necrosis factor-a (TNF-a), interleukin-1β (IL-1β) and histamine to attract more neutrophils and other immune cells to the injury site [2]. Both *in vitro* and *in vivo* studies have provided evidence that factors secreted by MCs enhance SCs activity and promote myoblast proliferation [70]. Under inflammatory conditions, immune cells recruited by muscle injury promote the induction of matrix metalloproteinases (MMPs) [71]. MMPs cleave collagens, laminin, and other extracellular matrix components and release the hepatocyte growth factor (HGF) from the sequestered sites to activate SCs in a nitric oxide-dependent manner [71]. Furthermore, damaged vascular endothelial cells release growth factors such as the vascular endothelial growth factor (VEGF) which further promotes SCs activation and proliferation in the muscle [37].

The infiltrated neutrophils release pro-inflammatory cytokines including TNF-a, IL-1β and interferon-γ (IFN-γ) to facilitate the removal of necrotic and apoptotic myofibers [55]. Typically, the concentration of neutrophils at the injured site peak within the first 6–24 h post-injury and return to baseline values after 72–96 h [72]. Subsequent to neutrophils, a second wave of immune cells, the macrophages infiltrate the injured muscle within a time-frame of 24–48 h after muscle microtrauma [73]. Of note, macrophages are considered a prominent regulator of skeletal muscle myogenesis [74,75]. Initially, the pro-inflammatory M1 macrophages are recruited, releasing cytokines such as TNF-a, IL-1β and IL-6 [76]. TNF-a promotes the activation of the NF-κB and p38 signaling pathways and upregulates proliferation and differentiation mechanisms [77]. Experimental evidence revealed that TNF-a receptor knockout mice exhibit impaired regeneration, highlighting the particularly important role of TNF-a in muscle regeneration [77]. IL-6 is also produced by M1 macrophages and research in animal models has shown that IL-6 ablation results in an attenuated migration, proliferation and differentiation of SCs [78]. In addition, M1 macrophages recruit T cells ($CD8^{+}$ and $CD4^{+}$) that infiltrate the injury site at ~72 h post-injury and remain elevated for up to 10 days [79]. T cells promote the release of multiple cytokines that in turn stimulate muscle SCs expansion [80]. Interestingly, the combination of four pro-inflammatory cytokines (IL-1α, TNF-α, IFN-γ) secreted by T cells stimulated SCs proliferation *in vivo* upon injury and promoted their serial expansion *in vitro* [70]. Moreover, M1 macrophages-derived fibroblast growth factors (FGFs), regulate SCs proliferation and migration

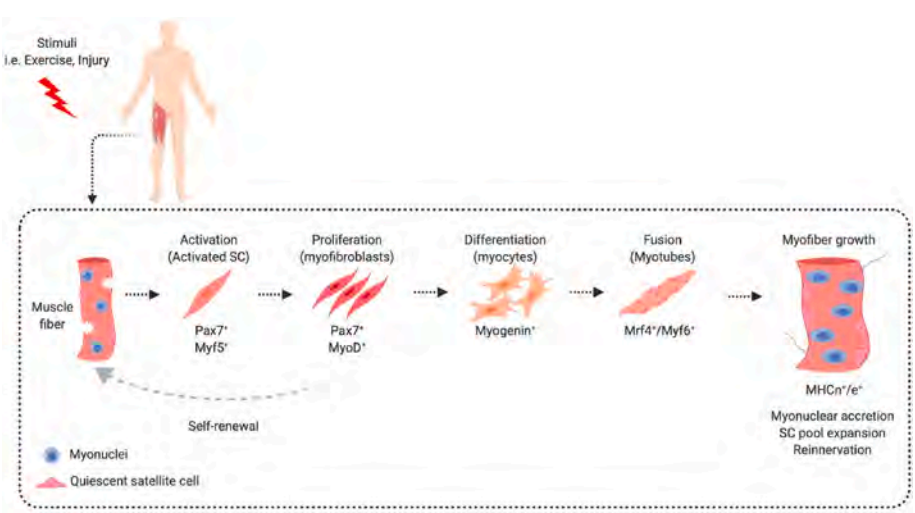

Fig. 1. The timeframe of skeletal muscle tissue regeneration following acute aseptic myotrauma. In response to stimuli, resident quiescent satellite cells (SCs) are activated and enter the myogenic programming. A part of the proliferating fibroblasts procced to differentiation into myocytes and another part return to quiescence in order to maintain the SCs pool (self-renewal). Differentiated myocytes fuse to form myotubes and repair the injured tissue. Beneath each state are markers to indicate that satellite cells undergo each phase before they get to their terminal phase.

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Table 1
The impact of acute damaging exercise on satellite cell response in human skeletal muscle.

Publication [Refs.]	Subjects	Age	Fitness status	Exercise protocol	Biopsy sampling timepoint	SCs related indices
Cermak et al. [58]	8 young men	23 ± 1	Recreationally active	300 kE EC at −180°/s	24-h post exercise	↑Pax7$^+$ cells (Type II fibers) ↑PCNA$^+$ cells (mixed fibers)
Crameri et al. [59]	8 young men	25 ± 3	Untrained	50 drop-jumps + 80 kE EC at −30°/s + 80 kE EC at −180°/s	4- and 8-days post exercise	↑N-CAM$^+$ cells (mixed fibers) ↑FA1$^+$ cells (mixed fibers)
Dreyer et al. [60]	10 young men	23–35	No resistance training	96 kE EC at – 60°/s	24-h post exercise	↑N-CAM$^+$ cells (mixed fibers)
	10 older men	60–75	No resistance training	96 kE EC at – 60°/s	24-h post exercise	↑N-CAM$^+$ cells (mixed fibers)
Hyldahl et al. [61]	7 young men	23 ± 2	Untrained	196 kE EC at −180°/s	24-h post exercise	↑Pax7$^+$ cells (Mixed fibers) ↑MyoD$^+$ cells (Mixed fibers)
McKay et al. [67]	8 young men	22 ± 1	No lower-body resistance training	300 kE EC at −180°/s	24-, 72- and 120-h post exercise	↑Pax7$^+$ cells (mixed fibers) ↑PCNA$^+$ cells (mixed fibers)
McKay et al. [63]	12 young men	21 ± 2	No lower-body resistance training	300 kE EC at −180°/s	24-h post exercise	↑Pax7$^+$ cells (mixed fibers) ↑N-CAM$^+$ cells (mixed fibers) ↑C-Met$^+$ cells (mixed fibers)
Mikkelsen et al. [64]	8 young men	23 ± 3	Well trained	200 kE EC at −120°/s	8-days post exercise	↑Pax7$^+$ cells (mixed fibers) ↑N-CAM $^+$ cells (mixed fibers) ↔Ki67$^+$ cells (mixed fibers)
Nederveen et al. [66]	29 young men	21 ± 0.5	Recreationally active	300 kE EC at −180°/s	6-, 24- and 72-h post exercise 24-h post exercise 6-, 24-, 72- and 96-h post exercise	↑Pax7$^+$ cells (Mixed and Type II fibers) ↑Pax7$^+$/MyoD$^+$ cells (Mixed fibers) ↑MyoD, MRF4 and Myogenin mRNA
O'Reilly et al. [65]	8 young men	21 ± 2	No lower-body resistance training	300 kE EC at −180°/s	24-, 72- and 120-h post exercise	↑N-CAM$^+$ cells (mixed fibers)
Toth et al. [68]	12 young men	21 ± 2	Untrained	300 kE EC at −180°/s	24-h post exercise	↑Pax7$^+$ cells (mixed fibers) ↑Myf5 mRNA (mixed fibers)

Pax7, paired-box protein 7; Myf5, myogenic factor 5; MyoD, myogenic determination factor; MRF4, myogenic regulatory factor 4; KE, knee extensors; EC, eccentric contractions; CFPE, capillary-to-fiber perimeter exchange index; ↑, increase; ↔, no change.

[44,45,66]. *In vitro* experiments suggest that various FGFs elicit a profound SCs proliferation response following injury [81].

During the inflammatory phase, muscle performance deteriorates depending on the type, intensity, duration and total volume of the exercise stimulus [82]. Mild performance deterioration results in minimum strength loss (<20%), limited inflammation and fast recovery (12–48 h) [2]. In moderate performance deterioration (20%–50%) CK increases in serum (>1000 U/l), the inflammatory response is more intense and is characterized by leukocyte infiltration in the injured area and the recovery phase lasts from 5 to 7 days [2]. In severe performance deterioration (>50%), histological analyses have revealed extensive necrosis in myofibers which is accompanied by a pronounced accumulation of neutrophils in the muscle tissue, soreness and swelling. In this scenario, systemic CK levels may exceed 10,000 U/l [2].

In the course of the inflammatory cascade macrophages progressively alter their phenotypic identity from pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages (day 2 to day 4) [75]. The predominant role of M2 macrophages is to resolute the inflammatory milieu by producing anti-inflammatory cytokines (e.g. IL-4, IL-10, IL-13). Additionally, M2 macrophages promote SCs differentiation to myotubes, thus initiating the late stage of myogenesis and muscle healing [55]. At this stage, IL-4 promotes myotube formation during the late fusion of myoblasts into myotubes and IGF-1 stimulates myotube hypertrophy [83,84]. Previous *in vitro* observations identified the mechanistic pillar between macrophage phenotype and stages of myogenesis. Specifically, culture of SCs with M1 macrophages increased their proliferative capacity but decreased myogenin levels [79]. On the other hand, culture of SCs with M2 macrophages upregulated myogenin levels and enhanced SCs fusion to form myotubes [85]. During this healing phase, muscle performance is diminished. Particularly in intense EIMD, disruption of calcium homeostasis leads to muscle protein breakdown even during the early phase of recovery [86]. Of note, 2–4 days post EIMD muscle strength reaches its nadir with concomitant conversion of M1 macrophages to M2 [87].

Altogether, the inflammatory process following muscle trauma is strictly interconnected with SCs function and crucial in promoting muscle regeneration and proper tissue healing (Fig. 2).

4. Redox status in the satellite cell niche

The redox status could be defined as the balance between oxidants (or pro-oxidants) and antioxidants [88]. When this equilibrium is disrupted and tilts towards an oxidized state resulting in excess production of ROS and in the disruption of redox signaling and control, oxidative stress occurs [11]. It has to be stressed, though, that oxidants and pro-oxidants along with antioxidants play fundamental roles in maintaining proper cellular function. ROS, as the main subcellular oxidizing agents, although seemingly act detrimentally on key cellular biological processes, it is established that they also serve as important signaling molecules [89–91]. Therefore, the maintenance of proper cellular function and state is an outcome of a highly sophisticated molecular network, where ROS and antioxidants are equally necessary. Cellular homeostasis relies on a delicate balance between oxidant and pro-oxidant molecules and an optimal level of ROS [92]. Myogenic stem cells are equipped with refined antioxidant systems, extremely sensitive to redox changes inside their niche [93]. Low ROS levels favor cell survival, signal transduction, mitochondrial function and biogenesis, SCs differentiation and muscle growth whereas high ROS levels are detrimental, promoting cell apoptosis, autophagy, mitochondrial disfunction and impaired SCs differentiation, muscle healing and myofiber growth [94–96]. Low ROS levels (e.g. H_2O_2) may activate the ERK and c-Jun N-terminal kinase (JNK) intracellular signaling pathways to promote myoblast proliferation in a dose- and time-dependent manner [97]. Excess ROS can lead to abnormal differentiation and apoptosis of

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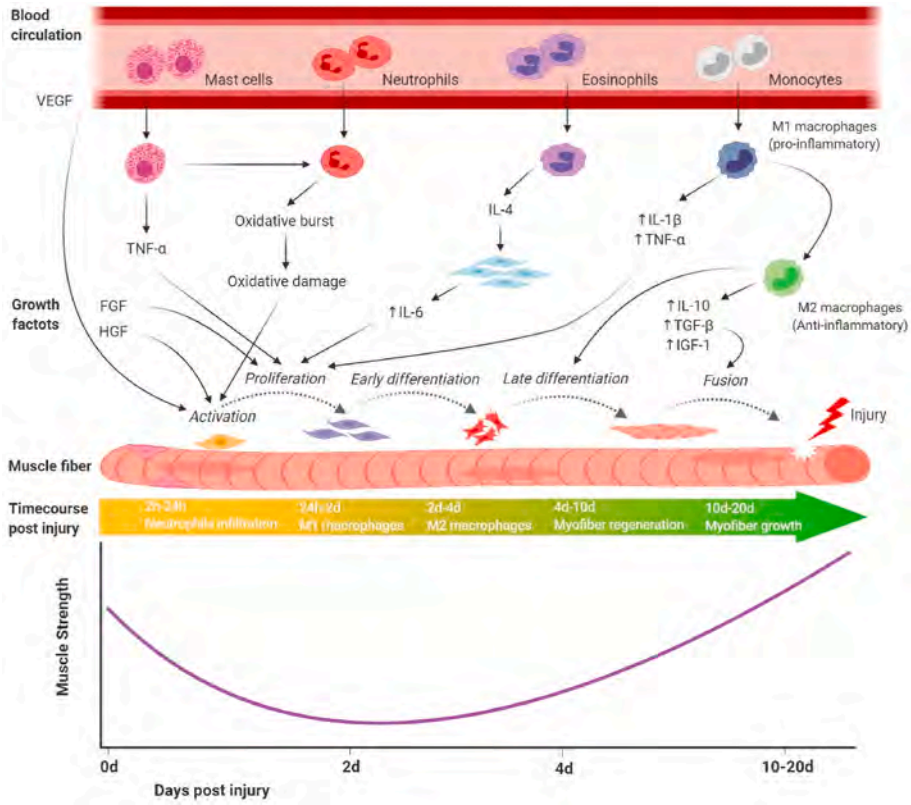

Fig. 2. The interaction between satellite cells (SCs), inflammation and muscle strength recovery during regeneration. Following injury, immune cells subpopulations are secreted inside the SCs niche. They promote the induction of several cytokines, growth factors and cell types which are closely implicated in the tissue regeneration process by targeting the stages of myogenic advancement. During the early events of the healing phase, muscle strength decreases, however, upon myofiber regeneration and growth muscle strength recovers.

stem cells through the activation of p38 and p53 signaling pathways and compromise hematopoietic stem cells self-renewal capacity and engraftment [98]. Furthermore, following transplantation, increased ROS in mesenchymal stem cells reduce their engraftment potential and induce apoptotic mechanisms [98]. Although the precise effects of ROS on signaling pathways during the myogenic process have not been fully elucidated, ROS levels appear to increase during reprogramming and to cause DNA damage in pluripotent stem cells, a phenomenon that can be mitigated by the addition of the non-specific antioxidant N-Acetylcysteine (NAC) [99].

Evidence emerged during the past two decades suggests that ROS produced physiologically by cells are important signaling molecules, acting through mechanisms such as post-translational redox modifications of cysteine thiols on proteins [10]. Post-translational redox modifications such as S-glutathionylation (most common) involves the formation of mixed disulphides between GSH and cysteine thiol groups of proteins that results in their activation or deactivation [100] (Fig. 3). GSH is crucial for SCs antioxidant defense under oxidative conditions, mainly by promoting cell survival mechanisms [101]. Collectively, endogenous antioxidants, which include enzymes and small molecules such as TRX, glutathione reductases, PRX and GSH are considered fundamental for the control of redox signaling networks and myogenic programming (Fig. 4).

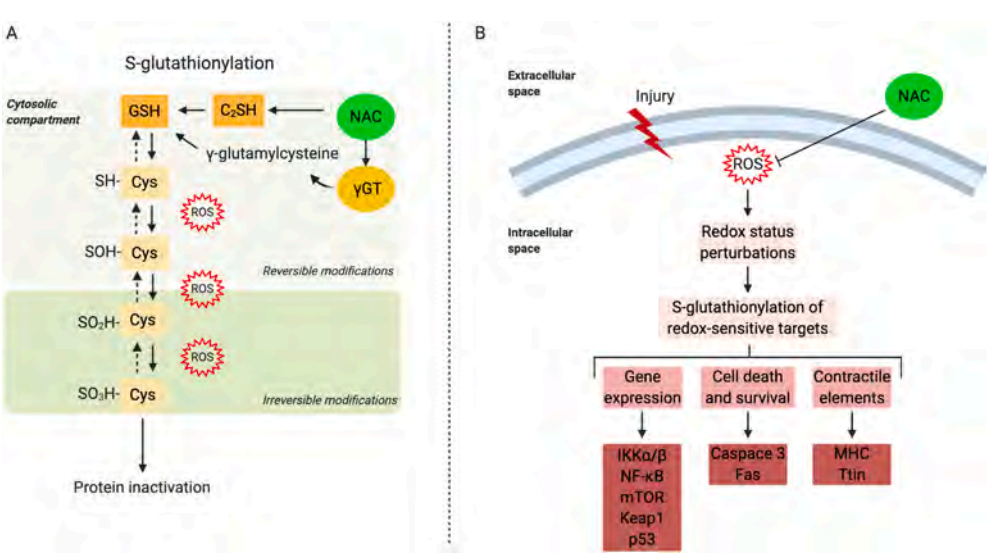

Fig. 3. Post-translational S-glutathionylation biochemistry and its impact in redox-sensitive cellular processes. Thiol oxidation of redox-sensitive cysteine residues results in reversible and/or irreversible modifications in multiple targets promoting their inactivation, depending on the degree of oxidation (A). N-acetylcysteine (NAC) replenishes glutathione (GSH) bioavailability mainly through the γ-glutamylcysteine. Acute muscle injury promotes the production of reactive oxygen species (ROS) and the induction of S-glutathionylation of several redox-sensitive targets however, NAC supplementation may mitigate the rise of ROS and protect the cysteine residues of proteins from oxidation, thus enhancing their functionality (B).

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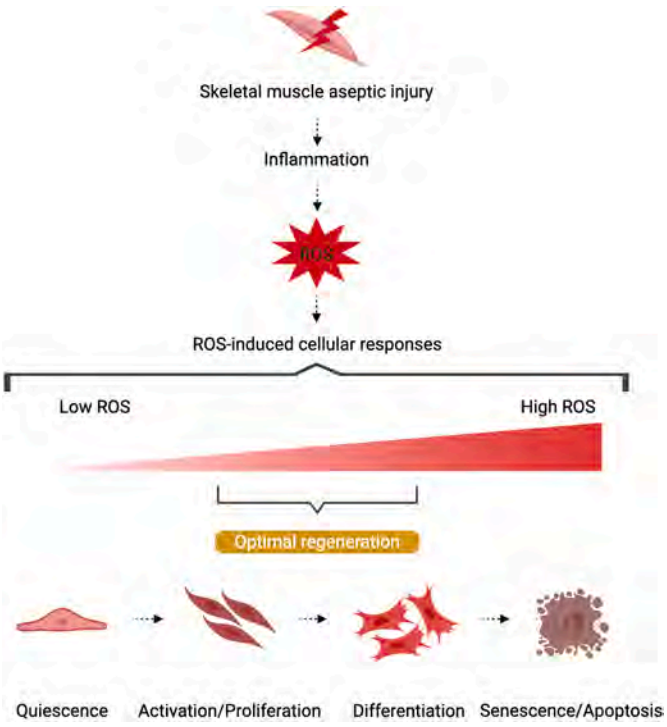

Fig. 4. The effect of redox status on satellite cells (SCs) homeostasis and function. Skeletal muscle aseptic injury results in the onset of inflammation and the production of reactive oxygen species (ROS). Proper muscle regeneration relies on the capacity of SCs to proliferate, differentiate and fuse with damaged myofibers. Low ROS levels promote SCs survival and self-renewal, while facilitating proliferation and differentiation mechanisms. On the other hand, high ROS levels lead to premature, abnormal differentiation and cell death. ROS balance inside the SCs niche is mandatory for appropriate tissue regeneration and remodeling following injury.

5. Evidence of a redox-dependent regulation of satellite cells

Intrinsic and extrinsic cues in the SCs microenvironment are redox-dependent and decide the fate of myogenic precursor cells during the stages of muscle regeneration [102]. Redox-sensitive signaling pathways directly regulate redox sensors which then affect downstream factors involved in cellular homeostasis [103]. They include the forkhead homeobox type O family (FoxO), nuclear factor erythroid 2-related factor 2 (Nrf2), apurinic/apyrimidinic (AP), endonuclease1/redox factor-1 (APE1/Ref-1) and ataxia telangiectasia mutated (ATM) which monitor redox homeostasis under low ROS levels [101]. High ROS levels induce abnormal differentiation and/or senescence by redox-sensitive molecules such as the hypoxia-inducible factors and p38 [102]. Skeletal muscle SCs reside in a redox-sensitive niche as ROS levels mediate their status and activity [102]. Increasing evidence in the literature supports the notion that redox homeostasis is an important modulator in the self-renewal and differentiation process of stem cells [104,105]. Notably, ROS are thought to negatively affect myogenesis in numerous pathological conditions (e.g. muscular dystrophy, sarcopenia and muscle wasting) characterized by muscle microtrauma, persistent inflammation and impairment of SCs activity [106–108]. Depletion of intracellular GSH raised ROS levels immensely and promoted NF-κB activation that led to MyoD downregulation and decreased myogenic potential [109]. Specifically, in H_2O_2-treated C2C12 cells, ROS production was markedly elevated accompanied by a decrease in GSH stores which resulted in a significant reduction in MyoD mRNA levels during myoblast proliferation and of myogenin and MRF4 at the later stages of differentiation [109]. Furthermore, the redox-sensitive factor PIXT2 act as key regulator of the intracellular redox state, preventing DNA damage as cells undergo differentiation [110,111]. Accordingly, Vallejo et al. [112] showed that the c-isoform of PITX2 transcription factor can modify the myogenic potential of SCs of a dystrophic-deficient mouse by increasing cell proliferation and the number of myogenic committed cells by regulating micro-RNA 31 (miR-31). Altogether, the combined effects of ROS elevation and GSH depletion resulted in mitochondrial degradation and apoptosis [109]. Similarly, an extensive mitochondrial disruption was observed in oxidatively injured C2C12 murine myoblasts [113].

Previous studies have shown that increased ROS could lead to reduced myogenesis by lowering MyoD protein expression and viability and stemness of SCs following a rise in NF-κB signaling in SCs and myoblasts [114]. However, it must be noted that the ROS/NF-κB axis can regulate both positively and negatively the myogenic process as low amounts of ROS are needed for the initiation of the regeneration phase [115]. In this context, ROS impact cellular signaling via post-translational modifications and more specifically protein oxidations (intramolecular disulfide bridges, sulfonylamide bond formation, activation of tyrosine kinases, inhibition of phosphatases) of multiple target proteins. Oxidation of the cysteine thiol group residues is the most extensively characterized type of protein modification and cysteine oxidation, accounting for approximately 1.9% of all protein modifications by ROS [98]. In turn, the catalytic activity of antioxidant enzymes, such as PRX, catalase (CAT), and glutathione peroxidase (GPX) can also be modified by signaling molecules indicating an influential balance between cellular signaling and redox status [116]. Yet, antioxidant enzymes like SOD, CAT, GPX and heme oxygenase-1 (HO-1) seem to regulate myogenic proliferation and differentiation [19]. Numerous *in vivo* studies have reported beneficial protective effects for GPX, SOD1, HO-1 and CAT regarding oxidative damage and maintenance of myogenic precursor cells function [114,117].

Two major pathways in SCs biology, which are well-characterized as redox-sensitive and act antagonistically are the Notch and Wnt signaling pathways [118,119]. The Notch signaling pathway is instrumental for the maintenance of the quiescent state of SCs and regulates the proliferation and differentiation of myogenic precursor cells [119]. The Notch pathway is partly modulated by the ROS -producer NADPH oxidase 1 [120,121]. Dysregulation of this pathway leads to premature differentiation of myoblasts and reduction in SCs pool via impaired self-renewal [122]. Downstream events in this pathway lead to the expression of Hes1, Hey1 and HeyL genes which regulate quiescence and stemness sustainability through the upregulation of PAX7 and inhibition of activation genes [122]. Canonical Wnt signaling is implicated in SCs differentiation, while non-canonical signals regulate self-renewal and myofiber growth [123]. Wnt proteins bind to transmembrane Frizzled receptors (Fzd) which cooperate with Dishevelled (Dvl) and heterotrimeric G-proteins for downstream signaling. During canonical Wnt signaling phosphorylation of β-catenin by casein kinase I (CK1) leads to its translocation into the nucleus where it forms complexes with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors promoting the expression of MyoD protein and downregulating Pax7, thus promoting myogenic proliferation and differentiation [123]. Non-canonical Wnt signaling stimulation, especially Wnt7a/Fzd7 signaling, can lead to the PI3K (Phosphoinositide 3-kinase) activation, of which then activates the AKT/mTOR pathway resulting in increased protein synthesis [124,125]. The Wnt pathway can be modulated by TRX [interacts with Dishevelled (Dvl) inside the pathway] and selenium which influences several Wnt proteins through GPX1 [120].

TNF-α/NF-κB axis is known to inhibit myogenesis, an effect that is widely attributed to the oxidative activation of NF-κB and subsequent gene expression [115]. The redox regulation of the NF-κB family of transcriptional activators plays a central role in differentiation, adaptation, and apoptosis of muscle cells. Muscle damage, inflammation and oxidative stress can activate NF-κB through phosphorylation and release of its inhibitory protein IKKα/β, while ROS can regulate NF-κB via multiple mechanisms [126].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is also implicated

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in redox control of SCs [127]. Under normal conditions, Nrf2 is localized in the cytosol, bound to its inhibitor Kelch-like ECH-associated protein 1 (Keap1). ROS oxidize Keap1 and it dissociates from Nrf2, which then translocates to the nucleus, heterodimerizes with Maf proteins and binds to antioxidant responsive element (ARE) in order to induce expression of ARE-dependent genes [128]. ROS-dependent Nrf2 signaling plays a critical role in the regulation of SCs self-renewal and proliferation as it can upregulate MyoD and downregulate myogenin levels [129]. In corroboration, Takahata et al. [130] reported protective effects of Nrf2 in response to oxidative damage in undifferentiated mesenchymal C3H10T1/2 stem cells.

IGF-1, a peptide hormone with a complex post-transcriptional regulation, is known to promote muscle protein synthesis and is also correlated with myoblast survival through a protective mechanism against H_2O_2-induced cell death via PI3K/Akt and ERK1/2 MAPK pathways [131,132]. Acting in a synergistic manner with Sirtuin 1 deacetylase, it initiates myoblast proliferation [133]. *In vitro* experiments have demonstrated that during late differentiation phase, ROS-induced IGF-1 activation promotes myotube hypertrophy likely through the upregulation of the Akt/mTOR pathway [134]. Myofiber growth and tissue regeneration may be affected by antioxidant enzymes not only through changes induced in SCs viability and proliferation but also by regulation of the differentiation process per se. Notably, increased myotube formation was observed following GPX upregulation [135], whereas a reverse effect was evident in GPX-1-deficient mice [117] and in response to reduced GSH concentration [109]. Collectively, this piece of evidence suggests the presence of a strong interaction and association between redox-regulated molecules and pathways involved in skeletal muscle SCs fate during both quiescence and the regeneration process (Fig. 5).

6. The influence of pathophysiological conditions on the redox-dependent regulation of satellite cells

Several pathophysiological conditions such as aging, muscular dystrophies, sarcopenia and muscle wasting have been shown to significantly impair SCs homeostasis and function, while inflammation and excess ROS also negatively affect SCs responses [136–141].

During aging, the regenerative capacity of skeletal muscle is compromised, due to a decline in SCs number and impaired sensitivity to the damage stimuli [142]. In aged mice, it has been shown that ~10% of the resident SCs are susceptible to senescence following mitogen exposure, thus limiting the ability of aged SCs for regeneration [139]. In humans, advanced age is often accompanied by the development of a low-grade, chronic, systemic inflammation (termed *inflammaging*), which is characterized by a marked elevation in circulating inflammatory molecules (i.e. TNF-α, IL-6) and overproduction of ROS [143,144]. These inflammatory cues inside the SCs niche propagate a cytokine-mediated upregulation of several redox-sensitive targets such as NF-κB pathway, further mitigating SCs activation capacity (through MyoD inactivation) and regeneration potential [145]. Furthermore, redox-mediated signaling pathways such as p38, p16, JAK/STAT and Notch/Wnt are largely affected during aging and impact SCs function. Inhibition of p38 in aged SCs increase their proliferative capacity and self-renewal potential [146], while high ROS levels can cause DNA damage in SCs, leading to a p16-induced cellular senescence response [147]. Furthermore, upregulation of JAK/STAT signaling during aging contributes to altered SCs phenotypes and aging-associated impairments that can be reversed by transient downregulation of JAK/STAT pathway activity [148]. In aged muscle, Notch ligand Delta1 is reduced and SCs ability to induce Notch signaling is compromised, causing defects in maintenance, survival and proliferation of aged SCs [149]. Additionally, Wnt signaling upregulation during aging, via the complement protein C1q, perturbates its dynamic balance with Notch, and thus favors misdifferentiation at the expense of self-renewal, contributing to the age-associated exhaustion of the SCs pool [136]. Interestingly, this age-related reduction in SCs pool has been characterized as a major hallmark of sarcopenia [136]. Altogether, the molecular redox-mediated alterations in the microenvironment and within the SCs during aging seem to impair their maintenance and function, leading to aberrant skeletal muscle regeneration.

In muscle diseases such as muscular dystrophy and muscle wasting, characterized by muscle microtrauma, SCs function and regeneration potential are also diminished. The increase in ROS production and alterations in redox balance have been shown to correlate with the severity of the pathology in several muscular dystrophies, mitigating skeletal muscle healing potential [19]. Cellular mechanisms related to ROS signaling have been recently associated with SCs defects in disease models. Experiments in mice have shown that dystrophin-deficient SCs-derived myotubes are more susceptible to oxidative damage and

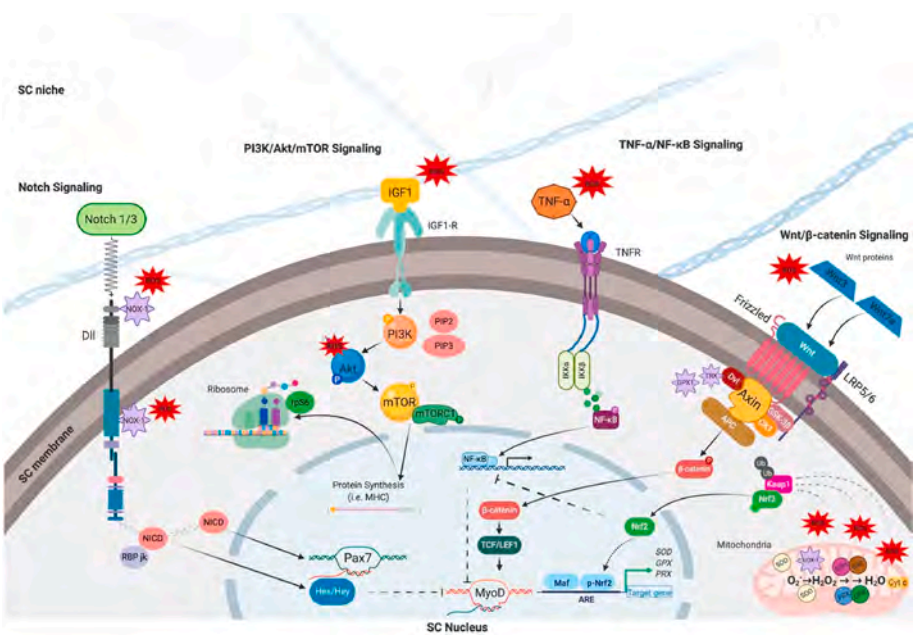

Fig. 5. Redox-sensitive signaling pathways involved in satellite cells (SCs) homeostasis and recovery following acute aseptic muscle microtrauma. Intracellular signaling pathways inside the SCs microenvironment orchestrate their activity and myogenic potential. Redox sensors and proteins sensitive to alterations in redox status can modulate quiescence, proliferation and differentiation through the induction of specific genes inside the SCs nucleus. Notch signaling promotes a quiescent state through the upregulation of paired box transcription factor 7 (Pax7) and downregulation of the myoblast determination factor (MyoD). Antagonistically, several Wnt proteins (Wnt/β-Catenin) induce the expression of MyoD and the proliferation of SCs. TNF-α/NF-κB pathway inhibits SCs proliferation by blocking MyoD expression. Nuclear factor erythroid 2-related factor 2 (Nrf2) dissociation from Kelch-like ECH-associated protein 1 (Keap1) and its translocation into the SCs nucleus induces the expression of antioxidant enzymes responsible for SCs maintenance and function, while blocking NF-κB. Phosphoinositide 3-kinase/Protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway is crucial during the late stages of tissue regeneration and remodeling, mainly via the induction of a hypertrophic response, thus promoting skeletal muscle growth.

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that this susceptibility was associated with the severity of the disease [150]. Interestingly, it has been reported that the susceptibility of *mdx* muscle to oxidative stress was accompanied by the alteration of p38 and JNK signaling pathways, both being redox-sensitive and involved in myogenic programming [151]. SCs derived from facioscapulohumeral muscular dystrophy (FSHD) patients also present increased susceptibility to oxidative damage compared to that from nonaffected muscles, a finding that was accompanied by morphological differentiation defects [152]. Epigenetic control has also been reported to interact with redox signaling and SCs function. It was shown that upregulation of the Polycomb group protein Bmi1 in mdx-derived SCs triggers a protective antioxidative response, limits DNA damage and enhances regeneration, thus improving the pathology [153]. Collectively, evidence suggests that disturbances in redox signaling negatively affect SCs intrinsic responses in the context of muscle diseases, and as such mitigate skeletal muscle's regeneration and healing potential.

7. Is muscle performance under redox regulation?

As mentioned, SCs are crucial regulators of muscle performance recovery kinetics following intense exercise. Therefore, a question may arise here as to whether muscle performance recovery is redox-dependent. So far, supplementation with vitamins (predominantly C and E) has been extensively utilized to alter redox status and improve performance in athletes; however, their effectiveness in enhancing performance and promoting molecular adaptations to exercise training is to date ambiguous (Table 2). In humans, Yfanti et al. [154] reported that vitamins C (500 mg/day) and E (400 IU/day) have no impact on endurance adaptive responses, mitochondrial biogenesis and antioxidant enzyme activity. In contrast, animal studies revealed that supplementation with vitamin C, E or their combination down-regulates biomarkers of mitochondrial biogenesis and the intramuscular antioxidant content [155]. In line with these findings, Ristow et al. [156] reported that supplementation with 1000 mg/day vitamin C combined with 400IU/day of vitamin E following 4 weeks of endurance training

Table 2
The effects of antioxidant administration on muscle performance, inflammation and redox status in humans.

Publication [Refs.]	Subjects	Fitness status	Supplementation protocol	Exercise regime	Effects on performance	Effects on inflammation	Effects on redox status
Bjornsen et al. [160]	34 elderly men	Untrained	12 weeks: 1000 mg Vt C + 235 mg Vt E per day	Resistance training	↓Muscle thickness ↑Muscle strength ↓Lean mass	N/A	N/A
Cobley et al. [164]	12 young men	Recreationally trained	6 days: 50 mg/kg/day NAC	Damaging exercise	↑YIRT-L-1 ↑Sprint	↑CK	N/A
Gomez-Cabrera et al. [155]	14 young men	Sedentary	8 weeks: 1000 mg/day Vt C	Endurance training	↔	N/A	N/A
He et al. [161]	22 young men	Moderately trained	2 weeks: 1000 mg Vt C + 400 IU Vt E per day	Downhill running	↓DOMS	↓CK	↑ORAC
McKenna et al. [165]	8 young men	Well-trained	125 + 25 mg/kg/h NAC before and during exercise	Endurance exercise	↑Na^+, K^+ pump activity ↑Performance	N/A	N/A
Medved et al. [167]	8 young men	Endurance trained	125 + 25 mg/kg/h NAC before and during exercise	Submaximal exercise	↓Fatigue	N/A	↑Muscle NAC ↔Blood GSH ↑TGSH/GSH ↑Muscle Cys
Michailidis et al. [87]	10 young men	Recreationally active	8 days: 20 mg/kg/day NAC	Damaging exercise	↑Short-term recovery	↓CRP, IL-6, CK	↑GSH/GSSG
Paschalis et al. [169]	36 young men	Recreationally active	30 days: 2 × 600 mg/day NAC	Performance testing	↑Performance	N/A	↓Isoprostanes F_2 ↑NADPH ↑GSH ↑SOD ↓PC
Paschalis et al. [170]	20 young men	Recreationally trained	30 days: 1000 mg/day Vt C	Endurance exercise	↑VO_{2max}	N/A	↓Isoprostanes F_2 ↓PC
Paulsen et al. [157]	54 young men and women	Recreationally trained	11 weeks: 1000 mg Vt C + 235 mg Vt E per day	Endurance training	↓PGC-1α ↓MAPK1 ↓CDC42 ↓COX4	N/A	↔
Paulsen et al. [159]	32 young men and women	Recreationally trained	10 weeks: 1000 mg Vt C + 235 mg Vt E per day	Resistance training	↓Muscle hypertrophy ↓Muscle strength	N/A	N/A
Ristow et al. [156]	40 young men	Trained and untrained	4 weeks: 1000 mg Vt C + 400 IU Vt E per day	Complex training	↓PGC-1α ↓PGC-1β ↓PPARγ	N/A	↓SOD1SOD2, GPX1 mRNA ↓TBARS
Sakelliou et al. [73]	10 young men	Recreationally active	8 days: 20 mg/kg/day NAC	Damaging exercise	N/A	↓HLA^+, ↓$11B^+$ cells ↓Neutrophils ↓Leukocytes	↓TBARS ↓GSSG ↓PC
Slattery et al. [166]	10 young men	Well-trained	9 days: 1.2 g/day NAC	Endurance exercise	↑Sprint	↓IL-6	↓Isoprostanes F_2 ↓TBARS ↑TAC
Theodorou et al. [158]	28 young men	Recreationally trained	9 weeks: 1000 mg Vt C + 400 IU Vt E per day	Eccentric training	↔	↔	↔
Yfanti et al. [154]	21 young men	Moderately trained	16 weeks: 500 mg Vt C + 400 IU Vt E per day	Endurance training	↔	N/A	↔

Vt, vitamin; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPARγ, peroxisome proliferator-activated receptor gamma; TBARS, thiobarbituric reactive substances; SOD1/1, superoxide dismutase 1,2; GPX1, glutathione peroxidase 1; COX4, cytochrome *c* oxidase 4; CDC42, cell division control protein 42 homolog; MAPK1, mitogen-activated protein kinase 1; DOMS, delayed onset of muscle soreness; CK, creatine kinase; ORAC, oxygen radical absorbance capacity; YIRT-L-1, Yo-Yo intermittent recovery test level 1; IL-6, interleukin 6; TAC, total antioxidant capacity; GSH, glutathione; GSSG, oxidized glutathione; TGSH, total glutathione; CRP, c-reactive protein; Cys, cysteine; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; ↑, increase; ↓, decrease; ↔, no effect; N/A, not applicable.

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attenuated the expression of several mitochondrial biogenesis-related mRNAs (PGC-1α, PGC-1β and PPARγ) and reduced the levels of TBARS in previously untrained male individuals. Similarly, in recreationally endurance trained men and women, supplementation with vitamin C and E (1000 mg/day and 235 mg/day, respectively) for 10 weeks, reduced mitochondrial biogenesis markers (PGC-1α, COX4, CDC42 and MAPK1), though no impact on redox status was observed [157]. In addition, several studies investigated the impact of vitamin C and E supplementation on redox status and performance adaptations induced by resistance exercise training. The work by Theodorou et al. [158] indicated that supplementation with vitamin C during 4 weeks of eccentric training had no effect either on performance or on redox status in recreationally trained young men. Conversely, Paulsen et al. [159] reported that administration of vitamins C and E ameliorated the increase in muscle strength and hypertrophy during a 10-week resistance exercise intervention in trained individuals. Likewise, vitamin C and E supplementation decreased muscle thickness and blunted the increase in lean mass following resistance training in healthy older males [160]. Nevertheless, other studies have reported positive effects of vitamin C and E supplementation. Characteristically, short-term ingestion of combined vitamin C and E supplementation (vitamin C: 1000 mg/d; vitamin E: 400 IU/d) not only attenuated the levels of CK and muscle soreness, but also enhanced muscle protection through an improved oxygen radical absorbance capacity (ORAC) following a second bout of exercise [161].

To this end, although indications exist that antioxidants can prevent protein oxidation during exercise [162], there is little or no evidence *in vivo* to suggest that antioxidant supplementation is sufficient to attenuate EIMD and/or accelerate recovery during the succeeding days. It is yet to be investigated whether changes in muscle performance appear in conjunction with alterations in redox status and SCs-mediated responses during the healing phase following myotrauma. Thus, we propose that data from interventional studies utilizing supplementation with antioxidants should be interpreted with caution as baseline redox status, the dose and length of the antioxidant supplementation as well as the choice of the redox biomarkers measured are critically implicated in the controversial cellular and physiological responses observed [163].

NAC, a thiol-based supplement and a precursor molecule for GSH, has been shown to exert positive effects on performance and skeletal muscle recovery [87]. NAC supplementation results in a significant preservation of muscle performance, via the upregulation of intramuscular GSH levels, and thus, it might be a useful strategy to enhance performance during short-term competitive situations in young recreationally trained men [164]. NAC may attenuate muscle strength decline, in part via improved K^+ regulation suggesting a role for ROS in muscle fatigue [165]. In a double-blind, randomized study, NAC supplementation improved cycling performance via an improved redox balance and promoted adaptive processes in well-trained athletes undergoing strenuous physical training [166]. These results are in agreement with previous observations in endurance athletes where NAC improved performance probably through an enhanced availability of muscle cysteine residues and GSH, possibly by promoting an optimal redox environment [167]. Only few studies have investigated the effects of redox status alterations on the recovery and healing processes following intense exercise in human muscle. In a double blind, crossover, randomized trial, NAC supplementation improved muscle GSH/GSSG ratio throughout recovery and attenuated the elevation of inflammatory markers of muscle damage (CK activity, C-reactive protein, proinflammatory cytokines), NF-κB phosphorylation, and the reduction of muscle strength during the first 2 days of recovery [87]. Of particular interest, NF-κB is associated with the early phase of regeneration and its down-regulation is required for proper SCs activation (via MyoD activation) [168]. In another study, NAC supplementation efficiently altered muscle's redox status following a very intense eccentric exercise protocol, as evident by the attenuated decline of reduced glutathione in erythrocytes, a diminished rise of plasma protein carbonyls as well as an enhanced antioxidant activity observed during recovery [73]. The authors also reported that NAC reduced biomarkers of muscle damage and inflammation (CK activity, CRP, pro-inflammatory cytokines, adhesion molecules) and protected muscle strength during recovery [73]. Furthermore, the rise of HLA^+ and $11B^+$ macrophages, as well as intramuscular macrophage infiltration have been shown to decrease in response to NAC supplementation under conditions of EIMD [73,87]. Altogether, these findings provide strong evidence for a mechanistic pillar between inflammation, redox status and performance in human skeletal muscle following EIMD.

Recent reports suggest that responses to antioxidant supplementation may be individualized [163]. Specifically, in the investigation by Paschalis et al. [169], thirty-six participants were classified in three experimental groups according to their basal GSH levels in blood (low, moderate and high GSH group), and were supplemented with NAC daily (2 × 600 mg), for 30 consecutive days. NAC supplementation restored baseline glutathione levels and redox homeostasis, reduced systemic oxidative stress and improved performance significantly in the low glutathione group [169]. Similarly, a previous work by the same group revealed that low vitamin C levels were associated with decreased physical performance and increased oxidative stress. However, daily vitamin C supplementation (3 × 333 mg) for 30 days, reduced oxidative stress markers and increased exercise performance only in those with low basal vitamin C levels [170]. These findings may explain the discrepancies often observed between studies investigating the effects of antioxidant supplementation on muscle performance. Although in this section we predominantly present indirect evidence of a redox-dependent regulation of performance recovery, we assume that these findings provide valuable information regarding the interconnection between muscle performance, redox status and the role of SCs. Studies that are directly investigating the relationship between redox status, SCs biology and performance in humans are currently lacking.

8. The impact of nutritional and pharmacological interventions on satellite cells-mediated skeletal muscle regeneration

In an attempt to ameliorate the detrimental effects of ROS-induced oxidative damage in muscle tissue and to enhance the myogenic potential as well as the capacity of skeletal muscle to promote healing, a number of *in vivo* and *in vitro* studies have utilized antioxidant and anti-inflammatory supplementation both in basal conditions and following myotrauma (Table 3). In regards to antioxidant manipulation two studies showed that administration of 20 mg/kg/day of proanthocyanidolic oligomer (PCO) orally for 14 days pre- and post-contusion injury resulted in increased SCs number and fetal myosin fibers ($MHCf^+$), improved muscle ultrastructural recovery, enhanced antioxidant capacity and reduced TNF-α levels compared to placebo in adult male Wistar rats [21,22]. *In vitro* and *in vivo* ursolic acid (UA) supplementation at rest (*in vitro*: 10 μM UA + DMSO; *in vivo*: 200 mg/kg) for 7 days resulted in increased $Pax7^+$ and $myogenin^+$ cells, increased myonuclei and myofiber content and reduced SCs apoptosis while cell death was evident [20]. In aged mice, Trolox (a Vitamin E analog) supplementation (*in vitro*: 100 μM 20% FBS containing Ham's F10 medium; *in vivo*: 30 mg/kg/day for 2 weeks intraperitonially) at rest, led to decreased autophagy and senescence of SCs and lowered myostatin mRNA expression levels [171]. Interestingly, improved SCs viability was accompanied by a decrease in mitochondrial ROS production in SCs following supplementation with Trolox [171]. Furthermore, Zhang et al. [172] reported a beneficial effect of nicotinamide riboside (NR) supplementation (400 mg/kg/day for 6 weeks) at rest and following cardiotoxin-induced muscle damage including a pronounced increase in the muscle regeneration and SCs number ($Pax7^+$ cells) 14 days post-injury. In the same study, *mdx* mice treated with the same dosage of NR also exhibited improved muscle regeneration and upregulated SCs content ($Pax7^+$ cells) [172]. Likewise, Hollinger et al. [173] reported that quercetin (plant flavonoid)-enriched diet (0.2% quercetin for 6

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Table 3
The impact of nutritional and pharmacological interventions on myogenic response, regeneration, inflammation and redox status *in vitro* and *in vivo*.

Publication [Refs.]	Species	Condition	Compound administration protocol	Effects on myogenic response	Effects on muscle damage/regeneration	Effects on inflammation	Effects on redox status
Bakhtiari et al. [20]	Isolated SCs	Culture	7 days: UA + DMSO in serum	↑Myogenin$^+$ cells ↓SCs apoptosis ↑Pax7$^+$ cells	N/A	N/A	N/A
	Male C57BL/6 mice	Resting	7 days: 200 mg/kg/day UA	↑Pax7$^+$ cells	↑Myofibers ↑Nuclei ↑Mb	N/A	N/A
Dorchies et al. [175]	*Mdx* primary cells	Oxidative-induced damage	48 h: GTP + EGCg in serum	↑Myogenisity ↑Cell viability	N/A	↑GSH/GSSG ↑GSH	N/A
Hollinger et al. [173]	*Mdx* mice	Resting	6 months: 0.2% quercetin/day	↔Myf5, MyoD myogenin, mRNA	↑Muscle morphology ↔MHCe$^+$ mRNA ↓Muscle fibrosis	↓TNF-α	N/A
Kruger et al. [21]	Male Wistar rats	Contusion-induced damage	14 days: 20 mg/kg/day PCO	↑Pax7$^+$ cells	↑Regeneration ↔CK	↑Macrophages ↓Neutrophils ↔ TNF-α ↔IL-1β ↓IL-6	↑ORAC
Laura Garcia-Prat et al. [171]	Aged mice	Resting	2 weeks: Trolox, 30 mg/kg/day (*in vivo*), 100 μM in serum (*in vitro*)	↓Myostatin mRNA ↓SCs senescence ↓SCs autophagy	N/A	N/A	↓SCs mt-ROS
Lim et al. [174]	CHQ5B cells	SIPS induction	24 h: 50 mg/ml TRF	↑SCs Proliferation ↓Myf5, Myf6, myostatin mRNA ↑SCs renewal	N/A	N/A	N/A
Mackey et al. [176]	Endurance-trained athletes	36-km race	4 days before race: 100 mg/day Indomethacin	↓NCAM$^+$ cells ↔FA1$^+$ cells	↔Central nuclei ↔MHCe$^+$	N/A	N/A
Mackey et al. [177]	Young adults	ES–induced injury	14 days: 1200 mg/day Ibuprofen	↑Notch1$^+$ cells ↔Ki67$^+$ cells ↑Pax7$^+$ cells	↔CK, LDH, Mb ↔Regeneration ↓MHCe$^+$	↔CD68 mRNA	N/A
Messina et al. [108]	*Mdx* mice	Resting	5 weeks: 8,5 mg/kg/day flavocoxid	↑Myogenin$^+$ SCs	↑Muscle morphology ↓CK	↓JNK-1, TNF-α ↓P-p38	N/A
Mikkelsen et al. [64]	Young adults	Damaging exercise	7.5 h: 50 mg/ml indomethacin	↓Pax7$^+$, NCAM$^+$ cells ↔ Ki67$^+$ cells	↔Central nuclei	↑CD16$^+$ cells	N/A
Myburgh et al. [22]	Male Wistar rats	Contusion-induced muscle damage	14 days: 20 mg/kg/day PCO	↑Pax7$^+$, CD56$^+$, CD34$^+$ cells	↑MHCf$^+$ myofibers	↑Macrophages ↓Neutrophils ↓TNF-α ↔IL-6	↑ORAC
Whitehead et al. [126]	*Mdx* mice isolated myofibers	Stretched contractions	20 Mm NAC in serum	N/A	↓Central nuclei ↑Muscle force ↑β-dystroglycan ↑Utrophin	↓Muscle damage ↓NF-κB	↓ROS
Zhang et al. [172]	Aged C57BL/6J mice	Resting and CTX-induced damage	6 weeks: 400 mg/kg/day NR	↑ Pax7$^+$	↑Regeneration	N/A	N/A
	Mdx mice	Resting and CTX-induced damage	6 weeks: 400 mg/kg/day NR	↑Pax7$^+$ cells ↑Beta-Gal$^+$ cells	↑Regeneration	N/A	N/A

SCs, satellite cells; PCO, proanthocyanidolic oligomer; UA, ursolic acid; CK, creatine kinase; H&E: hematoxylin and eosin staining; IL-1β, interleukin-1β; IL-6, interleukin-6; FA1, fetal antigen 1; JNK, c-Jun N-terminal kinases; TNF-α, tumor necrosis factor alpha; ORAC, oxygen radical absorbance capacity; MHCe, embryonic myosin heavy chain; mt-ROS, mitochondrial reactive oxygen species; LDH, lactate dehydrogenase; Mb, myoglobin; i.p., intraperitonially; TRF, tocotrienol-rich fraction; NR, nicotinamide riboside; CTX, cardiotoxin; SIPS, stress-induced premature senescence; ES, electrical stimulation; GTP, green tea polyphenol blend; EGCg, epigallocatechin gallate; eMHC, embryonic myosin heavy chain; NAC, N-acetylcysteine; DHE, Dihydroethidium; P-p38, phosphorylated-p38; ↑, increase; ↓, decrease; ↔, no effect; N/A, not applicable.

months) impoved muscle morphology and reduced fibrosis and TNF-α levels, at basal state, in *mdx* mice. In a model of stress-induced premature senescence (SIPS) induced by H_2O_2, tocotrienol-rich-fraction (Vitamin E isomer) treated CHQ5B cells (50 mg/ml for 24 h post-SIPS) demonstrated increased myoblast proliferation and self-renewal and decreased myostatin mRNA expression levels [174]. Moreover, primary cell cultures (from *mdx* mice) treated for 48 h with green tea polyphenol (GTP) blend and epigallocatechin gallate (EGCg), both classes of catechins, exhibited improved myogenisity (desmin$^+$ immunoreactivity) and cell viability with concomitant upregulation in GSH availability and GSH/GSSG ratio [175].

A few human studies have investigated the effect of anti-inflammatory based supplementation on SCs kinetics (the propagation from a quiescent to an activated/proliferative state) after muscle damaging stimuli, providing equivocal results. Indomethacin supplementation (100 mg/day orally for 4 days) prior to a 36-km race (intense exercise) decreased NCAM$^+$ cells during recovery while no effect was observed in newly formed myofibers and central nuclei count [176]. Muscle infusion with indomethacin (50 mg/ml, 2 μl/min, 7.5 h) prior to and after 200 maximal eccentric contractions of the quadriceps muscles resulted in decreased Pax7$^+$/NCAM$^+$ cells at day 8 of recovery and no alteration in the number of activated myogenic cells (Ki67$^+$ cells), while CD16$^+$ cells increased at day 8 post-exercise [64]. In contrast to these observations, supplementation with ibuprofen (1200 mg/day orally, 14 days before injury and for 30 days post-injury) increased Pax7$^+$ and Notch1$^+$ cells (in all fiber types) at day 7 following electrical stimulation-induced muscle injury, while no alteration was observed in Ki67$^+$ cells (activated SCs) [177]. Ibuprofen failed, however, to alter CD68 mRNA levels, CK, LDH and myoglobin responses, whereas embryonic (MHCe$^+$) and neonatal (MHCn$^+$) fibers decreased at 30 days post-injury [177].

Interestingly, studies examining the effect of antioxidant and/or

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pharmaceutical compounds on SCs activity following intense exercise protocols in skeletal muscle are lacking. It appears that antioxidant and anti-inflammatory treatment ameliorates ROS production, preserves short-term muscle performance following EIMD and expedites muscle healing possibly via upregulation of SCs activation, proliferation and differentiation. Refined intrinsic and extrinsic mechanisms related to redox status in SCs seem to coordinate this process and the redox-specific molecular mechanisms are yet to be determined.

9. Conclusions

Strong evidence from *in vivo* and *in vitro* experiments suggests an interplay between skeletal muscle SCs, inflammation and ROS in response to acute and/or chronic muscle microtrauma characterized by an elevated inflammatory status. Although low levels of ROS are beneficial and necessary for proper cell function through redox-sensitive signaling cascades, excessive ROS production leads to irreversible post-translational modifications (mainly thiol oxidation) and deactivation of key signaling molecules which may be detrimental for the successful tissue healing during regeneration. Taking into consideration the complexity of redox regulations, human studies investigating the effect of redox disturbances and ROS-induced SCs kinetics in conditions characterized by increased inflammation and deterioration of muscle performance are lacking. Evidence from cell lines and animal models indicate that the use of substances with antioxidant properties (i.e. vitamins, polyphenol extracts, NAC, NR) increase SCs viability and function under physiological stress or in pathological conditions such as aging and muscular dystrophy. It is therefore plausible to speculate that antioxidant treatment may offer a valuable nutritional and/or therapeutic strategy in order to enhance skeletal muscle SCs kinetics (by upregulating key survival and action mechanisms) under inflammatory conditions and potentially lead to improved muscle regeneration and performance. Future studies need to examine the SCs response in humans using aseptic models of muscle trauma, in combination with nutritional/pharmaceutical supplements able to alter redox status, in order to elucidate the redox-dependent regulation of SCs kinetics [178]. Although the redox-regulated pathways involved in SCs responses are limited to observations in animal studies, it would be of great importance to examine these redox-sensitive molecular pathways related to SCs responses (i.e. Notch/Wnt, Nrf2, TNF-α/NF-κB and IGF-1/Akt/mTOR signaling) using randomized, well-controlled, human clinical trials. Moreover, key redox sensors, antioxidant molecules and protein/lipid oxidation-modification (i.e. DNA methylation) must be assessed in relationship to SCs kinetics following myotrauma. Results from such studies will provide valuable insight into the impact of redox status during recovery from aseptic muscle trauma/injury and potential nutritional/pharmaceutical strategies to enhance muscle repair and remodeling in health and disease.

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Abbreviations

Akt	Protein kinase B
APC	adenomatous polyposis coli protein
ARE	antioxidant responsive element
CAT	catalase
CK	creatine kinase
CK1	casein kinase 1
Cyt C	cytochrome C
DII	Notch delta ligand
Dvl	dishevelled protein
EIMD	exercise-induced muscle damage
Fas	cell death receptor
FGF	fibroblast growth factor
GPX-1	glutathione peroxidase-1
GSH	glutathione
GSK-3β	glycogen synthase kinase 3 beta
GTP	green tea polyphenol
HGF	hepatocyte growth factor
IFN-γ	interferone-γ
IGF-1	insulin-like growth factor-1
IGF1-R	insulin-like growth factor 1-receptor
IKKα/β	inhibitor of nuclear factor kappa-B kinase subunit alpha/beta
IL-10	interleukin-10
IL-1β	interleukin-1β
IL-6	interleukin-6
Keap1	Kelch-like ECH-associated protein 1
LRP5/6	Low-density lipoprotein receptor-related protein 5/6
M1	pro-inflammatory macrophages
M2	anti-inflammatory macrophages
MCs	mast cells
MHC	myosin heavy chain
$MHCn^+/e^+$	myosin heavy chain neonatal/embryonic
MMPs	matrix metalloproteases
Mrf4	myogenic regulatory factor 4
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
Myf5	myogenic factor 5
Myf6	myogenic factor 6
MyoD	myoblast determination factor
NAC	N-acetylcysteine
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	Notch intracellular domain
NOX-1	NADPH oxidase 1
NR	nicotinamide riboside
Nrf2	nuclear factor erythroid 2-related factor 2
ORAC	oxygen radical absorbance capacity
Pax7	paired box transcription factor 7
Pax3	paired box transcription factor 3
PI3K	Phosphoinositide 3-kinase
PIP2/PIP3	phosphatidylinositol (4,5)-bisphosphate/ phosphatidylinositol (3,4,5)-trisphosphate
PRX	peroxiredoxin
RBP-jk	recombination signal binding protein jk
SCs	satellite cells
SH	cysteine residues
SIPS	stress-induced premature senescence
SO2H	sulfinic acid
SO3H	sulfonic acid
SOD	superoxide dismutase
SOH	sulfenic acid
TGF-β	transforming growth factor beta
TNFR	tumor necrosis factor receptor
TNF-α	tumor necrosis factor-alpha
TRX	thioredoxin
UA	ursolic acid

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VEGF vascular endothelial growth factor
VEGF vascular endothelial growth factor

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Trials

STUDY PROTOCOL Open Access

The redox-dependent regulation of satellite cells following aseptic muscle trauma (SpEED): study protocol for a randomized controlled trial

Konstantinos Papanikolaou[1], Dimitrios Draganidis[1], Athanasios Chatzinikolaou[2], Vassiliki C. Laschou[1], Kalliopi Georgakouli[1], Panagiotis Tsimeas[1], Alexios Batrakoulis[1], Chariklia K. Deli[1], Athanasios Z. Jamurtas[1] and Ioannis G. Fatouros[1*]

Abstract

Background: Muscle satellite cells (SCs) are crucial for muscle regeneration following muscle trauma. Acute skeletal muscle damage results in inflammation and the production of reactive oxygen species (ROS) which may be implicated in SCs activation. Protection of these cells from oxidative damage is essential to ensure sufficient muscle regeneration. The aim of this study is to determine whether SCs activity under conditions of aseptic skeletal muscle trauma induced by exercise is redox-dependent.

Methods/design: Based on the SCs content in their vastus lateralis skeletal muscle, participants will be classified as either high or low respondents. In a randomized, double-blind, crossover, repeated-measures design, participants will then receive either placebo or *N*-acetylcysteine (alters redox potential in muscle) during a preliminary 7-day loading phase, and for eight consecutive days following a single bout of intense muscle-damaging exercise. In both trials, blood samples and muscle biopsies will be collected, and muscle performance and soreness will be measured at baseline, pre-exercise, 2 and 8 days post exercise. Biological samples will be analyzed for redox status and SCs activity. Between trials, a 4-week washout period will be implemented.

Discussion: This study is designed to investigate the impact of redox status on SCs mobilization and thus skeletal muscle potential for regeneration under conditions of aseptic inflammation induced by exercise. Findings of this trial should provide insight into (1) molecular pathways involved in SCs recruitment and muscle healing under conditions of aseptic skeletal muscle trauma present in numerous catabolic conditions and (2) whether skeletal muscle's potential for regeneration depends on its basal SCs content.

Trial registration: ClinicalTrials.gov, ID: NCT03711838. Registered on 19 Oct 2018.

Keywords: Muscle stem cells, Redox potential, Antioxidants, Cell signaling, Tissue regeneration

* Correspondence: ifatouros@pe.uth.gr
[1]School of Physical Education, Sport Sciences and Dietetics, University of Thessaly, Karies, 42100 Trikala, Greece
Full list of author information is available at the end of the article

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Background

Skeletal muscle stem cells (satellite cells – SCs) are required for muscle repair, remodeling and growth [1]. Under conditions of increased transcriptional activity, such as in response to skeletal muscle injury, SCs are activated to promote healing [2]. SCs can be identified by their location (beneath the basal lamina of myofibers) and by the expression of transcriptional proteins, such as paired box protein (Pax7). Each stage of the regeneration process is partly controlled by myogenic regulatory factors (MRFs) which include myogenic factor 5 (Myf5), myogenic determination factor (MyoD), myogenin and myogenic factor 4 (Myf4), all orchestrating skeletal muscle's myogenic programming [3, 4].

Recent data suggest a redox-regulated link between SCs and myogenesis [5]. Muscle injury triggers the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)-mediated apoptosis to control stem-cell survival and maintain stemness [6]. SCs' antioxidant reserves protect these cells from oxidative damage whereas antioxidant enzymes, glutathione peroxidase 3 (GPX-3), superoxide dismutase 2 (SOD-2) and thioredoxin (Trx-1) regulate the homeostatic programming of quiescent SCs [7, 8]. Redox-sensitive signaling pathways, such as Notch and Wnt/β-catenin, are also critical for multiple stages of myogenesis including activation and proliferation of SCs. Interestingly, several Wnt proteins have been shown to regulate SCs activation, proliferation and differentiation [9, 10].

Skeletal muscle trauma is observed in numerous catabolic conditions, characterized by elevated proteolysis and muscle wasting, such as cancer, cachexia and muscular dystrophy, which result in physical capacity impairment and a deteriorated quality of life [11, 12]. In the absence of SCs, injured skeletal muscle abolishes its ability to regenerate or regenerates very poorly in response to muscle injury [13]. Flow cytometric analysis in isolated SCs revealed that the number of $Pax7^+$ cells/mg of muscle in G2/M, G0/G1 and S phases of the cell cycle increased by 202%, 32% and 59%, respectively, at 24 h post injury [14]. Under these conditions, SCs rapidly migrate to the injured area, differentiate into mature myoblasts and contribute to myofiber repair [15]. Exercise-induced muscle damage (EIMD) results in an aseptic muscle microtrauma and a pronounced inflammatory cascade characterized by leukocyte immobilization, macrophage infiltration, cytokine production and increased oxidative stress (OXS) [16, 17]. A study incorporating a muscle damage protocol (300 maximal eccentric contractions) on an isokinetic dynamometer, showed an increase in SCs content even at 180 h post exercise [18]. These marked similarities between aseptic EIMD and trauma makes eccentric exercise a valuable model to investigate the redox-dependent intracellular regulation of SCs involvement in skeletal muscle's healing potential.

Only two studies have examined the effectiveness of antioxidant supplementation on skeletal muscle healing and the inflammatory response in primary cultured cells and rat gastrocnemius muscle demonstrating that polyphenol administration enhances SCs activation and evokes an earlier appearance of M2 macrophages promoting an anti-inflammatory phenotype [19, 20]. However, no studies have investigated, so far, the effect of redox status perturbations on SCs responses and myogenic potential following aseptic trauma in human skeletal muscle. A powerful antioxidant that has been in clinical and sports performance practice for several decades is *N*-acetylcysteine (NAC). NAC is a thiol donor, non-specific antioxidant with a binary antioxidant role: Firstly, NAC directly scavenges a number of reactive oxygen species (ROS) and secondly, it produces reduced cysteine (Cys) by deacetylation, which supports the biosynthesis of endogenous reduced glutathione (GSH) via the activity of γ-glutamylcysteine synthase [21]. GSH plays a pivotal role in cellular metabolism and especially in terms of stem cells' oxidative defense, activity, survival and self-renewal under conditions of increased OXS and ROS production [22]. Low to moderate ROS levels control physiological cellular signaling pathways and facilitate muscle growth and development while high ROS levels impair myogenesis and cause cell death through apoptotic and/or necrotic mechanisms (e.g., NF-kB signaling) [23]. We hypothesize that NAC administration following aseptic muscle trauma, characterized by severe inflammation and high levels of ROS, will alleviate the increase in oxidative stress by decreasing ROS activity levels, resulting in an optimal intracellular redox environment, favoring the activity (activation, proliferation, differentiation and fusion) of SCs and thus facilitating muscle's regenerative process and adaptation following muscle injury. Thus, the objectives of this clinical trial are to examine the effects of redox status perturbation (via NAC administration) on the intracellular pathways responsible for SCs responses following aseptic muscle trauma induced by damaging exercise.

Methods/design

Study overview and design

The methods and ethics of the present study have been approved by the Institutional Review Board of the University of Thessaly (ref. number 1387) and procedures are in accordance with the Declaration of Helsinki, as revised in 2013. The SpEED study incorporates a randomized, two-trial (NAC vs placebo), cross-over, double-blind, repeated-measures design. Participants who will fulfill the inclusion criteria will have their body mass, height, resting metabolic rate (RMR), body composition, muscle strength,

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soreness and maximal oxygen consumption (VO_{2max}) measured. Since protein and antioxidant consumption as well as systematic physical activity (PA) and/or exercise may affect SCs responses and/or redox status, participants' daily nutrient intake (via a 7-day diet recall with emphasis on protein and antioxidant intake) and PA levels (via accelerometry instrumentation) will be monitored for a week before the inception of the study [24–26]. Then, a 2-week adaptation period (only before the first trial) will be applied to adjust (through a balanced diet, using RMR values and daily energy expenditure measurements) protein and antioxidant intake at the levels required by current Recommended Dietary Allowances (RDAs) (0.8–1 g of protein/kg/day; 900 mg/day of vitamin A; 90 mg/day of vitamin C; 600 IU/day of vitamin D; 15 mg/day of vitamin E; 11 mg/day of zinc; 400 mg/day of magnesium and 55 mg/day of selenium) [27, 28]. This dietary protocol will be designed and implemented by a registered dietician. During the same time-frame, familiarization with experimental procedures will be provided.

Evidence exists that skeletal muscle growth and myogenic potential is directly related to its basal SCs content [24, 29]. As such, volunteers will provide a resting (they will abstain from any exercise or intense PA for at least 5 days prior to sampling) muscle biopsy sample (baseline sampling) from their vastus lateralis muscle (of their dominant limb) and based on its SCs content will be stratified to either high (HR) or low (LR) respondents. Participants will be stratified as HR or LR using a K-means cluster analysis. Blood sampling and performance measurements will also be performed at baseline.

Following stratification to the HR and LR groups, volunteers of each group will then perform two trials in a randomized order: (1) NAC ingestion and (2) placebo ingestion. NAC or placebo will be consumed before (a 7-day loading phase) and after (immediately post exercise and for eight consecutive days thereafter) an intense eccentric exercise protocol. During both trials, participants will follow the balanced daily dietary protocol of the adaptation period. However, daily dietary intake will be recorded and analyzed during each trial in an attempt to minimize deviations from the prescribed diet. A 4-week washout period will be implemented between trials (dietary intake during this period will also be adjusted according to that applied during the adaptive period). During the entire experimental period, participants will be asked to abstain from any strenuous PA or exercise. Muscle biopsies and blood samples will be collected after overnight fasting before the exercise protocol (pre-exercise sampling) as well as at 2 and 8 days post exercise. Muscle strength and delayed onset of muscle soreness (DOMS) will be evaluated at the same time points. All measurements and collection of biological samples will be performed at the same time of day, in both trials, to prevent circadian rhythm variations. Figure 1 shows the Consolidated Standards of Reporting Trials (CONSORT) diagram of the study and Fig. 2 illustrates the experimental flowchart for the clinical trials.

The primary outcomes of the study are the SCs-specific markers measured in muscle ($Pax7^+$, $MyoD^+$ cells per type I/II myofibers), the macrophages' markers measured in muscle (cluster of differentiation 11b marker ($CD11b^+$), cluster of differentiation 206 marker ($CD206^+$) cells per myofiber), the myogenesis-related markers measured in muscle (Myf5, MyoD, myogenin, MRF4 and myostatin mRNA expression levels), the cell signaling markers measured in muscle (GPX-3, SOD-2, Trx-1, insulin-like growth factor 1 (IGF-1), Notch1, Wnt3 protein expression levels), and the oxidative stress markers measured in muscle (GSH, oxidized glutathione (GSSG), protein carbonyls (PC) and malondialdehyde (MDA)). The secondary outcomes are the oxidative stress markers measured in blood (total antioxidant capacity (TAC) in serum, GSH, GSSG, catalase (CAT) and hemoglobin (Hb) in red blood cell lysates), and the inflammatory markers measured in blood (C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-8, IL-10), blood creatine kinase activity (CK) as a marker of muscle damage and serum cortisol). Other outcomes include body mass, height, body mass index (BMI), total and regional fat mass, lean mass and body fat, RMR, number of steps/day and time spent at sedentary, light, moderate, vigorous and moderate-to-vigorous PA, total daily energy intake and expenditure, daily intake of carbohydrate, fat, protein, vitamin A, vitamin C, vitamin D, vitamin E, selenium, zinc and magnesium, VO_{2max}, knee extensors' (KE) maximal eccentric and concentric peak torque and DOMS.

Participants recruitment and screening

We will initially recruit 40–60 young men. In organized meetings, participants will be informed by the investigators about the purpose of the study, the experimental procedures and all the possible risks and benefits associated. Participants will be recruited via media advertisements and posters. All volunteers will complete a health history questionnaire and a written consent form will be acquired from each participant by the investigators. All personal information and data obtained will be confidential and only the researchers of the study will have access to the database. Participants will be included in the study if they (1) are healthy, non-smokers, aged 18–30 years; (2) have a BMI of 18.5–24.9 kg/m^2; (3) abstain from any vigorous PA during and ≥ 4 weeks prior to the study; (4) have no recent history of musculoskeletal injury, lower limb trauma and metabolic diseases; and (5) they refrain from consumption of alcohol, caffeine, any type of nutritional supplements, non-steroidal anti-

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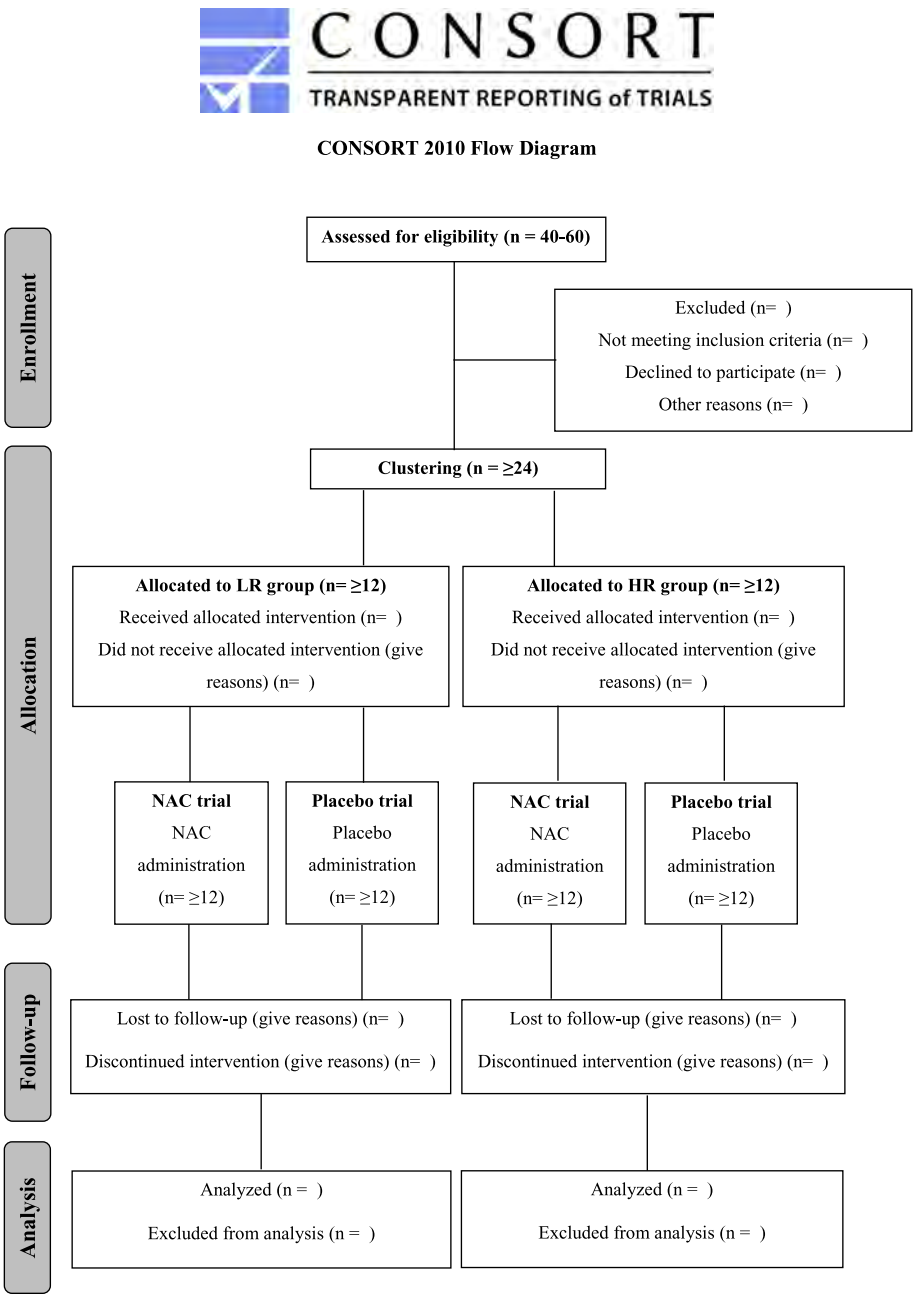

Fig. 1 The Consolidated Standards of Reporting Trials (CONSORT) diagram of the study

inflammatory drugs (NSAIDs) and medication before (≥ 6 months) and throughout the experimental period.

Exclusion criteria

- Allergies or intolerance to NAC
- Recent febrile illness
- Use of anti-inflammatory medication
- Use of medication interacting with muscle metabolism

Exercise protocol

Participants will perform a protocol consisting of 300 eccentric unilateral maximal contractions (20 sets, 15 repetitions/set, 30-s rest between sets) of the quadriceps muscles on an isokinetic dynamometer (Cybex 770, USA) at a speed of 30°/s. A different limb will be used in each trial. Before the protocol a standard warm up will precede involving 8-min cycling on a cycle ergometer (Monark 834, 154 ERGOMED C, Sundbyberg, Sweden) at a speed of 70 rpm/min and at 50 W, followed by 5-

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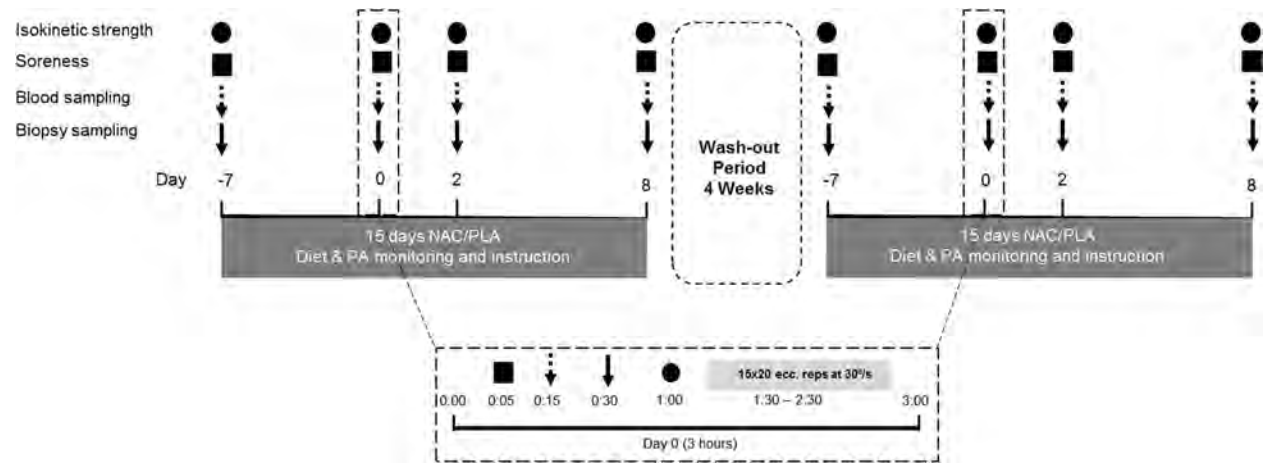

Fig. 2 The experimental flowchart for the clinical trials

min stretching exercises. KE will be isolated using straps in the shoulders, hips and thigh. This protocol has been described in the literature to effectively induce a significant level of skeletal muscle damage and myofibrillar disruption as documented with electron microscopy and immunohistochemistry [30, 31].

Supplementation protocol

Participants will consume either NAC or placebo in a random order according to a double-blind, crossover design. A dosage of 40 mg NAC/kg/day will be administered orally in three doses (equally distributed), in order to maximize cysteine levels (for glutathione synthesis) in the circulation and skeletal muscle, primarily due to NAC fast rate of clearance (~ 6 h post ingestion) [32]. According to a model proposed by Reid in 2001, there is an optimal intracellular redox status required for optimum muscle function and force production [33]. Hence, when NAC is supplemented orally as an exogenous antioxidant (glutathione precursor), under conditions of increased oxidative stress and ROS production, it is plausible to administer a moderate dose for optimal scavenging of ROS, which according to recent studies, represents an absolute dose of ~ 1.2 to 5 g/day and evokes a pronounced improvement in muscle function and performance [34–37]. Larger doses of NAC supplementation may buffer physiological levels of cellular oxidants and may result in impaired muscle force production and performance deterioration [38]. With our dose of 40 mg/kg/day we will reach an absolute dose of approximately ~ 2.8 g of NAC for an average participant weighted ~ 70 kg. Additionally, this dosage has been shown to successfully increase total thiol levels in plasma [39]. NAC in a powder form will be diluted in a 250-ml drink containing 248 ml water and 2 ml of natural, non-caloric, flavoring-sweetener containing sucralose (Flavdrops, My Protein, Cheshire, UK). The placebo supplement will be prepared to be identical to NAC in terms of taste and smell apart from the NAC content. In both trials, each participant will be asked 15 times (once a day) if they realize whether the drink they consumed was the placebo or the experimental one. Responses will be recorded, and correct or incorrect answers will be measured. A research assistant will perform the randomization and assign participants to the interventions (NAC vs placebo) during the clinical trials using an online, computer-based, third-party, semi-automatic randomization system. The same research assistant will have full access to this list and will monitor the presence of any adverse side effects in both trials via questionnaires [30]. Both investigators and participants will be blinded to supplementation condition. Possible adverse reactions to oral NAC supplementation include an upset stomach, nausea, stomach and/or intestinal gas, sleepiness, metallic taste, light-headedness, redness of eye, face, or hands, and cough [39].

Anthropometric measurements

Standing body mass and height will be measured on a beam balance equipped with stadiometer (Beam Balance-Stadiometer, SECA, Vogel & Halke, Hamburg, Germany) while participants wear light clothing as described previously [40]. BMI will be calculated as mass per height squared. Dual energy X-ray absorptiometry (DXA) scanner (GE Healthcare, Lunar DPX-NT, Singapore) will be utilized for body composition assessment. On each testing day the equipment will be calibrated using a LS phantom in accordance to standard procedures. Participants will be asked to remain still and they will be scanned in the supine position using the total body analysis under scanning conditions automatically selected by the software (standard, thick, thin scanning). Total and regional fat mass (g), lean mass (kg) and body fat (% and kg) values

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will be obtained. GE enCORE software will be utilized for all DXA scans and analyses.

Resting metabolic rate

For RMR assessment, resting VO_2/VCO_2 values will be measured in the morning (07:00–09:00) after overnight fasting utilizing an open-circuit type indirect calorimeter with a ventilated hood system (Vmax Encore 29, BEBJO296, Yorba Linda, CA, USA) and the 24-h RMR will be calculated as previously described [41].

Physical activity assessment

Habitual PA will be monitored over a 7-day period using the ActiGraph, GT3X+ accelerometers (ActiGraph, Pensacola, FL, USA). Participants will be taught, by an experienced researcher, how to wear the adjustable belt on the waist with the accelerometer monitor on the right side of the hip and they will be asked to wear it throughout the day for seven consecutive days, apart from bathing, swimming and sleeping. To be included in the analysis, participants will have to complete four full days of wearing time (i.e., ≥ 4 days with ≥ 10 wear hours/day). From the data obtained, non-wear time will be calculated and daily activity levels and sedentary time will be expressed as steps per day and time spent at sedentary, light, moderate, vigorous and moderate-to-vigorous PA [42, 43]. ActiLife 6 software will be used to initialize accelerometers and download data using a 60-s epoch length.

Dietary intake analysis

Participants will be instructed by a registered dietitian on how to estimate food/fluid servings and sizes and how to complete a 7-day diet recall before and during both trials and the washout period to ensure that they will follow the same dietary regimen. Specifically, participants will be provided with colored images showing different food portions and detailed instructions that they will use to weight their food. When possible, the name of the brand and/or manufacturer will be recorded. Diet recalls will be analyzed using the Science Fit Diet 200 A (Science Technologies, Athens, Greece) dietary software for data regarding total energy (kcal), carbohydrate, fat, protein (g/kg/day and g/day), vitamin A (mg/day), vitamin C (mg/day), vitamin D (IU/day), vitamin E (mg/day), selenium (mg/day), zinc (mg/day) and magnesium (mg/day).

Maximal oxygen consumption

VO_{2max} will be measured using open-circuit spirometry with an automated pulmonary gas exchange system (Vmax Encore 29, BEBJO296, Yorba Linda, CA, USA) via the breath-by-breath analysis during a graded exercise test on a treadmill (Stex 8025 T, Daegu, Korea) until volitional fatigue, according to procedures previously described [44]. Briefly, following a standard warm-up (8 min of low-intensity running on a treadmill) each participant will complete a graded exercise test protocol at a starting speed of 8–10 km/h (depending on participants' fitness training history), with an increase of 1 km/h in the running speed every 2 min. During the test VO_2/VCO_2 values will be measured in 20-s intervals. Criteria for terminating the test include: (1) participant reached a level of volitional fatigue, (2) predicted maximum heart rate reached and/or surpassed, (3) respiratory quotient values ≥ 1.10 and (4) plateau in VO_2 values. VO_{2max} will be calculated from the averaged VO_2 measures during the final minute of the test.

Muscle strength and soreness

KE maximal eccentric and concentric peak torque of the exercised limb will be measured on an isokinetic dynamometer (Cybex 770, Rosemont, IL, USA) at 60°/s as described elsewhere [45]. DOMS of KE of the exercised limb will be evaluated by palpation of the belly and distal region after participants have performed three full-squat repetitions. Then, participants will rate their soreness level on a scale from 1 to 10 (1 = no pain, 10 = extremely sore). DOMS assessments will be carried out by the same investigator [46].

Blood sampling and biochemical assays

Following an overnight fasting blood samples will be drawn from the antecubital vein by venipuncture with a 20-gauge disposable needle equipped with a Vacutainer® tube holder (Becton Dickinson, Franklin Lakes, NJ, USA) with the participants in a supine position. For serum separation, blood samples will be allowed to clot at room temperature and then will be centrifuged (1370 g, 10 min, 4 °C). The supernatant will be aliquoted into eppendorf tubes for subsequent analysis of CRP, TNF-α, IL-6, IL-8, IL-10 (inflammation), CK activity (muscle damage), TAC (oxidative stress) and cortisol (hormonal response). Another blood portion will be collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes and will be centrifuged at 1370 g, 10 min, 4 °C to collect the plasma. Plasma samples will be used for the measurement of PC (protein oxidation) and MDA (lipid peroxidation). Packed erythrocytes (RBCs) will be obtained after lysis of the plasma samples for the measurement of GSH, GSSG, CAT and Hb (RBCs' redox status). All samples will be aliquoted in multiple eppendorf tubes and stored at − 80 °C until analysis. A small portion of whole blood (~2 ml) will be collected in tubes

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containing EDTA for a complete blood count analysis on an automated hematology analyzer (Mythic 18, Orphee SA, Geneva, Switzerland). All assays will be performed in duplicate.

Muscle biopsy sampling

Percutaneous needle muscle biopsies will be obtained after an ~ 10 h overnight fast (baseline) using the Bergstrom technique with the application of manual suction, from the mid-portion of the vastus lateralis muscle under local anesthetic (xylocaine 1%), by a registered surgeon [47]. After the biopsy, no antibiotics, pain killers or anti-inflammatory drugs will be administered to participants. Volunteers who will receive any type of pharmaceutical drugs and/or analgesics in the biopsy site, in the rare case of excess bleeding or pain, will be excluded from the analyses. Subjects will be asked to refrain from any PA at least 96 h prior to muscle biopsy sampling. Subsequent muscle biopsies (pre exercise, 2 and 8 days post exercise) will be spaced 5 cm apart to diminish a repeated biopsy effect. Upon excision, adipose tissue and blood will be carefully removed and muscle samples suited for histology will be aligned and immediately be mounted in optimal cutting temperature (OCT) compound, immersed in nitrogen-cooled isopentane and stored at − 80 °C. Embedded samples will be sectioned (7 μm) at − 20 °C using a cryostat, placed on glass slides and stored at − 80 °C. Muscle samples suited for mRNA, Western blotting, muscle thiols and OXS analyses will directly be frozen in liquid nitrogen, and stored at − 80 °C.

Histological analyses

Sections will be stained with hematoxylin and eosin (H&E) in order to quantify damaged myofibers [48]. Myofibers indicating loss of the physiological outline, sarcolemmal damage, mononuclear cell infiltration and centrally located nuclei will be expressed as a percentage of the total number of myofibers.

Immunofluorescence

Muscle cross-sections (7 μm) will be allowed to air dry at room temperature for 30 min. For fiber-type-specific SCs analyses, samples will be stained with appropriate primary and secondary antibodies against specific antigens such as, Pax7, MyoD, myosin heavy chain type II, and laminin as described previously [49–51]. For fiber-type-specific M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophage quantification, muscle cross-sections will be stained with appropriate primary and secondary antibodies against $CD11b^+$, $CD206^+$ and laminin as described previously [52]. Nuclei will be visualized with 4′,6-diamidino-2-phenylindole (DAPI) contained in the mounting media prior to coverslipping. The specificity of staining will be verified using negative controls. Slides will be viewed using an Olympus BX41 microscope equipped with appropriate filters and a high-resolution fluorescent camera. Images will be captured and analyzed using the Image-Pro Plus v6.0 software. All images will be obtained with the 20X objective. Myofiber cross-sectional area (CSA), fiber type distribution (% type I and II fibers), myonuclei content ($DAPI^+$ cells), fiber-type-specific SCs content and activation status ($Pax7^+$ and $MyoD^+$ cells per myofiber) and M1 ($CD11b^+/CD206^-$cells) and M2 ($CD11b^+/CD206^+$ cells) macrophage content will be determined. The SCs content and activation will be determined via the colocalization of Pax7 and DAPI and/or the colocalization of Pax7, MyoD and DAPI within the laminin border. M1 and M2 macrophage content will be determined via the colocalization of CD11b, CD206 and DAPI.

Quantitative RT-PCR

Total RNA will be isolated from 10–20 mg of frozen muscle tissue using the NucleoSpin RNA Plus kit (Machery-Nagel, Bethlehem, PA, USA), according to the manufacturer's instructions, at a final volume of 80–120 μL. RNA concentration (ng/ml) and purity (260/280) will be measured spectophotometrically (Hitachi UV/VIS; Hitachi Instruments Inc., Tokyo, Japan). Then samples will be reverse transcribed using a PrimeScript 1st strand cDNA synthesis kit (Takara Mountain View, CA, USA) in 20-μl reaction volumes, according to the manufacturer's protocol. Quantitative RT-PCR reactions will run in triplicates containing RT Sybr Green qPCR Master Mix. Primers for Myf5, MyoD, myogenin, MRF4, myostatin and gyceraldehyde 3-phosphate dehydrogenase (GAPDH) will be purchased and mRNA expression levels will be calculated using the $2^{-\Delta\Delta Ct}$ method. Fold changes from baseline will be calculated using the $\Delta\Delta C_t$ method and normalization will be performed using the housekeeping gene GAPDH [53].

Western blotting

Changes in protein expression levels of GPX-3, SOD-2 and thioredoxin Trx-1 (related to SCs homeostasis), IGF-1, Notch1 and Wnt3 (related to SC mobilization) will be analyzed by immunoblotting. Muscle samples will be homogenized in lysis buffer and then centrifuged (13, 000 rpm, 4 °C, 10 min) and the supernatant will be collected. Total protein concentration will be determined using the Bradford method (Bradford Protein Assay; Bio-Rad). Twenty milligrams of protein will be loaded in gradient precast gels (Mini-PROTEAN TGX Gels; Bio-Rad, Hercules, CA, USA) and will be subjected to SDS-PAGE electrophoresis at room temperature. Afterwards, proteins will be transferred to trans-blot stacks using the

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Trans-blot Turbo transfer system (Bio-Rad, Hercules, CA, USA), blocked for 1 h and incubated with primary antibodies overnight at 4 °C. Membranes will be washed in tris-buffer saline (TBS-T) solution and will be incubated with appropriate secondary antibodies for 1 h at room temperature. Following another washing step (in TBS-T), membranes will be visualized by chemiluminescence and quantified using densitometry. Normalization will be performed with the housekeeping protein GAPDH.

Muscle thiols and OXS markers

Muscle samples will be homogenized in phosphate buffer saline (PBS) containing protein inhibitors as described previously [54]. After homogenization, the samples will be centrifuged (12,000 g, 4 °C, 30 min) and the supernatant will be collected. GSH, GSSG, PC and MDA levels will be measured as indices of muscle's redox status. All measurements will be performed spectophotometrically (Hitachi UV/VIS; Hitachi Instruments Inc., Tokyo, Japan) as described elsewhere [54]. All assays will be performed in duplicate.

Statistical analyses and Power calculation

A preliminary power analysis (based on previous studies that used NAC administration to investigate its effects on EIMD), using the G*Power 3.0.10 program, showed that a minimum number of 10 participants per group is needed to obtain statistical meaningful results among repeated measurements [30]. Specifically, power calculation was performed for a two-way repeated-measures analysis of variance (ANOVA), within-between interaction test and input variables included: effect size, 0.55; α error, 0.05; power, 0.95; number of groups (LR and HR), 2; correlation among repeated measures, 0.5 and non-sphericity correction, 1. However, the total number of participants depends also on potential dropouts according to the following formula: $n' = n/(1 - d)$ [55].

Thus, the final number of participants to be recruited with a dropout rate of 15% would be $n' = 10/(1–0.15) = 11.8$. Therefore, ≥ 12 participants per group (LR vs HR) will be selected from the initial sample ($N = 40–60$) via k-means clustering to participate in the clinical trial (NAC vs placebo).

A k-means cluster analysis will be utilized to efficiently define two separate groups of subjects (LR and HR groups) from the total sample ($N = 40–60$), based on the SCs content of their vastus lateralis muscle of their dominant leg [24]. This type of analysis requires a relatively large initial sample size ($N = \geq 40$), is a form of partitional clustering and is a multivariate method used to identify homogeneous groups (i.e., clusters) of cases based on a common trait [29, 56].

All analyses and reporting of the results will comply with the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) Statement for reporting randomized clinical trials (RCTs) [57]. Results of participant's baseline characteristics and outcome variables (primary, secondary and other) will be summarized using descriptive statistics and will be expressed as mean (standard deviation) or median (range) for continuous variables. Data normality will be examined using the Kolmogorov-Smirnov and the Shapiro-Wilk tests. If our data sets follow normal distribution, parametric tests will be applied. Baseline comparisons on the LR and HR groups (anthropometrics, body composition, strength, VO_{2max}, dietary profile, PA) will be performed using a one-way ANOVA test. Time- and trial-effect comparisons within and between trial (NAC or placebo) in the LR and HR groups will be analyzed using a two-way repeated-measures ANOVA test with a Bonferroni correction for pairwise comparisons. If the data normality is violated, non-parametric tests will be applied. Baseline comparisons on the LR and HR groups will be performed using a Kruskal-Wallis test. Time-effect comparisons within trial (NAC or placebo) in the LR and HR groups will be analyzed using a Friedman test accompanied by Wilcoxon signed-rank test for pairwise comparisons. Trial-effect comparisons between trials (NAC vs placebo) in the LR and HR groups will be analyzed using a Kruskal-Wallis test accompanied by a Mann-Whitney *U* test for pairwise comparisons. Pearson's correlation analysis will also be used to examine possible relations among variables. Correlation coefficients of $r < 0.2$, $0.2 < r < 0.7$ and $r > 0.7$ will be defined as small, moderate and high, respectively. The level of statistical significance will be set at $p < 0.05$. Effect sizes (ES) and confidence intervals (95% CI) will be calculated on results of all dependent variables using the Hedge's *g* method, corrected for bias. ES will be interpreted as none, small, medium-sized and large for values 0.00–0.19, 0.20–0.49, 0.50–0.79 and ≥ 0.8, respectively. Multiple-imputation analysis will also be utilized to handle missing data during data collection and sensitivity analyses will be executed to evaluate the robustness of the results [58]. Statistical analyses will be performed using the SPSS 20.0 software (IBM Corp., Armonk, NY, USA).

Discussion

The present study is designed to assess the impact of redox status on SCs responses and the mechanisms (hormonal regulation, M1 and M2 macrophages,

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intracellular signaling) associated with their mobilization and function following aseptic skeletal muscle trauma induced by exercise. Nutritional supplementation or medications have been shown to affect SCs biology under traumatic conditions. Hydrolyzed whey protein supplementation and anti-inflammatory medication (ibuprofen) results in increased SCs response and expedites skeletal muscle recovery [25, 48]. Antioxidant supplementation (vitamin C, vitamin E, NAC or combined antioxidants) may attenuate loss of muscle force production and reduce muscle soreness and lipid peroxidation levels but it may also delay recovery suggesting a potential redox-associated mechanism involved in muscle healing [54, 59]. However, this possibility has not been explored in the human skeletal muscle. More specifically, there is no information regarding the redox-dependent mechanism of SCs mobilization and action in human skeletal muscle. It is well established in the literature that thiol oxidation is a major post-translational oxidative modification affecting the cysteine residues in multiple proteins [60]. On the other hand, increased ROS can oxidize GSH leading to irreversible modification [61]. In this sense, low levels of GSH may not only attenuate the antioxidant defense leading to SCs damage but it may also alter cellular redox status drastically. Furthermore, SCs activity is also determined by redox-sensitive cues (cytokines, immune cells, signaling molecules) emerging from the surrounding microenvironment [62]. Supplementation with a powerful GSH precursor, such as NAC, could not only foster GSH levels and thus muscle's antioxidant potential but also will change myofiber redox balance which is crucial for the redox-dependent regulation of intracellular signaling pathways mediating pro- and anti-inflammatory response to muscle trauma [30]. Our research hypothesis states that NAC-induced change of muscle's redox status will upregulate SCs availability and mobility under conditions of increased oxidative stress and inflammation in human skeletal muscle. This is of great importance as muscle injury is present in several clinical conditions characterized by increased muscle wasting, atrophy and sepsis that result in physical deterioration and poor quality of life such as in many types of cancer, cachexia, muscular dystrophies, etc. [11, 12]. Consumption of NAC, a potent thiol-based antioxidant, upregulates GSH/GSSG and reduces the respiratory burst and MAPK- and NF-kB-mediated pro-inflammatory cytokine release during inflammation produced by muscle injury [30, 63]. Information derived from this study will elucidate the redox-dependent regulation of intracellular signaling pathways involved in SCs regulation and muscle healing in human skeletal muscle. Therefore, the results of the proposed study should provide information about possible nutritional and/or pharmaceutical interventions to promote SCs function and increase skeletal muscle's healing potential.

Trial status

The trial has not yet commenced. Participant recruitment is ongoing.

Additional file

Additional file 1: Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) 2013 Checklist: recommended items to address in a clinical trial protocol and related documents. (DOCX 50 kb)

Abbreviations

BMI: Body mass index; CAT: Catalase; CD11b: Cluster of differentiation 11b marker; CD206: Cluster of differentiation 206 marker; CK: Creatine kinase; CRP: C-reactive protein; CSA: Cross-sectional area; DAPI: 4′,6-diamidino-2-phenylindole; DOMS: Delayed onset of muscle soreness; DXA: Dual energy X-ray absorptiometry; EDTA: Ethylenediaminetetraacetic acid; EIMD: Exercise-induced muscle damage; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GPX-3: Glutathione peroxidase 3; GSH: Reduced glutathione; GSSG: Oxidized glutathione; H&E: Hematoxylin and eosin; Hb: Hemoglobin; HR: High respondents; IGF-1: Insulin-like growth factor 1; IL-10: Interleukin 10; IL-6: Interleukin 6; IL-8: Interleukin 8; KE: Knee extensors; LR: Low respondents; M1: Pro-inflammatory-phenotype macrophages; M2: Anti-inflammatory-phenotype macrophages; MAPK: Mitogen-activated protein kinase; MDA: Malondialdehyde; MRFs: Myogenic regulatory factors; mRNA: Messenger RNA; Myf4: Myogenic factor 4; Myf5: Myogenic factor 5; MyoD: Myogenic determination factor; NAC: *N*-acetylcysteine; NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells; OCT: Optimal cutting temperature; OXS: Oxidative stress; PA: Physical activity; Pax7: Paired box protein; PBS: Phosphate buffer saline; PC: Protein carbonyls; RBCs: Red blood cells; RCT: Randomized clinical trial; RDA: Recommended dietary allowance; RMR: Resting metabolic rate; ROS: Reactive oxygen species; RT-PCR: Real-time polymerase chain reaction; SCs: Satellite cells; SOD-2: Superoxide dismutase 2; TAC: Total antioxidant capacity; TBS: Tris-buffer saline; TNF-α: Tumor necrosis factor alpha; Trx1: Thioredoxin 1; VO_{2max}: Maximal oxygen consumption

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Authors' contributions

KP, IGF, AZJ: conception of the study and protocols. DD, AC, VCL, CKD: assisted in further development of the protocol. KG, PT, AB: planned the analyses. All authors drafted and approved the final manuscript.

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Availability of data and materials

All primary and secondary outcome data will be published in data depositories. The study adheres to the SPIRIT 2013 Checklist (Additional file 1).

Ethics approval and consent to participate

The methods and ethics of the present study have been approved by the Institutional Review Board of the University of Thessaly (ref. number 1387). A written consent form will be acquired from each participant by the investigators.

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Consent for publication
All authors gave their consent for publication.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]School of Physical Education, Sport Sciences and Dietetics, University of Thessaly, Karies, 42100 Trikala, Greece. [2]School of Physical Education and Sport Sciences, Democritus University of Thrace, 69100 Komotini, Greece.

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ORIGINAL CONTRIBUTION

Skeletal muscle and erythrocyte redox status is associated with dietary cysteine intake and physical fitness in healthy young physically active men

Konstantinos Papanikolaou[1] · Athanasios Z. Jamurtas[1] · Athanasios Poulios[1] · Panagiotis Tsimeas[1] · Dimitrios Draganidis[1] · Nikos V. Margaritelis[2] · Ioannis Baloyiannis[3] · Constantinos Papadopoulos[4] · Apostolos Sovatzidis[5] · Chariklia K. Deli[1] · Anastasia Rosvoglou[1] · Kalliopi Georgakouli[6] · Theofanis Tzatzakis[1] · Michalis G. Nikolaidis[2] · Ioannis G. Fatouros[1]

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Abstract

Purpose To investigate the association between redox status in erythrocytes and skeletal muscle with dietary nutrient intake and markers of physical fitness and habitual physical activity (PA).

Methods Forty-five young physically active men were assessed for body composition, dietary nutrient intake, muscle strength, cardiorespiratory capacity and habitual PA. Blood and muscle samples were collected to estimate selected redox biomarkers. Partial correlation analysis was used to evaluate the independent relationship of each factor with redox biomarkers.

Results Dietary cysteine intake was positively correlated ($p<0.001$) with both erythrocyte ($r=0.697$) and muscle GSH (0.654, $p<0.001$), erythrocyte reduced/oxidized glutathione ratio (GSH/GSSG) ($r=0.530$, $p=0.001$) and glutathione reductase (GR) activity ($r=0.352$, $p=0.030$) and inversely correlated with erythrocyte protein carbonyls (PC) levels ($r=-0.325$; $p=0.046$). Knee extensors eccentric peak torque was positively correlated with GR activity ($r=0.355$; $p=0.031$) while, one-repetition maximum in back squat exercise was positively correlated with erythrocyte GSH/GSSG ratio ($r=0.401$; $p=0.014$) and inversely correlated with erythrocyte GSSG and PC ($r=-0.441$, $p=0.006$; $r=-0.413$, $p=0.011$ respectively). Glutathione peroxidase (GPx) activity was positively correlated with step count ($r=0.520$; $p<0.001$), light ($r=0.406$; $p=0.008$), moderate ($r=0.417$; $p=0.006$), moderate-to-vigorous ($r=0.475$; $p=0.001$), vigorous ($r=0.352$; $p=0.022$) and very vigorous ($r=0.326$; $p=0.035$) PA. Muscle GSSG inversely correlated with light PA ($r=-0.353$; $p=0.022$).

Conclusion These results indicate that dietary cysteine intake may be a critical element for the regulation of glutathione metabolism and redox status in two different tissues pinpointing the independent significance of cysteine for optimal redox regulation. Musculoskeletal fitness and PA levels may be predictors of skeletal muscle, but not erythrocyte, antioxidant capacity.

Trial registration Registry: ClinicalTrials.gov, identifier: NCT03711838, date of registration: October 19, 2018.

Keywords Glutathione · Cysteine · Antioxidant enzymes · Redox regulation · Muscle function · Physical activity

✉ Ioannis G. Fatouros
ifatouros@pe.uth.gr

1 Department of Physical Education and Sports Science, University of Thessaly, 42100 Trikala, Greece

2 Department of Physical Education and Sports Science, Aristotle University of Thessaloniki, 62110 Serres, Greece

3 Department of Surgery, University Hospital of Larissa, School of Medicine, University of Thessaly, 41500 Larissa, Greece

4 First Department of Neurology, Aeginition Hospital, School of Medicine, National and Kapodistrian University of Athens, 11528 Athens, Greece

5 Department of Nephrology, University Hospital of Alexandroupolis, School of Medicine, Democritus University of Thrace, 68100 Alexandroupolis, Greece

6 Department of Nutrition and Dietetics, University of Thessaly, 42100 Trikala, Greece

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Introduction

Glutathione is a ubiquitously distributed antioxidant consisting of the three amino acids cysteine, glycine and glutamic acid, exerting its biological antioxidant activity via the thiol group of cysteine residues [1]. The tripeptide is mainly present in its reduced (GSH) and oxidized form (glutathione disulfide, GSSG), and the ratio between these (GSH/GSSG) is used as an index of cellular redox status [2]. Glutathione acts as a direct scavenger of free radicals (reactive oxygen and nitrogen species, RONS) as well as a substrate for the glutathione peroxidase (GPx) enzyme family [3]. GPx catalyzes the reduction of hydrogen peroxide (H_2O_2) to water through the oxidation of GSH to GSSG, which is reduced back to GSH by the activity of glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate (NADPH) [4]. Through its antioxidant properties, GSH is essential for numerous intracellular and physiological processes including redox signaling, immunoregulation, muscle metabolism and function and, thus, low glutathione availability may have important physiological implications [5–7].

Evidence suggests that genetics play an important role in GSH deficiency or sufficiency as mutations in the GSH synthase gene could severely impact its concentration [8, 9]. Furthermore, nutrition and especially cysteine intake is also a significant determinant for GSH bioavailability and redox status. Human and animal studies have revealed that inadequate sulfur amino acid intake may result in low cysteine and GSH levels [10, 11]. In fact, reduced dietary cysteine intake is associated with decreased erythrocyte GSH concentration and elevated plasma lipid peroxidation levels in young individuals [12]. Furthermore, recent studies indicate that individualized antioxidant interventions targeting in reversing specific antioxidant inadequacies (i.e., N-acetylcysteine for glutathione deficiency or vitamin C for vitamin C deficiency) could result in improved redox profile and physiological function [13], while the presence of oxidative stress per se does not justify the general use of antioxidants [14].

Beyond the dietary regulation, physical activity (PA) has been also considered a crucial determinant of redox status. Increased PA levels have been associated with enhanced systemic redox status and reduced oxidative stress both in youth [15] and elderly [16] populations. Nevertheless, the relationship between tissue-specific (i.e., skeletal muscle) redox status and PA remains to be elucidated. Studies in humans have shown that low levels of GSH and other antioxidants (i.e., vitamin C, vitamin E) are linked to increased oxidative stress and impaired muscle performance, a phenomenon that is reversible via specific antioxidant supplementation [17, 18]. However, previous reports suggest that general use of antioxidants should be treated with caution as it may interfere with physiological adaptations to exercise (i.e., mitochondrial biogenesis and glucose uptake) [19, 20]. Moreover, under physiological conditions, oxidative stress has been shown to promote a signaling cascade, necessary for optimal muscle regeneration following exercise-induced muscle damage [21].

Despite the pivotal role of redox status in cellular homeostasis and muscle function, there is lack of data regarding the relationship between tissue-specific (particularly in skeletal muscle) redox status constituents (i.e., GSH state, antioxidant enzyme activity) and dietary cysteine intake, as well as physical fitness and PA profile. Therefore, the aim of this investigation was to examine whether erythrocyte and skeletal muscle redox status is associated with (i) dietary nutrient intake, (ii) lower limb muscle strength, (iii) cardiorespiratory capacity and (iv) habitual PA levels in healthy young physically active men. We hypothesized that decreased redox status in erythrocytes and skeletal muscle would be independently associated with lower dietary cysteine intake, physical fitness and PA levels.

Methods

Participants and experimental design

This study is part of a primary randomized controlled trial, with its purpose and methodology described in detail elsewhere [22]. In this work, data upon screening/baseline secondary variables (redox status, dietary nutrient intake, physical fitness and PA) are presented. Figure 1 depicts the timeframe diagram of the study. A total of 48 healthy young men 18–30 years volunteered and underwent baseline medical screening. Participation in this investigation was secured if participants: (a) abstained from smoking; (b) refrained from unaccustomed and/or heavy exercise ≥ 4 weeks before the study; (c) had no recent history of musculoskeletal injury, febrile illness, lower limb trauma and metabolic diseases and (d) abstained from the consumption of alcohol, caffeine, nutritional supplements, and medication (i.e., non-steroidal anti-inflammatory drugs) before (≥ 6 months) and throughout the study. Following recruitment, participants who met the inclusion criteria (n = 45) underwent an assessment of body mass, body height, body composition, lower limb muscle strength and cardiorespiratory fitness. Thereafter, participants were given accelerometers and diet recalls to assess their habitual PA and nutrient intake, respectively, during a 7-day period. Thereafter, participants were instructed to refrain from any strenuous PA and/or exercise for 5 days and a resting blood and muscle biopsy sample was collected early in the morning following overnight fasting. Participants' anthropometric characteristics, dietary intake,

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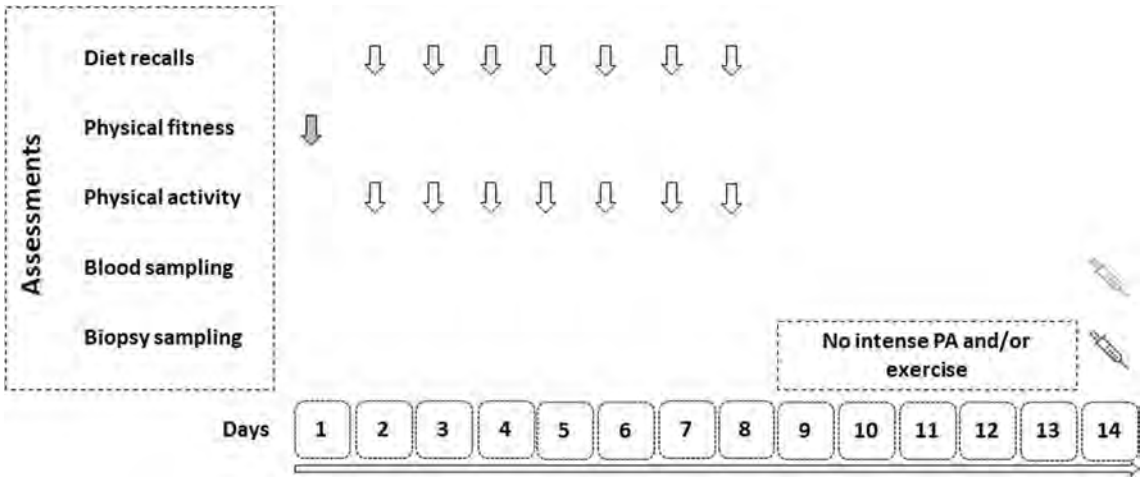

Fig. 1 The timeframe diagram of the study. *PA,* physical activity

muscle strength, cardiorespiratory capacity, PA and redox status are shown in Table 1. The study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The overall study was approved by the Institutional Review Board of the University of Thessaly (#1387/2018) and was pre-registered at clinicaltrials.gov (ID: NCT03711838).

Body composition

Body height was measured with a stadiometer (Stadiometer, SECA, Vogel & Halke, Hamburg, Germany) as described [23], while body mass and composition (total, fat and lean mass) were measured by dual emission X-ray absorptiometry (DXA, GE Healthcare, Lunar DPX NT, Diegem, Belgium) as described before [24].

Dietary nutrient intake

Participants were instructed by a registered dietician on how to complete a 7-day diet recall by precisely estimating food/fluid servings and sizes. Colored food images were also provided, illustrating different portion amounts that they could use to estimate their food volume [25]. Participants were asked to describe their dietary intake in as much detail as possible (i.e., the name of the manufacturer for commercially available products). Diet recalls were analyzed using the Science Fit Diet 200A (Science Technologies, Athens, Greece) dietary software and the following parameters were estimated: energy (kcal/day), protein (g/day and % of total energy/day), carbohydrates (g/day and % of total energy/day), fat (g/day and % of total energy/day), cereals (g/day), legumes (g/day), vitamin C (mg/day), vitamin E (mg/day), selenium (μg/day), zinc (mg/day), cysteine (g/day), glutamic acid (g/day), glycine (g/day) and methionine (g/day). From the estimated parameters, the following variables were also calculated: total cysteine, glutamic acid and glycine intake (Cyst-GA-Gly in g/day), sulfur amino acid (SAA) intake (cysteine and methionine in g/day), cysteine/energy (g/kcal/day), cysteine/protein (g/g/day) and cysteine/SAA (g/g/day). Unusual foods in participants' diets, not established in the database, were analyzed by substituting these foods with appropriate equivalents (according to their nutrient category, composition and quantity) which were designed and validated by a registered dietician, to produce complete dietary analysis reports.

Muscle strength

Maximal knee extensors' eccentric peak torque (Nm) at 60°/s was measured using an isokinetic dynamometer (Cybex Norm 770, Ronkonkoma, New York) after a familiarization session as previously described [26]. Maximal strength (kg) in back squat (one-repetition maximum, 1RM) was determined using standardized procedures as previously described [27] and following familiarization.

Cardiorespiratory capacity

Maximal oxygen consumption (VO_2max) was assessed during a graded exercise test on a treadmill (Stex 8025 T, Korea) by open-circuit online spirometry, using an automated pulmonary gas exchange system (Vmax Encore 29, BEBJO296, Yorba Linda, CA, USA) via breath-by-breath analysis as previously described [28] and expressed as ml/kg/min.

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Table 1 Participants' anthropometric characteristics, dietary nutrient intake, physical fitness, physical activity and redox status

Anthropometrics		
Age (years)	21.40 (19.95/22.80)	[18.00–28.10]
Body mass (kg)	73.66 ± 10.32	[50.70–93.40]
Height (m)	1.76 ± 0.07	[1.60–1.93]
BMI (kg/m^2)	23.65 ± 2.37	[17.75–28.33]
Body fat (%)	19.48 ± 7.57	[7.10–35.30]
Lean mass (kg)	54.99 ± 6.43	[39.12–69.60]
Dietary intake		
Total energy (kcal/day)	2245.05 (1842.41/2636.59)	[1201.49–4753.59]
Protein (g/kg/day)	1.27 (1.06/1.65)	[0.60–3.00]
Protein (% of total energy/day)	17.30 ± 2.96	[10.98–25.00]
Carbohydrates (g/day)	255.47 ± 84.47	[100.31–502.68]
Carbohydrates (% of total energy/day)	44.10 (40.60/47.90)	[26.16–58.36]
Fat (g/day)	88.92 (71.64/114.94)	[43.40–227.30]
Fat (% of total energy/day)	37.22 (33.87/40.52)	[26.16–71.95]
Cereals (g/day)	373.90 ± 13.52	[215.60–589.40]
Legumes (g/day)	21.40 (0.00/37.50)	[0.00–84.00]
Vitamin C (mg/day)	89.83 (65.62/132.76)	[8.68–646.52]
Vitamin E (mg/day)	7.63 (5.81/10.10)	[1.35–20.35]
Selenium (μg/day)	125.76 (101.06/150.67)	[67.05–227.81]
Zinc (mg/day)	13.15 ± 4.44	[6.50–23.40]
Methionine (g/day)	2.47 ± 0.94	[0.40–4.80]
Cysteine (g/day)	1.50 (1.05/2.20)	[0.28–3.30]
Glutamic acid (g/day)	16.55 (14.95/20.38)	[4.32–36.57]
Glycine (g/day)	3.69 ± 1.16	[0.48–6.60]
SAA (g/day)	4.10 ± 1.63	[0.68–8.10]
Physical fitness		
KE eccentric peak torque (Nm)	274.11 ± 55.24	[180.00–400.00]
1RM back squat (kg)	105.00 (100.00/116.25)	[64.70–170.00]
VO_2max (ml/kg/min)	50.18 ± 4.82	[34.60–60.80]
Physical activity		
Steps count (*n*)	7248.56 ± 2553.15	[818.00–11,771.00]
ST (min/day)	459.20 ± 132.45	[101.00–751.00]
LPA (min/day)	256.33 ± 94.12	[46.50–505.00]
MPA (min/day)	45.85 ± 18.58	[3.80–81.20]
VPA (min/day)	2.60 (0.67/6.60)	[0.00–17.20]
VVPA (min/day)	0.00 (0.00/0.80)	[0.00–12.80]
MVPA (min/day)	51.56 ± 21.44	[4.60–101.40]
Erythrocyte redox status		
GSH (μmol/g Hb)	4.48 ± 1.16	[2.44–7.07]
GSSG (μmol/g Hb)	0.25 ± 0.09	[0.11–0.49]
GSH/GSSG ratio	20.37 ± 7.69	[5.63–38.49]
PC (nmol/g Hb)	2.10 (1.04/3.53)	[0.29–5.61]
Skeletal muscle redox status		
GSH (nmol/mg protein)	19.07 ± 5.70	[8.80–37.21]
GSSG (nmol/mg protein)	0.82 (0.56/1.30)	[0.32–2.36]
GSH/GSSG ratio	24.77 ± 12.83	[5.24–57.75]
GPx (U/mg protein)	0.0117 ± 0.0023	[0.0070–0.0170]
GR (U/mg protein)	0.0018 ± 0.0005	[0.0010–0.0030]

Data are presented as mean ± SD or median and 25%/75% intervals in parenthesis with ranges in brackets ($n = 45$)

BMI body mass index, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *GPx* glutathione peroxidase, *GR* glutathione reductase, *KE* knee extensors, *LPA* light physical activity, *MPA* moderate physical activity, *MVPA* moderate-to-vigorous physical activity, *PC* protein carbonyls, *SAA* sulfur amino acids, *ST* sedentary time, *VO_2*max maximal oxygen consumption, *VPA* vigorous physical activity, *VVPA* very vigorous physical activity, *1RM* 1-repetition maximum

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Habitual physical activity

PA was assessed using the tri-axial GT3X + accelerometers (ActiGraph, Pensacola, FL, USA). Participants were instructed on how to wear the elastic belt containing the accelerometer around their waist with the device aligned on the right hip and they were asked to wear it throughout the day, except for bathing and sleep, for 7 consecutive days. Data were analyzed if participants had 5 days with ≥ 10 wear hours/day. Non-wear time was calculated according to the algorithms developed by Choi et al. [29] for vector magnitude (VM) data and categorized as intervals of counts/minute (cpm). Daily activity and sedentary time were estimated according to VM data and expressed as steps/day and time/day in sedentary (< 199 cpm), light (200–2689 cpm), moderate (2690–6166 cpm), vigorous (6167–9642 cpm), very vigorous (> 9643 cpm) and moderate-to-vigorous (2690–6167 cpm) PA [30]. ActiLife 6 software was used to set up accelerometers and download data in 60-s epoch length intervals.

Blood sampling and redox assays

Blood samples were collected from an antecubital arm vein using a 20-gauge disposable needle equipped with a vacutainer tube holder after overnight fasting. Samples were collected into tubes with ethylenediaminetetraacetic acid (EDTA) that were immediately centrifuged for plasma separation (1370 g, 4 °C, 10 min). Subsequently, plasma was discarded and an equal volume of distilled water was added to the packed erythrocytes. Tubes were vigorously mixed and centrifuged (4000 g, 4 °C, 15 min), and the resultant erythrocyte lysate was collected for subsequent analysis of GSH, GSSG, protein carbonyls (PC) and hemoglobin (Hb). Erythrocyte lysates were stored at − 80 °C in multiple aliquots until assayed. Hb was measured spectrophotometrically using a commercially available kit (Zafiropoulos, Greece). Erythrocyte GSH, GSSG and PC were determined spectrophotometrically as described previously [31] and normalized to Hb concentration.

Muscle biopsy sampling and redox assays

Muscle biopsy samples were obtained from the middle portion of vastus lateralis muscle under sterile conditions and application of local anesthetic (xylocaine 1%), using the Bergstrom needle technique modified for manual suction, as previously described [23]. Muscle samples were separated from any visible fat and connective tissue residues, immediately frozen in liquid nitrogen and stored at -80 °C for further analysis of GSH and GSSG concentration as well as GPx, and GR enzyme activity.

Prior to analyses, muscle samples were homogenized in ice-cold phosphate-buffered saline (PBS, 0.01 M, 1 mM EDTA, pH: 7.4) containing a protease inhibitors cocktail (1 mM leupeptin, 1 mM aprotinin, 1 mM PMSF) and the lysate was centrifuged (15,000 g, 4 °C, 15 min) to obtain a clear supernatant. Total protein concentration was determined using the Bradford assay (Bradford Protein Assay, Bio-Rad).

Muscle GSH and GSSG concentrations were determined spectrophotometrically as described previously [31]. Briefly, for GSH, 50 µl of muscle homogenate were treated with 5% trichloroacetic acid (TCA) and centrifuged (15,000 g, 4 °C, 5 min), and the supernatant was collected. Then, 20 µl of the supernatant were mixed with 660 µl of phosphate buffer (PB, 67 mM, pH 7.95) and 330 µl of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, 1 mM). The samples were incubated in the dark at room temperature for 15 min, and the absorbance was read at 412 nm. GSH concentration was normalized to total protein content.

For GSSG, 50 µl of muscle homogenate were treated with 5% TCA, centrifuged (15,000 g, 4 °C, 5 min) and neutralized to pH 7.0–7.5 with NaOH. Thereafter, 1 µl of 2-vinylpyridine was added, and the samples were incubated for 2 h at room temperature with vortexing every 15 min. 5 µl of sample were mixed with 600 µl of PB (143 mM, 6.3 mM of EDTA, pH 7.5), 100 µl of nicotinamide adenine dinucleotide phosphate (NADPH, 3 mM), 100 µl DTNB (10 mM) and 194 µl of distilled water and the samples were incubated for 10 min at room temperature. Following the addition of 1 µl of glutathione reductase, the change in absorbance at 412 nm was read for 1 min. GSSG concentration was normalized to total protein content.

GPx activity was determined as previously described [32]. Briefly, 500 µl PB (100 mM, 1 mM EDTA, pH 7), 100 µl GR (0.24 U), 100 µl GSH (10 mM) and 100 µl of the muscle homogenate (diluted 1:10 in PBS) were mixed and the solution was incubated at room temperature for 10 min. Then, 100 µl NADPH (1.5 mM in 0.1% $NaHCO_3$) solution was added and the samples were incubated at room temperature for 3 min. Afterward, 100 µl of tert-butyl hydroperoxide were added to the samples and the decrease in absorbance at 340 nm was monitored for 5 min. GPx activity was calculated based on the molar extinction coefficient of NADPH (6200 l/mol/cm) and normalized to total protein concentration.

GR activity was determined as previously originally described [33] and modified [34]. Briefly, for the samples, 700 µl PB (200 mM, 1 mM EDTA, pH 7.5), 250 µl 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, 3 mM in PBS), 50 µl NADPH (2 mM in PBS) and 50 µl GSSG (20 mM in PBS) were mixed. The reaction started by the addition of 25 µl of

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the muscle homogenate (diluted 1:2 in PBS) and the increase in absorbance was monitored at 412 nm for 1 min. For the standard, muscle homogenate was replaced by 25 µl of GR (1 U/ml) solution. GR activity was calculated based on the absorbance change of the standard sample and normalized to total protein concentration.

Statistical analyses

Data normality was evaluated using the Shapiro–Wilk test. The relationship between selected variables was tested using bivariate or partial correlation analysis to exclude the effects of confounding variables in the relationships examined. Specifically, (i) the relationship between redox status and dietary nutrient intake was controlled for physical fitness and PA levels, (ii) the relationship between redox status and physical fitness was controlled for dietary nutrient intake and PA levels and (iii) the relationship between redox status and PA was controlled for dietary nutrient intake and physical fitness level. For partial correlation analysis, the variables included in the dietary nutrient intake, physical fitness and PA sets were the following: dietary nutrient intake (cysteine, glutamic acid and glycine), physical fitness (knee extensors peak torque, 1RM squat and VO_2max), PA (step count, sedentary time, light PA, moderate PA, vigorous PA, very vigorous PA and moderate-to-vigorous PA). When at least one of the variables (i.e., in each pair of variables that were examined for relationship) violated the assumptions of parametric analyses, non-parametric (Spearman) partial correlation analysis was utilized. This type of analysis has been used to assess the relationship between non-normally distributed variables while controlling for confounding factors [35, 36]. The variables in which normal distribution was violated were the following: age, energy, protein (g/kg/day), carbohydrates (g/day), fat (g/day and % of total energy/day), legumes, selenium, vitamin C, vitamin E, cysteine, glutamic acid, Cyst-GA-Gly, cysteine/energy, cysteine/protein, 1RM back squat, vigorous PA, very vigorous PA, erythrocyte protein carbonyls and muscle oxidized glutathione. The magnitude of the correlations was considered as minimal, small-, medium- and large-sized for values 0.1–0.2, 0.2–0.5, 0.5–0.8 and > 0.8, respectively, according to Cohen's d criteria. Linear regression analysis was also conducted and 95% confidence bands of the best-fit line were estimated. Differences in cereal and legume intake were examined using an independent samples *T* test and a non-parametric Mann–Whitney test, respectively. The level of statistical significance was set at $p < 0.05$. Data are presented as means ± SD for normally distributed variables and as median with 25%/75% intervals in parenthesis for non-normally distributed variables. Statistical analyses were performed using the SPSS software (IBM SPSS Statistics, version 25.0).

Results

Participants' anthropometric characteristics, dietary profile, physical fitness, physical activity and redox status

Overall, participants' daily dietary macro- and micronutrient intake was within the safe and recommended range (Table 1) for the age, gender and PA level [37, 38]. From the total sample, the proportion of underweight, normal and overweight participants, according to their BMI score, was 2.2, 68.9 and 28.9%, respectively. Moreover, participants' PA level was at the upper limit of the current recommendations adopted by WHO for the adult population aged 18–64 (150–300 min of moderate PA per week) [39]. However, it should be noted that participants' PA profile exhibited a substantial level of variability, and in some cases, it was twice as high as some measures of average PA in this age group.

The independent relationship between redox status and dietary nutrient intake

Figure 2 depicts the correlation between redox status and dietary nutrient intake. Dietary cysteine intake was positively correlated with both erythrocyte and muscle GSH ($r = 0.697$, $p < 0.001$ and $r = 0.654$, $p < 0.001$, respectively), erythrocyte GSH/GSSG ratio ($r = 0.530$, $p = 0.001$), and muscle GR activity ($r = 0.352$, $p = 0.030$), while it was inversely correlated with erythrocyte PC ($r = -0.325$, $p = 0.046$). Glutamic acid and glycine intake as well as the total intake of cysteine, glutamic acid and glycine (Cyst-GA-Gly) showed no significant correlation with redox biomarkers (Table 2). Total SAA intake was positively correlated only with erythrocyte and muscle GSH ($r = 0.566$, $p < 0.001$ and $r = 0.374$, $p = 0.027$, respectively) (Table 2). The correlations between dietary cysteine intake and redox biomarkers with and without the adjustments for confounders are presented in supplementary file 1.

The independent relationship between redox status and physical fitness

Figure 3 illustrates the correlation between redox status and physical fitness markers. Knee extensors' eccentric peak torque was positively correlated with muscle GR enzyme activity ($r = 0.355$, $p = 0.031$). 1RM in back squat exercise was positively correlated with erythrocyte GSH/GSSG ratio ($r = 0.401$, $p = 0.014$) and inversely correlated with erythrocyte GSSG ($r = -0.441$, $p = 0.006$) and PC ($r = -0.413$, $p = 0.011$). No other significant correlations were detected between redox status and physical fitness variables. The correlations between

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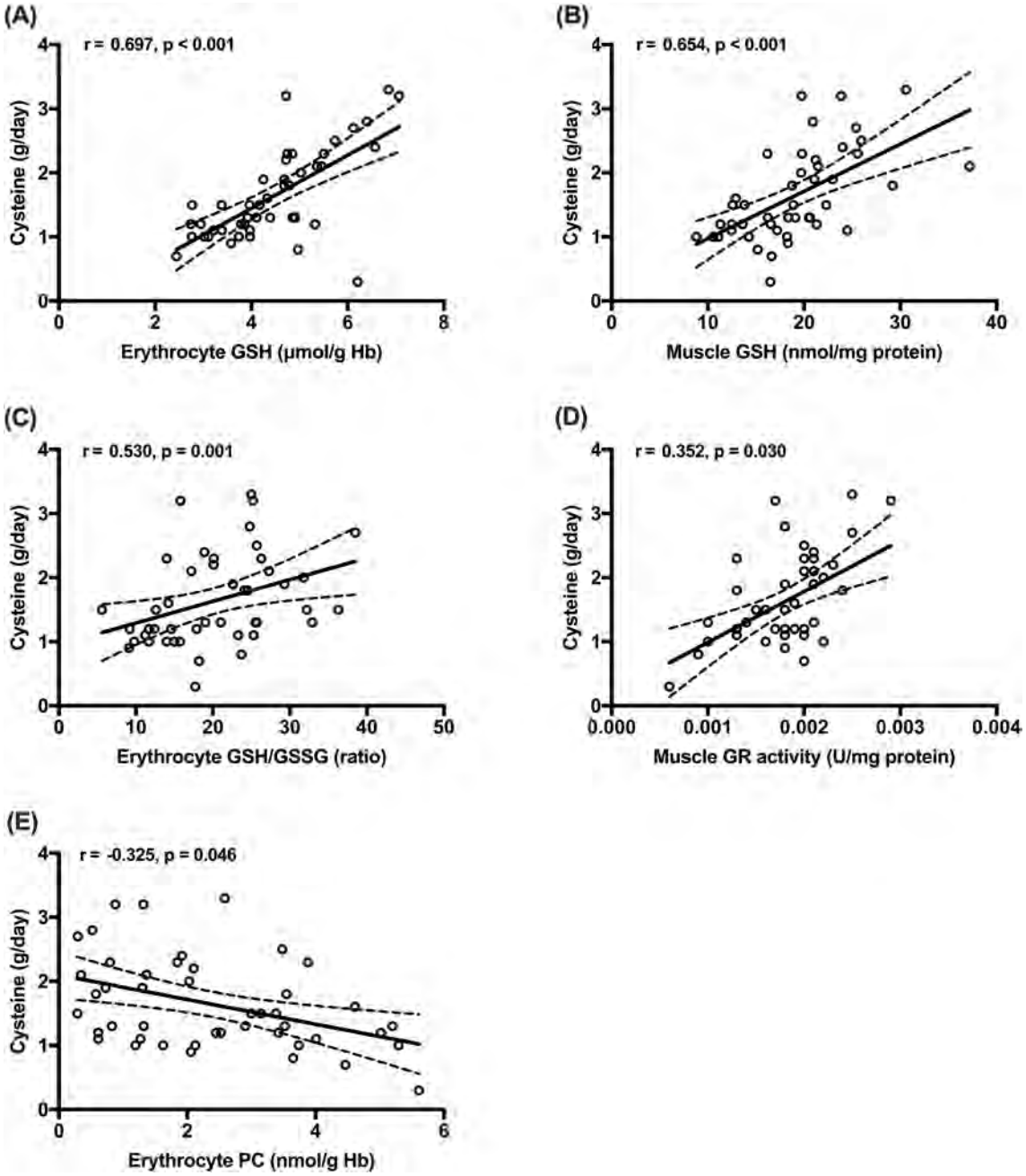

Fig. 2 Partial correlation and linear regression analyses between dietary cysteine intake vs erythrocyte (**A**) and muscle (**B**) glutathione, erythrocyte reduced/oxidized glutathione ratio (**C**), muscle glutathione reductase enzyme activity (**D**) and erythrocyte protein carbonyls (**E**), ($n = 45$). Correlations were adjusted for physical fitness and PA variables. *GSH* glutathione, *GR* glutathione reductase, *GSSG* oxidized glutathione, *PC* protein carbonyls

physical fitness and redox biomarkers with and without the adjustments for confounders are presented in supplementary file 1.

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Table 2 Partial correlations between glutamic acid, glycine, Cyst-GA-Gly and SAA dietary intake with redox biomarkers

Glutamic acid vs redox biomarkers	
Erythrocytes	
Glutamic acid (g/day) and GSH (μmol/g Hb)	$r = -0.007$; $p = 0.965$
Glutamic acid (g/day) and GSSG (μmol/g Hb)	$r = 0.123$; $p = 0.475$
Glutamic acid (g/day) and GSH/GSSG (ratio)	$r = 0.011$; $p = 0.951$
Glutamic acid (g/day) and PC (nmol/g Hb)	$r = 0.194$; $p = 0.258$
Skeletal muscle	
Glutamic acid (g/day) and GSH (nmol/mg protein)	$r = -0.031$; $p = 0.857$
Glutamic acid (g/day) and GSSG (nmol/mg protein)	$r = 0.206$; $p = 0.228$
Glutamic acid (g/day) and GSH/GSSG (ratio)	$r = -0.204$; $p = 0.233$
Glutamic acid (g/day) and GPx activity (U/mg protein)	$r = 0.246$; $p = 0.148$
Glutamic acid (g/day) and GR activity (U/mg protein)	$r = -0.002$; $p = 0.992$
Glycine vs redox biomarkers	
Erythrocytes	
Glycine (g/day) and GSH (μmol/g Hb)	$r = -0.029$; $p = 0.867$
Glycine (g/day) and GSSG (μmol/g Hb)	$r = 0.204$; $p = 0.226$
Glycine (g/day) and GSH/GSSG (ratio)	$r = -0.162$; $p = 0.339$
Glycine (g/day) and PC (nmol/g Hb)	$r = 0.205$; $p = 0.231$
Skeletal muscle	
Glycine (g/day) and GSH (nmol/mg protein)	$r = -0.186$; $p = 0.270$
Glycine (g/day) and GSSG (nmol/mg protein)	$r = 0.252$; $p = 0.138$
Glycine (g/day) and GSH/GSSG (ratio)	$r = -0.307$; $p = 0.064$
Glycine (g/day) and GPx activity (U/mg protein)	$r = -0.079$; $p = 0.641$
Glycine (g/day) and GR activity (U/mg protein)	$r = -0.113$; $p = 0.507$
Cyst-GA-Gly vs redox biomarkers	
Erythrocytes	
Cyst-GA-Gly (g/day) and GSH (μmol/g Hb)	$r = 0.084$; $p = 0.624$
Cyst-GA-Gly (g/day) and GSSG (μmol/g Hb)	$r = 0.149$; $p = 0.386$
Cyst-GA-Gly (g/day) and GSH/GSSG (ratio)	$r = 0.057$; $p = 0.741$
Cyst-GA-Gly (g/day) and PC (nmol/g Hb)	$r = 0.182$; $p = 0.288$
Skeletal muscle	
Cyst-GA-Gly (g/day) and GSH (nmol/mg protein)	$r = -0.001$; $p = 0.995$
Cyst-GA-Gly (g/day) and GSSG (nmol/mg protein)	$r = 0.209$; $p = 0.221$
Cyst-GA-Gly (g/day) and GSH/GSSG (ratio)	$r = -0.199$; $p = 0.245$
Cyst-GA-Gly (g/day) and GPx activity (U/mg protein)	$r = 0.213$; $p = 0.211$
Cyst-GA-Gly (g/day) and GR activity (U/mg protein)	$r = -0.018$; $p = 0.917$
SAA vs redox biomarkers	
Erythrocytes	
SAA (g/day) and GSH (μmol/g Hb)	**$r = 0.566$; $p < 0.001$**
SAA (g/day) and GSSG (μmol/g Hb)	$r = 0.268$; $p = 0.119$
SAA (g/day) and GSH/GSSG (ratio)	$r = 0.227$; $p = 0.189$
SAA (g/day) and PC (nmol/g Hb)	$r = -0.145$; $p = 0.405$
Skeletal muscle	
SAA (g/day) and GSH (nmol/mg protein)	**$r = 0.374$; $p = 0.027$**
SAA (g/day) and GSSG (nmol/mg protein)	$r = 0.194$; $p = 0.265$
SAA (g/day) and GSH/GSSG (ratio)	$r = 0.035$; $p = 0.842$
SAA (g/day) and GPx activity (U/mg protein)	$r = -0.104$; $p = 0.553$
SAA (g/day) and GR activity (U/mg protein)	$r = 0.319$; $p = 0.062$

Data are presented as correlation coefficients and p values ($n = 45$). Correlations were adjusted for physical fitness and PA profile variables

Cyst-GA-Gly total dietary intake of cysteine, glutamic acid and glycine, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *GPx* glutathione peroxidase, *GR* glutathione reductase, *PC* protein carbonyls, SAA total dietary intake of cysteine and methionine

Significant correlations and the corresponding p-values are presented in bold

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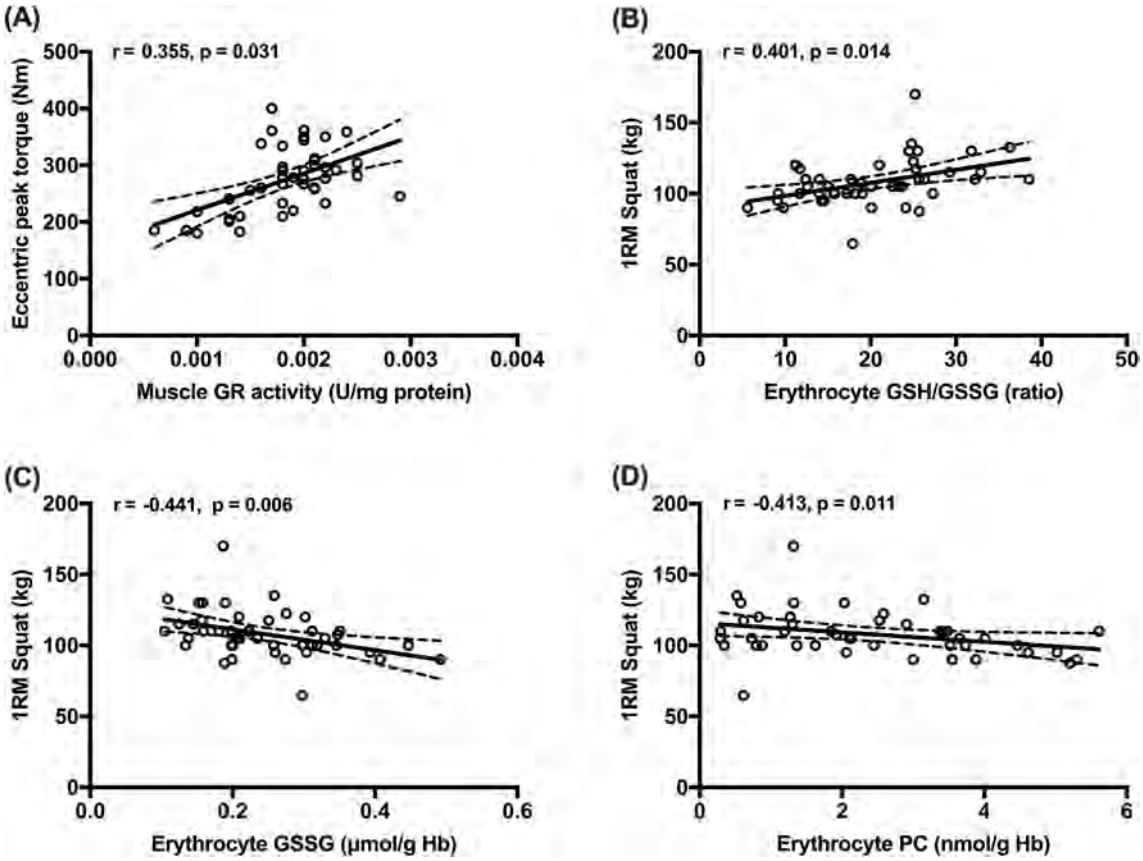

Fig. 3 Partial correlation and linear regression analyses between muscle glutathione reductase enzyme activity vs knee extensors eccentric peak torque (**A**), 1-repetition maximum in squat exercise vs erythrocyte reduced/oxidized glutathione ratio (**B**), erythrocyte oxidized glutathione (**C**) and erythrocyte protein carbonyls (**D**), ($n=45$). Correlations were adjusted for dietary nutrient intake and PA variables. *GSH* glutathione, *GR* glutathione reductase, *GSSG* oxidized glutathione, *PC* protein carbonyls, *1RM* one-repetition maximum

The independent relationship between redox status and physical activity

Figure 4 presents the correlation between redox status and PA levels. Muscle GPx enzyme activity was positively correlated with step count ($r=0.520$, $p<0.001$) light PA ($r=0.406$, $p=0.008$), moderate PA ($r=0.417$, $p=0.006$), moderate-to-vigorous PA ($r=0.475$, $p=0.001$), vigorous PA ($r=0.352$, $p=0.022$), and very vigorous PA ($r=0.326$, $p=0.035$), while muscle GSSG inversely correlated with light PA ($r=-0.353$, $p=0.022$). No other significant correlations were noted between redox status and PA variables. The correlations between PA and redox biomarkers with and without the adjustments for confounders are presented in supplementary file 1.

Discussion

In this investigation, we examined for the first time, the independent relationship between dietary nutrient intake, muscle strength, cardiorespiratory capacity and PA profile with erythrocyte and skeletal muscle redox status in healthy young, physically active men. Our findings suggest that (i) reduced dietary cysteine intake is linked to impaired glutathione metabolism and elevated protein oxidation in erythrocytes and skeletal muscle, (ii) upregulated redox status is associated with increased lower limb muscle strength, (iii) higher PA levels are associated with enhanced antioxidant enzyme activity in skeletal muscle and (iv) redox status markers in both tissues were not associated with cardiorespiratory capacity (VO_2max).

Dietary cysteine intake exhibited a meaningful correlation with erythrocyte and skeletal muscle GSH concentration as well as with overall redox status (GSH/GSSG ratio). Similarly, previous human studies showed a positive association between dietary cysteine intake and erythrocyte GSH concentration [12, 40]. In contrast, no association was detected between glutamic acid, glycine (both constituent amino acids for GSH synthesis) and the total dietary intake of cysteine, glutamic acid and glycine (Cyst-GA-Gly) with GSH metabolism, which corroborates previous observations, showing that cysteine is the "rate-limiting" amino acid

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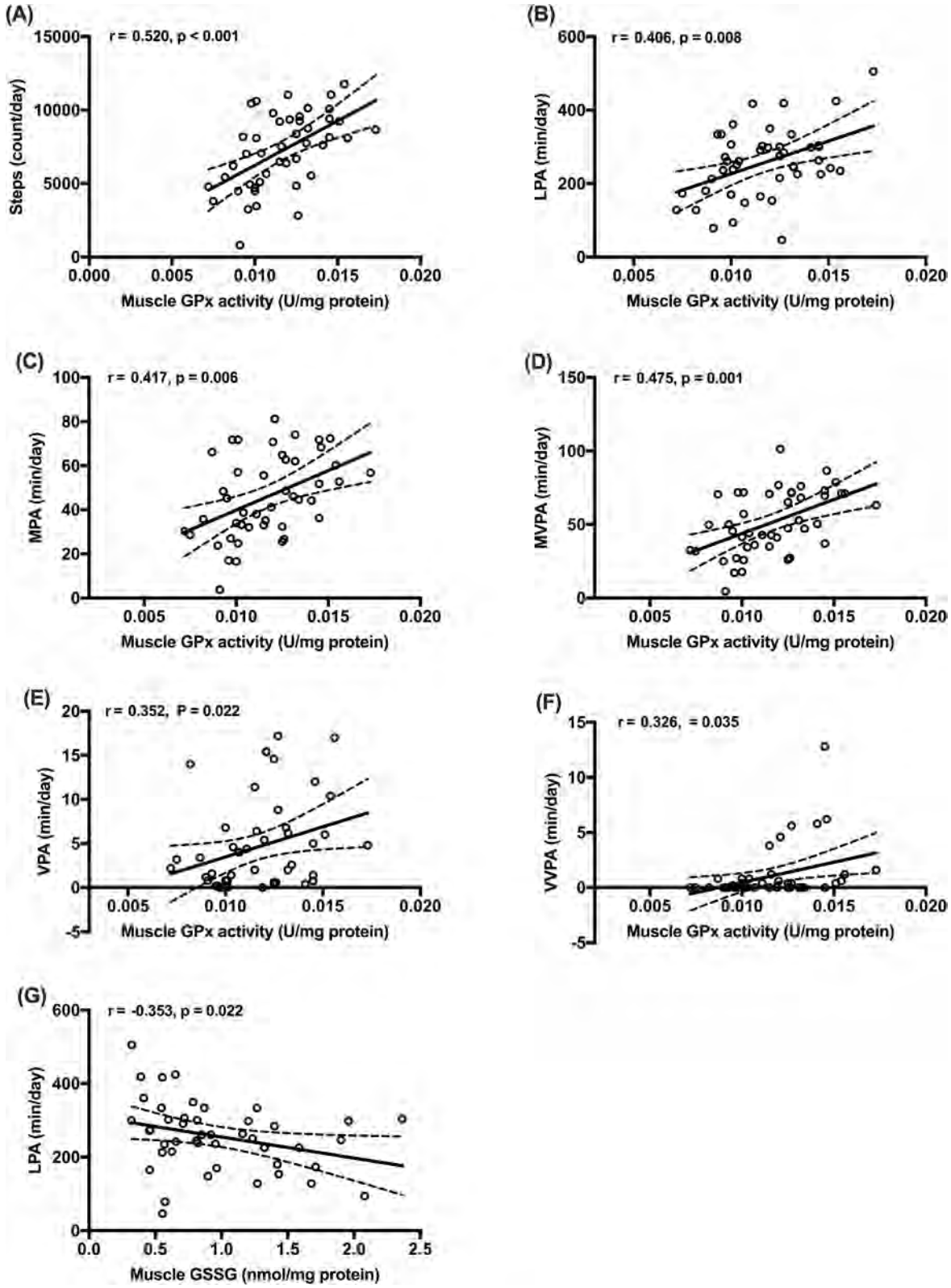

for GSH synthesis [12]. It seems that, in young adults of increased PA, increased dietary cysteine intake is associated with higher intramuscular GSH concentration and turnover (via GR enzyme activity). Previous in vitro and animal studies indicated that GSH levels are linked to cysteine availability in a dose-dependent manner [11, 41]. Reduced cellular

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◂**Fig. 4** Correlation and linear regression analyses between muscle glutathione peroxidase enzyme activity vs step count (**A**), light physical activity (**B**), moderate physical activity (**C**), moderate-to-vigorous physical activity (**D**), vigorous physical activity (**E**) and very vigorous physical activity (**F**) as well as muscle oxidized glutathione vs light physical activity (**G**), ($n = 45$). Correlations were adjusted for dietary nutrient intake and physical fitness variables. *GPx* glutathione peroxidase, *GSSG* oxidized glutathione, *LPA* light physical activity, *MPA* moderate physical activity, *MVPA* moderate-to-vigorous physical activity, *VPA* vigorous physical activity, *VVPA* very vigorous physical activity

GSH/GSSG ratio in cysteine-depleted media of HT29 cell culture was reversed upon re-addition of the amino acids to the culture media [42]. Moreover, in healthy young men, sulfur amino acid depletion over a 4-day period negatively affected plasma levels of free cysteine, while sulfur amino acid repletion immediately recovered cysteine/cystine redox status [10]. To date, there is no consensus statement regarding the recommended dietary intake (RDI) for cysteine; nevertheless, a previous study proposed the consumption of ~ 21 mg/kg/day of dietary cysteine for adult healthy men [43]. In our study, participants' average dietary cysteine intake corresponds to approximately 22,3 mg/kg/day which is slightly above the recommended value indicated in the aforementioned study. However, it should be noted that the significant trends observed in the present study may also refer to the "theoretical normal range" of dietary cysteine intake. Additionally, it has been suggested that SAA intake surplus (> 2–3 times of RDI) is considered safe and may confer health and/or performance benefits, via upregulation in GSH synthesis and turnover [44]. The literature on human SAA intake consistently refers to the combination of methionine and cysteine intake, as methionine conversion to cysteine through the methionine cycle and the transsulfuration pathway can supply ~ 100% of the requirements under resting conditions [45, 46]. In accordance, erythrocyte GSH fractional synthesis rate and concentration did not change with increasing cysteine intakes in healthy young men receiving an adequate diet (protein intake of 1 g/kg/day and methionine intake of 14 mg/kg/day) [47]. However, in some cases, defects in enzyme and metabolite activity interacting with methionine metabolism may compromise the methionine–cysteine conversion [48]. Moreover, it has been suggested that dietary cysteine has a sparing effect of ~ 64% on methionine requirement in adult men [48, 49]. Nevertheless, cysteine has been characterized as a "conditionally indispensable" amino acid [50], requiring a dietary source to provide enough precursor when basal endogenous synthesis cannot meet the metabolic need, [51]. Thus, it could be speculated that in individuals with increased PA and/or fitness status, the metabolic requirement for cysteine may exceed the endogenous conversion rate from methionine as a result of an adaptive response to systematic PA and upregulation of the cysteine-dependent GSH enzymes [52]. Interestingly, our results revealed a significant independent correlation between dietary cysteine intake with GSH metabolism and overall redox status, while in contrast, we did not detect similar partial associations when substituting cysteine with total SAA intake, except for GSH in both tissues (Table 2). A possible explanation for this discrepancy in the observed associations (cysteine vs. SAA intake), which warrants further investigation, may be the increased fitness status of our cohort, as a result of a higher PA level, which may have resulted in an upregulated metabolic need for cysteine, due to a chronic effect of PA, despite the fact that samples were collected at a resting state. Based on these observations, dietary cysteine intake seems to represent the major dietary determinant factor of overall GSH metabolism, justifying its physiological relevance and assessment separately from total SAA intake in young men with increased PA.

It is evident that future clinical studies with more participants incorporating advanced statistical and analytical tools (e.g., isotope amino acid tracers) and controlling for relevant factors such as sex, age and fitness status are needed to confirm the validity of the associations observed in the present investigation in the context of dietary cysteine monitoring, to distinguish between possible redox deficiencies accompanied by impaired GSH metabolism.

Regarding protein intake, previous reports showed that low protein diets may alter GSH synthesis rate and decrease antioxidant capacity as a result of a limited amino acid dietary intake [40]. Here, participants' median dietary protein intake (1.27 g/kg of body weight) was within the safe levels for young men with increased PA [37, 53]. However, contrary to a previous report [12], we did not detect a significant correlation between total protein intake and redox biomarkers, which may be attributed to some participants' high degree of variability in total protein intake (ranging from 0.6 to 1 g/kg/day) during the 7-day assessment period, as a result of a more plant-based diet protocol. Indeed, 18% of our cohort (8 out of 45 participants), when compared to the total sample, consumed a higher amount of legumes (median values: 47.5 vs 15.7 g/day, $p < 0.001$), mainly including beans, chickpeas and lentils and lower amounts of cereals (mean values: 304.4 vs 391.3 g/day, $p = 0.002$), mainly comprising of pasta, rice, oats and quinoa. Of note, such dietary plans (high legume/low cereal plant-based diet) may result in high concentrations of saponins, isoflavones and other phytochemicals which can potently exert redox effects by improving the antioxidant profile and reducing oxidative damage in men and women [54, 55] through direct (Nrf2 activation) and indirect (citric acid cycle enzymatic activity) mechanisms [56]. In this study, participants with lower cysteine intake (plant-based diets) exhibited redox status imbalances. Moreover, we did not detect any significant correlation between legume intake and redox biomarkers

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(supplementary file 2); thus, the possible increased amount of phytochemicals in the participants' diet may not have influenced antioxidant status and the validity of the presented correlations.

To provide further insight into the causal role of dietary cysteine consumption in GSH metabolism, additional analyses was performed, and cysteine consumption was corrected for energy, protein and SAA intake. Following correction, associations between cysteine intake and GSH status remained significant (except for antioxidant enzymes), thus substantiating, for the first time, the functional/causal role of dietary cysteine intake in redox regulation, irrespective of other macro- and micro-nutrient consumption (supplementary file 2). Interestingly, cysteine intake was positively correlated with muscle GR enzyme activity, which may be attributed to a cysteine-dependent increase in GSH synthesis and/or turnover. GR is an essential component of the intracellular redox system, reducing GSSG back to GSH, thus assisting in optimal redox balance [57]. Indeed, defects in the enzyme activity are linked with several pathologies [58, 59]. A cysteine-induced increase of GSH levels through diet may positively impact skeletal muscle antioxidant enzyme capacity and redox regulation. Moreover, higher cysteine intake was inversely correlated with erythrocyte protein oxidation, which is in line with a negative association between cysteine intake and plasma F_2-isoprostanes previously observed in young adults [12]. In fact, specific antioxidant deficiencies (i.e., GSH, vitamin C) have been linked with oxidative stress development and impairment of physical function, which can be inverted by targeted antioxidant supplementation without significant side-effects reported [17, 18]. In light of the above, our results provide further insights into the significance of dietary cysteine intake in maintaining an optimal redox environment in erythrocytes as well as in skeletal muscle. Based on the partial correlation analysis, it is evident that dietary cysteine consumption per se is a significant determinant factor in relation to redox status and oxidative stress, irrespective of the individual's physical fitness and PA profile.

Redox biomarkers were also correlated with lower limb strength performance. Specifically, knee extensors' eccentric peak torque was positively correlated with muscle GR enzyme activity while 1RM in back squat exercise was positively correlated with erythrocyte GSH/GSSG ratio and negatively correlated with muscle GSSG and erythrocyte PC levels. A recent study revealed that decreased levels of cysteine intake and erythrocyte GSH are associated with reduced maximal isometric handgrip strength [12]. Our findings further corroborate that the aforementioned disturbances in redox homeostasis may be linked to impairments in skeletal muscle performance, especially in lower limb muscle groups. It has become apparent that redox regulation is a crucial modulator of several physiological functions including muscle contractile activity and adaptation to exercise [60, 61]. An optimal redox equilibrium is required for normal muscle function as an exceedingly oxidized or reduced environment may result in lower muscle performance [62, 63]. Since low cysteine and GSH levels are associated with elevated oxidative stress and impaired muscle function, it has been hypothesized that a potential mechanism may involve an RONS-induced oxidative modification of specific cysteine and methionine residues in the actin–myosin junction, resulting in contractile inhibition and as such a decline of muscle function [64]. Furthermore, alterations in Ca^{2+} sensitivity have been also implicated in skeletal muscle function and fatigue development [65, 66]. In isolated muscle fibers exposed to H_2O_2, GSH and myoglobin addition partially prevented muscle force reduction via a rise in Ca^{2+} sensitivity, which was accompanied by the S-glutathionylation of cysteine residues on the troponin I (fast) protein [66, 67]. In humans, supplementation with N-acetylcysteine attenuated fatigue development during repeated bouts of exhaustive exercise [68, 69] and ameliorated performance decline 2 days after a muscle-damaging protocol of the knee extensors [70]. Of note, only mild side-effects (mainly gastrointestinal) were reported during these studies. However, it was documented that prolonged antioxidant supplementation hampers late strength recovery following exercise by interfering with key anabolic pathways (i.e., Akt–mTOR axis) [7, 70]. It is evident that in skeletal muscle, redox signaling and mild levels of oxidative stress are indispensable modulators of the inflammatory/regenerative phase following injury, through the induction of muscle stem cells' proliferation and differentiation [21, 71]. It seems that, under physiological conditions, non-targeted use of antioxidants blunts redox signals and muscle's ability to regenerate. Specifically, it was shown that N-acetylcysteine treatment impairs myofiber growth and fusion at the late restorative phase (30 days) following muscle injury in mice [72], while Nrf2 over-activation (through antioxidant supplementation) hampers myoblasts differentiation in vitro [73]. Nevertheless, despite the established link between cysteine oxidation and muscle function, our analyses revealed that lower limb muscle strength is also independently associated with glutathione metabolism and oxidative stress. However, from a physiological point of view, it is plausible to note that muscle function and strength can be redox-dependent and not vice versa [74].

To our knowledge, this is the first study to provide direct evidence regarding the association between redox status and objectively assessed (via accelerometers) PA profile. The use of accelerometry to objectively quantify PA profile has been documented to be a reproducible and valid methodological approach across the life-span [24, 75]. Our results indicate that PA, independent of its intensity, is associated with increased GPx enzyme activity in skeletal muscle, while light

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PA is inversely correlated with muscle GSSG concentration. Of note, we did not observe any association between GSH and PA variables. By incorporating partial correlation analysis, our results show that PA levels independently influence muscle antioxidant capacity, regardless of nutritional and physical fitness status. Regarding the relationship between PA/exercise and redox status, several reports have indicated that regular PA/exercise can exert antioxidant effects via an adaptive response characterized by the upregulation of antioxidant status (i.e., trolox-equivalent antioxidant capacity) and key antioxidant enzymes in skeletal muscle (i.e., superoxide dismutase) [52, 76]. In healthy prepubertal children, higher fitness levels (assessed by questionnaires) positively correlated with selected erythrocyte redox status biomarkers (GSH, GSSG, GSH/GSSG ratio) [77]. Furthermore, a recent cross-sectional study investigated the influence of self-reported PA on redox status and nitric oxide bioavailability in prepubertal children. It was shown that engagement in regular PA increased nitric oxide bioavailability in children of healthy muscle mass, while systemic antioxidant capacity and insulin sensitivity were enhanced in children with obesity [15]. In the elderly, data suggest that moderate-to-vigorous PA and/or endurance training induces an adaptive increase in systemic antioxidant enzyme activity and decreases lipid peroxidation in both women and men [16, 28]. Additionally, research from our group has shown that men with elevated chronic systemic inflammation and increased oxidative stress perform less steps and spent less time in moderate-to-vigorous PA per day compared to their healthy counterparts [24]. Collectively, these data suggest that increased PA positively impacts redox status, and especially muscle antioxidant enzyme activity in young physically men with increased PA. It appears that the maintenance of high PA levels may positively regulate redox status by reducing GSH oxidation, with significant implications for health and disease prevention.

Study limitations

At this point, it should be noted that the design and results of this observational investigation cannot establish a cause-and-effect relationship between redox status, cysteine intake, muscle strength and PA levels, and the results can only be applied in young, healthy, men with increased PA. Moreover, alternatives to causality for the associations observed with dietary cysteine intake may include the interference of tissue inflammation/dysfunction markers not measured in the present study.

Conclusion

In summary, our findings support the conclusion that decreased dietary cysteine intake is associated with impaired GSH metabolism and increased levels of protein oxidation in erythrocytes. Moreover, redox status disturbances are linked to decreased lower limb muscle strength performance, while a higher PA level, independent of its intensity, is associated with increased muscle antioxidant capacity, which may be implicated in the treatment of several pathologies and chronic diseases characterized by dysfunctional antioxidant status. Further research, with appropriately designed large-scale clinical trials, applying sensitive statistical analyses and varying relevant factors such as age, sex and physical condition is warranted to confirm the consistency of the associations observed in the present study and elucidate the mechanisms of redox regulation by dietary cysteine and PA/exercise.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00394-023-03102-2.

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Author contributions KP, IGF and AZJ: designed the study; IB, CP and AS: performed muscle tissue sampling and medical monitoring; CKD: performed blood sampling; KG: performed dietary nutrient analyses; AP, PT and TT: performed anthropometric and physical fitness assessments; DD: analyzed accelerometry data; AR and DD: performed biochemical redox assays; NVM and MGN: provided valuable insight on the experimental processes and analyses; KP: analyzed the data; KP and IGF: wrote the paper. All authors read and approved the final manuscript.

Data availability statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All experimental procedures were in accordance with the 1964 Declaration of Helsinki and its later amendments and were approved by the Institutional Review Board of the University of Thessaly (approval reference number: #1387/2018).

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