THE EFFECT OF ECCENTRIC EXERCISE AND IRON SUPPLEMENTATION ON BLOOD REDOX STATUS AND MUSCLE PERFORMANCE IN ADULTS AND CHILDREN

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The effect of eccentric exercise and iron supplementation on blood redox status and muscle performance in adults and children

ABSTRACT

Muscle-damaging eccentric exercise can lead to increased production of reactive oxygen and nitrogen species (RONS). The typical approach so far was to provide antioxidants to minimize RONS production and improve muscle recovery and performance, yet, the efficacy of such an approach is still under debate. On the other hand, the effect of substances such as iron, that are considered pro-oxidants and could alter the symptoms of muscle damage, has not yet been investigated. The aim of the present study was to examine if iron supplementation would alter symptoms of muscle damage, redox status indices, and muscle performance after eccentric exercise in healthy adults and children. In a randomized, double blind crossover study, that was conducted in two cycles, healthy adults (n = 15) and children (n = 11) received daily either the iron supplement (37mg of elemental iron) or the placebo for 3 weeks prior to, and up to 96 hours after an acute eccentric exercise bout (5 sets of 15 maximal eccentric contractions). Subjects performed the exercise during the first cycle with one leg, which was followed by a four-week washout period, before performing the exercise during the second cycle with the contralateral leg. Physiological parameters were estimated in both age groups at baseline, after 3 weeks of supplementation (pre-exercise), immediately after exercise, 24, 48, 72, and 96 hours after exercise. Blood samples in adults were collected at baseline, pre-exercise, 24, 48, 72, and 96 hours post-exercise, while in children at baseline, pre-exercise, and 72 hours following exercise. Iron supplementation resulted in improvement of iron status and elevated TBARS levels. Iron supplementation did not affect muscle damage, muscle performance and blood redox status after eccentric exercise, as exercise-induced modifications were similar in both groups. On the other hand, iron supplementation decreased iron concentration and increased transferrin saturation the days following eccentric exercise. Children were less susceptible to muscle damage compared to adults whose symptoms were more severe. Despite its pro-oxidant character, iron supplementation, as administered in the present study, did not alter redox responses or muscle function and performance after muscle damaging exercise.

Keywords: free radicals, pro-oxidants, muscle damage, aseptic inflammation, soreness

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1. INTRODUCTION

1.1. Oxidative stress

The term oxidative stress refers to the disturbance of the balance between the pro-oxidant and antioxidant mechanisms of the human body in favor of the former leading to potential damage (Halliwell & Gutteridge 2007). Such a disturbance results in increased production of reactive oxygen and nitrogen species (RONS), overwhelming antioxidants' response. In principal, oxidative stress can occur either due to defective antioxidant defense and diminished antioxidants, or due to excessive production of free radicals (Halliwell & Gutteridge 2007).

1.1.1. Free radicals

Any species capable of independent existence that contains one or more unpaired electrons in their atomic or molecular orbital is defined as free radical (Halliwell & Gutteridge 2007). Free radicals are unstable molecules and sometimes can be highly reactive, due to their effort to become stable, either by accepting, donating or sharing an electron (Halliwell & Gutteridge, 2007).

Free radicals are products of the physiological cell metabolism and in low to medium concentration are favorable for stress-induced cell response, signal transduction, gene transcription, cell proliferation, inflammation and apoptosis (Ji 2007, Ji 2008, Valko 2007, Reid 1992); free radicals in moderate quantities are also essential for muscle function and training adaptations (Gomez-Cabrera 2010, Gomez-Cabrera 2005, Reid 2001). However, excess generation can lead to oxidative stress disrupting that way the normal function of the human organism. Indeed, oxidative stress has been implicated in various pathological conditions involving cardiovascular disease (Elnakish 2013), cancer (Che 2015, Valko 2006), neurological disorders (Bhat 2015, Jenner 2003), ischemia/reperfusion (Valko 2007), diabetes (Kojda & Harrison 1999, Halliwell 2001), as well as muscle fatigue (Powers 2008) and ageing (McArdle 2002, Valko 2007).

Free radicals can be derived both by endogenous and exogenous sources. Endogenous sources include mitochondrial electron transport chain, several enzymes such as cyclooxygenase, NADPH oxidases and xanthine oxidase (XO), as well as auto-oxidation reactions and inflammation (Rahal 2014). Exogenous sources of free radicals include ultraviolet and ionizing radiation, chemical substances and drugs, intense exercise, smoking, as well as several environmental pollutants (Powers 2004, Rahal 2014). Additionally, diet can influence redox homeostasis; poor consumption of important antioxidant vitamins can negatively affect the overall antioxidant defense of the human body (Powers 2004).

When a free radical reacts with a molecule, a new radical is produced, and chain reactions begin, that could adversely affect the physiological function of important biomolecules, such as lipids (Halliwell & Chirico 1993), proteins (Grune 1997) or DNA (Dizdaroglu 2002). Free radicals are mainly distinguished in oxygen-centered reactive species (ROS) and nitrogen-centered reactive species (RNS). RONS are produced as intermediates in reduction-oxidation reactions leading from O_2 to H_2O (Halliwell 2007). ROS include superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), and hydroperoxyl radical ($HO_2^{\bullet}$), whilst RNS include nitric oxide radical ($NO^{\bullet}$) and $NO_2^{\bullet}$. ROS and RNS comprise not only the oxygen and nitrogen radicals but also some non-radical derivatives of O_2 and N_2, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), and peroxynitrate anion ($ONOO^-$), due to their high chemical reactivity and ability to further exacerbate oxidative stress. The mechanisms, by which some of the above RONS are produced, as well as the importance of their generation to either favoring or risking the integrity of the human body, are discussed in the subsequent paragraphs.

Superoxide radical: Superoxide is generated through the addition of one e^- to O_2. Superoxide anion, is considered the “primary” ROS, and can further interact with other molecules to generate “secondary” ROS, either directly or prevalently through enzyme- or metal-catalyzed processes (Valko 2007). The most important source of $O_2^{\bullet -}$ *in vivo* is the mitochondrial electron transport chain. About 1 to 3% of the O_2 reduced in mitochondria may form $O_2^{\bullet -}$ (Halliwell 2007, Muller 2000).

Superoxide radical formation:

$$O_2 + e^- \longrightarrow O_2^{\bullet -} \quad \text{(eq. 1)}$$

Superoxide can also be produced *in vivo* by NADPH oxidase (NOX) enzymes and xanthine oxidase (XO). Regarding NOX enzymes, they can be activated in several tissues such as lungs and adipose tissue (Halliwell 2007). NADPH oxidase can also be activated and contribute to the production of $O_2^{\bullet -}$ through oxidative burst during phagocytosis (Halliwell 2007, Murray 1993). Xanthine oxidase (XO) can also reduce O_2 to $O_2^{\bullet -}$. In cases of muscle injury and ischaemia-reperfusion, XO can promote the generation of $O_2^{\bullet -}$ and H_2O_2 by oxidizing the accumulated hypoxanthine during the reoxygenation period (White 2000). Additionally, the binding of O_2 to haem proteins haemoglobin and myoglobin can occasionally result in the release of $O_2^{\bullet -}$. It has been estimated that about 3% of the haemoglobin present in human erythrocytes undergoes oxidation every day and so these cells are exposed to a constant flux of $O_2^{\bullet -}$ (Halliwell 2007). Moreover, cytochrome P_{450}-dependent oxygenases, but also peroxisomes, produce $O_2^{\bullet -}$ (Valko 2006). Excess production of $O_2^{\bullet -}$ can damage proteins, lipids and DNA, and has been implicated in the pathophysiology of a variety of diseases (Valko 2007).

Hydroxyl radical ($OH^{\bullet}$): $OH^{\bullet}$ is of the most reactive free radical species known (Halliwell 2007 p42, 44). With an *in vivo* half-life of approx. 10^{-9}s (Valko 2007), their direct chemical action is strictly confined to the close vicinity of the site of its generation (Asmuss 2000), reacting with normal cells and destructing cellular enzymes and cellular membranes by lipid peroxidation, or provoking alteration of DNA molecules (Halliwell & Chirico 1993, Dizdaroglu 2002). In biological fluids, it can be generated mainly through Fenton (eq. 2) and Haber-Weiss (eq. 3) reactions in the presence of transition metal ions such as iron (Fe) or copper (Cu). Under stress conditions, $O_2^{\bullet -}$ releases iron from iron-containing proteins and facilitates $OH^{\bullet}$ production from H_2O_2.

Fenton reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad \text{(eq. 2)}$$

Haber-Weiss reaction:

$$O_2^{-} + H_2O_2 \xrightarrow{Fe} O_2 + OH^{\bullet} + OH^{-} \quad \text{(eq. 3)}$$

Hydroxyl radical can also derive from the reaction of HOCl with $O_2^{\bullet -}$ (Folkes 1995), from decomposition of NOO^- (Radi 1991), as well as ultra violet (UV) and ionizing radiation (Halliwell 2007). The importance of $OH^{\bullet}$ in oxidative stress is precisely due to its great reactivity. When $OH^{\bullet}$ reacts with an organic compound (lipid, protein or nucleotide), an oxidized molecule is generated and a chain reaction begins leading to the formation of other radical species such as $ROO^{\bullet}$, $RO^{\bullet}$, and $HO_2^{\bullet}$. These species are of the most abundant radicals in biological systems and are readily formed in any oxygen containing environment. Therefore, increased production of $OH^{\bullet}$ can subsequently lead to oxidation of lipids, proteins or DNA (Valko 2006).

Hydrogen peroxide (H_2O_2): As already been mentioned, H_2O_2 is not a free radical itself, yet it is included in ROS, due to its high chemical reactivity and ability to further exacerbate oxidative stress. It is continually produced *in vivo* in many tissues, and can be formed by the addition of hydrogen cations to $O_2^{\bullet -}$ a chemical procedure called dismutation. The formation is catalyzed by the enzyme superoxide dismutase (SOD) (Halliwell 2007).

Dismutation of superoxide

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad \text{(eq. 4)}$$

Although H_2O_2 is not very reactive, it is capable of inactivating a few enzymes directly through oxidation, such as the glycolytic enzyme glyceraldehyde-3-phospate dehydrogenase (G3PDH), as well as some protein phosphatases and caspases involved in apoptosis (Halliwell 1993). Furthermore, H_2O_2 crosses cell membranes and reacts with iron and copper (Fenton reaction) forming the very reactive $OH^{\bullet}$. What is more, H_2O_2 also interacts with heme proteins to cause oxidative damage (Halliwell 2007).

Nitric oxide radical ($NO^{\bullet}$): $NO^{\bullet}$ is synthesized *in vivo* in biological tissues by the action of specific enzymes, the nitric oxide synthases (NOSs), which convert L-arginine into citruline (Pryor 2006, Ghafourifar 2005). Nitric oxide is a typical example of the beneficial physiological role of free radicals, in several physiological processes, including neurotransmission (Knott 2009), blood pressure regulation (Knott 2009 Halliwell 1993), defense mechanisms, smooth muscle relaxation (Halliwell 1993) and immune regulation (Knott 2009, Valko 2007). Additionally, due to its high reactivity with other free radicals *in vivo*, $NO^{\bullet}$ is also considered a free radical scavenger. Thus the ability to scavenge $OH^{\bullet}$, or $RO_2^{\bullet}$, makes $NO^{\bullet}$ a powerful inhibitor of lipid peroxidation (Denicola 2002) protecting that way against atherosclerosis. On the other hand, $NO^{\bullet}$ can cause damage to proteins, lipids, and DNA (Rahal 2014), and has been implicated in many prevalent neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, and ischemic stroke (Knott 2009). Additionally, in cases of oxidative burst during inflammatory conditions, $NO^{\bullet}$ and the $O_2^{\bullet -}$ may react together to produce significant amounts of the much more oxidatively active molecule, peroxynitrite anion ($ONOO^-$), which is a potent oxidizing agent that can cause DNA fragmentation (Valko 2007) and lipid oxidation (Carr 2000).

1.2. Human body's antioxidant defense

Human body has developed a defense antioxidant system of enzymatic and non-enzymatic antioxidant mechanisms that depends on dietary consumption of antioxidant vitamins and metals and on the endogenous *in vivo* generation of antioxidant substances such as glutathione (Ji 1999, Aruoma 1994). The defense mechanisms comprise of: a) agents that catalytically remove reactive species (RS), such as superoxide dismutase (SOD), superoxide reductase, catalase and peroxidase enzymes; b) agents that decrease RS formation, such as mitochondrial uncoupling proteins, transferrins, albumin, haptoglobins, and heme oxygenases; c) proteins that protect biomolecules against oxidative damage by other mechanisms, e.g. chaperons; d) the physical "quenching" of RS, e.g. of singlet O_2 by carotenoids; e) "sacrificial agents" that are preferentially oxidized by RS to preserve more important biomolecules, such as GSH, α-tocopherol, bilirubin, ascorbate, urate, albumin. This antioxidant system preserves the balance between the favorable and the damaging effects of reactive species.

Taken into consideration all of the mechanisms mentioned above, a broad definition of antioxidant has been proposed, that is, any substance that when present at low concentrations compared with those of an oxidizing substrate significantly delays or prevents oxidation of that substrate (Halliwell 2007).

Additionally, to take into account all of the aforementioned antioxidant defenses, another more simplified definition is also proposed which defines as antioxidant any substance that delays, prevents or removes oxidative damage to a target molecule (Halliwell 2007).

1.2.1. Enzymatic antioxidant mechanisms

Enzymatic antioxidant substances are produced endogenously and play an important role on the maintenance of redox homeostasis in the human body. These substances convert RONS into less reactive molecules of oxygen and H_2O. Enzymatic

antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutaredoxin (GRX), and thioredoxin (TRX) (Konig 2001).

Superoxide dismutases (SODs): SODs consist a very important defense mechanism against $O_2^{\bullet-}$ formation which plays a major role in O_2 toxicity. SOD exists in three forms with different metals in their reactive center that are essential for the catalytic breakdown of $O_2^{\bullet-}$ (Powers & Jackson 2008). In humans, these forms are present as cytoplasmic CuZnSOD or SOD1, mitochondrial MnSOD or SOD2, and extracellular SOD (EC-SOD) or SOD3 (Halliwell 2007, Fattman 2003). The first form of SOD, CuZnSOD, is a dimer that contains copper and zinc in its reactive center and except for cytoplasm, it is also found in the space between inner and outer mitochondrial membranes. The second form of SOD, MnSOD, is a tetramer that contains manganese in its active site and is located in the mitochondria. The third form of SOD, EC-SOD, is a tetramer which similarly to CuZnSOD, contains copper and zinc in each subunit. It seems likely that *in vivo*, EC-SOD is bound to cell surfaces, largely by association with cell surface carbohydrates. All three forms of SOD catalyze the conversion of $O_2^{\bullet-}$ to O_2 and H_2O_2 (eq. 4).

Dismutation of superoxide

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad \text{(eq. 4)}$$

Catalase (CAT): Catalase, like SOD, catalyzes a dismutation reaction and direct decomposes H_2O_2 to ground-state O_2 (Halliwell 2007) (eq. 5).

The reaction mechanism of catalase

$$2H_2O_2 \xrightarrow{\text{CAT}} 2H_2O + O_2 \quad \text{(eq. 5)}$$

Catalase is a tetramer containing four subunits, each of which has Fe (III)-heme at its active site (Reid 1981). It is present in all organs, but especially concentrated in liver, erythrocytes and kidney. Subcellular, catalase is located mainly in peroxisomes and in small quantities in mitochondria and endothelial reticulum (Chance 1979, Antunes 2002). Catalase protects erythrocytes against H_2O_2 generated by dismutation of $O_2^{\bullet-}$ from hemoglobin autoxidation (Halliwell 2007).

Glutathione peroxidase (GPx): GPx enzymes also catalyze the reduction of H_2O_2 (eq. 6) or lipid hydroperoxides (eq. 7), using reduced glutathione (GSH) as the reductive substance.

The reaction mechanism of glutathione peroxidase

$$H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + 2H_2O \quad \text{(eq. 6)}$$

$$LOOH + 2GSH \xrightarrow{GPx} GSSG + 2H_2O + LOH \quad \text{(eq. 7)}$$

The GPx enzymes are widely distributed in tissues and are mostly specific for GSH as a hydrogen donor. It exists in four different types: a) the “classical” cytosolic GPx or GPx1, b) GPx2, mainly found in the cells lining the gastrointestinal tract, c) GPx3 that is found in plasma and other extracellular fluids, d) phospholipid hydroperoxide glutathione peroxidase or GPx4, found in lipid membranes and lipoproteins. The amount of GPx enzymes differs among human tissues; high concentration is found in the liver, kidney and whole blood, while moderate in lens and erythrocytes and low in blood plasma (Chance 1979).

Glutathione reductase (GR): The GR catalyzes the conversion of GSSG back to GSH using NADPH as a reductive agent (eq. 8), so that the normal high ratio of GSH to GSSG to be sustained (Halliwell 2007).

Regeneration of GSH by glutathione reductase

$$GSSG + NADPH + H^{+} \xrightarrow{GR} 2GSH + NADP^{+} \quad \text{(eq. 8)}$$

1.2.2. Non enzymatic antioxidant mechanisms

Non-enzymatic antioxidants, include nutritionally derived vitamins and provitamins (vitamin E, vitamin C, and β-carotene), flavonoids and polyphenols, proteins or peptides containing thiol groups (mainly glutathione), and various other low molecular weight compounds as coenzyme Q, uric acid, bilirubin (Konig 2001, Clarkson 2000), but also some blood components (Halliwell 2007). Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health (Valko 2007).

Glutathione: Glutathione is one of the most critical non-enzymatic antioxidant substances of the human organism, constituting the main regulator of endocytic redox homeostasis. Glutathione is a tripeptide, and in the human body can be synthesized by the amino L-cysteine, L-glutamic acid, and glycine. The sulfhydryl group (SH) of cysteine serves as a proton donor and is responsible for its biological activity (Halliwell 2007). Glutathione is a ubiquitous molecule that is produced in all organs, especially in the liver (Pastore 2003) which supplies 90% of the circulating GSH (Ji 1995). It is highly abundant in the cytosol, nuclei, and mitochondria and consists of the major soluble antioxidant in these cell compartments (Masella 2005). Glutathione has several protective roles against oxidative stress (Masella 2005). One of the main roles is being a cofactor of several detoxifying enzymes that participate

in the protection of the cell, such as glutathione peroxidase (GPx) and glutathione transferase. Another critical role of glutathione is the participation in the transport of amino acids through the plasma membrane. Additionally, glutathione scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase (eq. 6). Moreover, glutathione is able to regenerate the most important antioxidants, Vitamins C and E, back to their active forms. Glutathione exists in two forms, the reduced (GSH) and oxidized glutathione (GSSG). In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). In the presence of H_2O_2 and hydroperoxides, endocytic glutathione rapidly oxidizes to GSSG, but also rapidly reduces to GSH by the action of the enzyme glutathione reductase, using NADPH as an electron donor, in low stress conditions. If the cell is unable to reduce GSSG to GSH, the increase of GSSG can be used as an indicator of oxidative stress (Clarkson 2000), and the ratio of GSH/GSSG is used as a reliable measurement of oxidative stress (Ji 1995).

Uric acid: Uric acid is a powerful reduced agent, and the final product of purines catabolism, produced by hypoxanthine and xanthine by the action of the enzymes xanthine oxidase and xanthine dehydrogenase. The main antioxidant role of uric acid is to provide protection against lipid peroxidation by being a powerful scavenger of $OH^{\bullet}$, $O_2^{\bullet -}$ and singlet oxygen (Wayner 1987, Ames 1981). Additionally, uric acid also serves as a metal ions chelator, by binding iron and copper ions, minimizing that way the potential of metal ions-catalyzed free radical reactions (Halliwell 2007). Uric acid comprises the main regulator of total antioxidant capacity (TAC) of plasma (Bartosz 2003), which refers to the quantity of oxidant molecules that have been neutralized by all of the antioxidant substances (Bartosz 2003).

Bilirubin: Bilirubin is the end product of hemoglobin catabolism in the endocytic reticulum and next to uric acid, one of the most important plasma antioxidants. Bilirubin circulates in plasma bound to albumin, and effectively suppresses the oxidation of lipids and lipoproteins (Stocker 1987). About 80% of the bilirubin arises from the catabolism of haemoglobin from senescent red blood cells, by reticuloendothelial cells in the spleen, liver and bone marrow (Maines 1988), while

the rest comes from myoglobin, catalase and cytochromes. Additionally to its antioxidant properties, bilirubin is also used as an index of hemolysis, due to its derivation from erythrocytes catabolism (Baranano 2002).

Coenzyme Q_{10} (CoQ_{10}): Coenzyme Q_{10} is a fat-soluble, vitamin-like substance present in nearly all human tissues, with the highest concentrations in the heart, liver and kidney (Aberg 1992). Coenzyme Q_{10} can be synthesized endogenously, but is also supplied to the organism by several foods. Meat and fish are the richest source of dietary CoQ_{10}; vegetable oils are also quite rich, but dairy products are rather poor sources CoQ_{10} (Kubo 2008). Coenzyme Q_{10} is essential in mitochondrial electron transport process of cellular respiration and energy production (Ernster and Dallner 1995), and exists in three redox states; ubiquinone-10 is the fully oxidized form, while ubisemiquinone-10 and ubiquinol-10 are the partially oxidized and the reduced form respectively. Except for mitochondria, CoQ_{10} is also found in other cell membranes and in lipoproteins. Ubiquinol can scavenge $RO_2^{\bullet}$, and inhibit lipid peroxidation; it can also regenerate α-tocopherol from its radical in lipoproteins and membranes (Ernster and Dallner 1995).

Vitamin E: Vitamin E is the major lipid-soluble antioxidant in cell membranes and lipoproteins that protects against lipid peroxidation by acting directly with a variety of oxygen radicals, including singlet oxygen, lipid peroxide products, and the $HO^{\bullet}$, to form a relatively innocuous tocopherol radical (Clarkson 2000). Vitamin E inhibits lipid peroxidation due to its ability to scavenge lipid peroxyl radicals much faster than these radicals react with adjacent fatty acid side chains or membrane proteins (Halliwell 2007). Skeletal muscle contains approximately 30 to 50 nmol of vitamin E per gram of wet weight, whereas the concentration of vitamin E in the heart and liver amounts to 60 to 70 nmol/g (Ji 1995).

Vitamin C: Vitamin C is a water-soluble vitamin that represents a first-line antioxidant defense in plasma. Vitamin C can directly react with, and scavenge $O_2^{\bullet-}$, $OH^{\bullet}$, and singlet oxygen (Clarkson 2000, Cesari 2004), therefore being a powerful inhibitor of lipid peroxidation. Additionally, vitamin C inhibits lipid peroxidation induced by haemoglobin or myoglobin, but also by activated neutrophils and $ROO^{\bullet}$

(Halliwell 2007). Furthermore, vitamin C serves as an electron donor to vitamin E radicals produced in the cell membrane during oxidative stress to regenerate vitamin E in lipoproteins and membranes (Ji 1995, Clarkson 2000). Nevertheless, vitamin C in high concentrations may act as a pro-oxidant, as it can convert iron from its inactive ferric form (Fe^{+3}) to its active ferrous form (Fe^{+2}) (Powers 2004). Ferrous iron, in turn, can react with H_2O_2, and lead to the formation of the very reactive $HO^{\bullet}$ through Fenton reaction, which can initiate lipid peroxidation.

Carotenoids: Carotenoids are lipid-soluble antioxidants and precursors of vitamin A, or retinol. In blood they are located in the circulating lipoproteins, while in tissues (mostly in adipose tissue and liver) they occur within fat stores, in the hydrophobic interior of membranes and bound to hydrophobic domains of certain proteins (Halliwell 2007). Beta-carotene, which is considered the major carotenoid, is the most efficient "quencher" of singlet oxygen (In Clarkson 2000). It is particularly well-suited for scavenging $O_2^{\bullet-}$, $OH^{\bullet}$, and peroxyl radicals such as $ONOO^{-}$, acting that way against lipid peroxidation (Powers 2004). Other important carotenoids are lycopene and α-carotene.

Polyphenols: Phenols are a class of chemical compounds containing a hydroxyl group (–HO) attached to a benzene (aromatic) hydrocarbon group. Polyphenols consist of more than two aromatic rings binding to –HO groups (Halliwell 2007). Flavonoids such as quercetin, resveratrol, curcumin and catechins, are of the best studied phenols, which include several thousand compounds; they are found in high levels in onion, grapes and wine, teas, and many other plant products (Manach 2004). Flavonoids can act as scavengers of peroxyl radical, breaking that way chain reactions and inhibiting lipid peroxidation; they can also scavenge other RS such as $OH^{\bullet}$, $NO_2^{\bullet}$, ONOOH and HOCl (Halliwell 2008). Additionally, phenols have a chelating ability as they bind transition metal ions, especially iron and copper, decreasing their potential of generating free radicals through Fenton and Haber-Weiss reactions (Nijveldt 2001, Halliwell 2008). Moreover, they can inhibit the ability of myeloperoxidase to oxidize low-density lipoproteins (LDL), gaining that way a potential anti-atherosclerotic effect (Halliwell 2008).

Blood components: Transferrin (the iron-transport protein) and ferritin (the iron-storage protein) play an important role in maintaining free-iron levels low (Bacic 2008, Murray 1993), and hence restrain the conversion of H_2O_2 to $OH^{\bullet}$. Lactoferrin secreted by neutrophils in situations of inflammation, also acts as an antioxidant by binding any available iron ions, thus helping iron-dependent $OH^{\bullet}$ generation to be minimized (Halliwell 2007). Likewise, plasma haptoglobins and haemopexin, confer antioxidant properties, such that, in cases of release of haem and haem-proteins from damaged cells (e.g. lysed erythrocytes), they bind to haemoglobin and haem respectively (Halliwell 2007, Murray 1993), dropping their effectiveness in stimulating lipid peroxidation. Ceruloplasmin and albumin also assist in the antioxidant defense protecting important targets against lipid peroxidation (e.g. LDL), by binding to copper ions that could participate in Fenton chemistry. Ceruloplasmin binds 90% of the copper present in plasma while albumin carries the other 10% (Murray 1993). Additionally, albumin contains a –SH group; albumin–SH reacts with several RS such as $ONOO^{-}$, $NO_2^{\bullet}$, HOCl, and $RO^{\bullet}$, leading to the formation of less reactive molecules (Halliwell 2007).

The pro-oxidant character of antioxidants: Despite their favorable capacity of delaying, preventing or removing oxidative damage, it should be noted that under certain circumstances, non-enzymatic antioxidants may also become pro-oxidative agents. Flavonoids, as well as antioxidant vitamins have been reported to act as pro-oxidants when transition metals are available. For example, β-carotene, in the presence of increased partial pressure of oxygen, can be converted to a peroxyl radical, and vitamin C can form DNA-damaging genotoxins from lipid hydroperoxides in the presence of transition metal ions (Halliwell 2008).

1.3. Exercise-induced oxidative stress mechanisms

During exercise oxygen consumption is enhanced and during oxidative metabolism, most of the consumed oxygen is bound to hydrogen to form H_2O through oxidative phosphorylation in mitochondria. However, it has been estimated that an amount of 2-5% of the consumed oxygen does not fully reduced to H_2O, but it leaks out of the

respiratory chain, as both NADH-ubiquinone reductase and ubiquinone-cytochrome c reductase generate $O_2^{\bullet-}$ (Ji 1995). It is well-known that tissue and whole body oxygen consumption is increased dramatically during strenuous exercise that can result in a 20-fold whole body and in 100-fold muscle fiber oxygen consumption (Ji 1995). As a result of this increase, an augmentation of the $O_2^{\bullet-}$ production in the mitochondria occurs. The $O_2^{\bullet-}$ that is formed from the oxygen leakage at the respiratory chain reduces in H_2O_2 through the enzyme MnSOD, making possible the formation of $OH^{\bullet}$ through Fenton or Haber-Weiss reactions (Halliwell 2007) in which iron plays a critical role. Indirect data showing mitochondrial oxidative damage supports the hypothesis that mitochondria are a primary site of ROS generation during exercise. Indeed, mitochondrial lipid peroxidation is enhanced after exercise, accompanied by loss of protein thiol content and inactivation of oxidative enzymes (Ji 1999). Furthermore, both muscle and heart mitochondria from severely exercised animals demonstrated lower coupling and decreased GSH redox status (Chandwaney 1998). The mitochondrial theory of ROS production is also indirectly supported by the training adaptation of mitochondrial antioxidant enzymes, such as MnSOD and GPx (Ji 1999).

Neutrophils and macrophages comprise cells of the immune system that can produce great amounts of reactive oxygen species (Malm 1999). In cases of exercise that includes eccentric contractions and leads to muscle injury and inflammation, the activity of the above immunity cells increases significantly during, as well as after exercise through the catalytic action of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and myeloperoxidase (Peake 2005, Close 2005, Vollaard 2005). Neutrophils are the first cells to gather into the injured area and along with macrophages of the tissue clean the area through phagocytosis. A subsequent increase of oxygen consumption (oxidative burst) occurs, and the molecular oxygen initially reduces into superoxide anion and afterwards into hydrogen peroxide which in turn can form hypochloric acid (Pyne 1996). A number of reactions after the formation of hypochloric acid can lead to the production of $OH^{\bullet}$ either through Fenton chemistry or through the reaction of hydrogen peroxide

with hypochloric acid. Additionally, O_2^- that is produced by polymorphonuclear leukocytes can release iron from ferritin (Biemond 1984).

Cytokines that are released by neutrophils and the injured myofibers consist of another source of RONS. These cytokines activate RONS-generating enzymes such as xanthine oxidase (XO) and cyclooxygenase-2 (Ji 2007, Hellsten 1997). Additionally, in ischemic muscle contractions, isometric and sprinting exercise, exercise in a hypoxic environment or exercise with impaired blood flow due to vascular diseases, skeletal muscle may undergo a significant deficit of adenine nucleotides; in situations like these, XO may become an important pathway. Xanthine oxidase uses molecular oxygen as electron donor during purines degradation to uric acid, resulting in the production of $O_2^{\bullet-}$ and H_2O_2 anions (McCord & Fridovich, 1968). Indeed, high intensity exercise, as well as eccentric exercise lead to the activation of xanthine oxidase pathway, and the subsequent production of superoxide $O_2^{\bullet-}$ and H_2O_2 anions (Hellsten 1997).

The destruction of haemoglobin and myoglobin due to hemolysis that occurs after muscle-damaging eccentric exercise (Theodorou 2011) can also contribute to increased production of free radicals by releasing catalytic iron from erythrocytes, which in turn can participate in Fenton and Haber-Weiss reactions (Gutteridge 1986).

1.4. Eccentric exercise

The movement of human body is the product of skeletal muscles' action. Skeletal muscles are attached to the bones and their volitional contraction leads to force generation and movement of the body's skeleton. Each muscle contains many muscle fibers that comprise the muscle cells. Each muscle fiber consists of many myofibrils, and each myofibril of thick (myosin) and thin (actin) filaments placed in a repeated mode along the myofibril. The functional units of this repeated module are called sarcomeres and their bounds are separated by the Z bands. Thin filaments are attached to Z bands, while thick filaments lie in the central area of the sarcomere, known as A band. According to the sliding filament theory (Huxley 1996, Holmes 2000), muscle contraction results from the parallel sliding of actin filaments over myosin filaments by the binding of myosin heads to actin, while the two groups of filaments remain at relatively constant length.

Muscle contraction is the activation of tension-generating sites within muscle fibers (Vander 2001). Muscle contraction does not necessarily mean muscle shortening because muscle tension can be produced without changes in muscle length. More specific, muscle contraction can mainly distinguish in: a) isometric contraction, b) concentric contraction, c) eccentric contraction.

a*) Isometric contraction:* when muscle tension changes without any corresponding changes in muscle length, the muscle contraction is described as isometric. An example can be found when the muscles of the hand and forearm grip an object; the joints of the hand do not move, but muscles generate sufficient force to overcome the objects load and prevent the object from being dropped.

b) Concentric contraction: in concentric contraction, muscle tension is sufficient to overcome the load, and the muscle shortens as it contracts. This occurs when the force generated by the muscle exceeds the load opposing its contraction. In relation to the elbow, a concentric contraction of the biceps would cause the arm to bend at the elbow as the hand moved from the leg to the shoulder (a biceps curl), to lift an object.

c) Eccentric contraction: in eccentric contraction, the tension generated by the muscle is insufficient to overcome the external load and the muscle fibers lengthen as they contract. Eccentric contractions can occur involuntarily as it happens when attempting to move a weight too heavy for the muscle to lift, or voluntarily, when we try to relocate an object from a higher (e.g. the table) to a lower (e.g. the floor) position.

During the three types of contraction, cross-bridge cycle is continuously repeated. In isometric contraction, although myosin's heads are cross-bridged with actin's filaments, they cannot move them because of the heavy load of the muscle fibers. In contrast, during concentric and eccentric contractions there is a movement of actin filaments; cross-bridges are moving at an angle causing the shortening of sarcomeres towards A band, whereas the extension of sarcomeres towards the Z band, during concentric and eccentric contractions, respectively (Vander 2001).

Normally, during the activities of everyday life, and also during athletic actions, all three types of muscle contraction occur. Eccentric exercise can predominately be performed by walking or running downhill. Such an activity, allows the quadriceps muscles to work eccentrically when exerting a braking force to maintain or slow the pace. Level running can also be considered as a type of eccentric exercise such that, at the beginning of the stance phase quadriceps muscles are working eccentrically.

Eccentric exercise has been used as a means to develop muscle strength and size (Dudley 1991). Eccentric muscle contractions produce a greater muscle force than concentric or isometric contractions (Gault 2013, Stauber 1989). Indeed, muscles are reported to be approximately 40% stronger during eccentric than during concentric contractions, whereas exercise that incorporates both eccentric and concentric contractions produces greater gains in strength than concentric contractions alone (Colliander 1990). This is probably due to the fact that during eccentric contraction, some of the cross-bridging heads of myosin instead of performing their usual rotational movement, retract and remain attached to the actin filaments (Stauber 1989). In the same time, additional cross-bridges are activated during eccentric contraction and a greater number of activated cross-bridges compared to isometric

and concentric muscle contractions produce greater force (Stauber 1989). Except for greater amounts of force, eccentric exercise can also result to greater damage to the internal membrane system, intermediate filaments, Z discs and contractile proteins (Nosaka 2008), more intense muscle soreness (Paschalis 2010), as well as greater inflammation and oxidative stress (Nikolaidis 2007) compared to concentric exercise. The recruitment of fewer muscle fibers in eccentric contractions, to exert a given amount of force as compared to concentric contractions, account for the greater muscle damage with eccentric actions. Since the force-velocity relationship indicates that each individual muscle fiber can exert a larger force while being stretched than it can while being shortened (Holmes 2006) and fewer fibers are activated during eccentric contractions, larger forces per muscle fiber are developed during eccentric actions thereby resulting in greater damage.

Nevertheless, despite the greater muscle injury provoked by eccentric exercise, recent research has also shown favorable effects on several health aspects (Theodorou 2013). Additionally, training adaptations has been shown to be greater with eccentric exercise than concentric exercise in respect with body strength, lipidemic profile, and insulin resistance (Nikolaidis 2008, Paschalis 2010b, Paschalis 2011). Paschalis et al. (2011) report that 30 min of eccentric exercise weakly for eight week improved health risk factors (muscle strength and performance, REE, lipid oxidation as well as decreased insulin resistance and blood lipid profile). Moreover, eccentric exercise is also characterized by a lower metabolic demand than concentric exercise (Gault 2013). For example, when walking or running downhill, oxygen consumption is reported to be lower compared to level walking or running, at the same absolute speed (Gault 2013). Therefore, eccentric exercise could be adapted from sensitive populations such as the elderly, obese, or those suffering from cardiovascular diseases and diabetes, as an exercise module for ameliorating the symptoms of the disease and improving their quality of life.

1.4.1. Eccentric exercise: a valuable model for studying muscle injury

Skeletal muscle injury is manifested in several debilitating diseases that are characterized by marked proteolysis and muscle wasting such as cancer (Al-Majid 2008), muscular dystrophy (Tidball 2010), rheumatoid arthritis (Stavropoulos-Kalinoglou 2014), and sepsis (Fehrenbach 2006). Muscle injury causes tissue disruption and subcellular damage followed by cytokine release and a rapid invasion of leukocyte subpopulations into the muscle (Peake 2005). This inflammatory response is followed by a muscle repair/regeneration phase (Peake 2005, Cantini 2002) and muscle recovery (Smith 2008). Intense eccentric exercise induces muscle micro-trauma characterized by loss of muscle cell integrity and a marked inflammatory response evidenced by leukocyte infiltration, edema, hyperthermia, protein release into the plasma and oxidative stress (Jamurtas 2013, Childs 2001, Gleeson 1995, Theodorou 2011, Paschalis 2007, Goldfarb 2005), but not sepsis (Fehrenbach 2006). The remarkable resemblances between exercise-induced aseptic inflammation and muscle trauma make eccentric exercise a valuable tool to investigate muscle inflammation and oxidative stress in humans.

1.5. Iron

Iron is one of the most abundant elements, essential for the completion of numerous important biological functions, including electron transfer reactions, gene regulation, binding and transport of oxygen, regulation of cell growth and differentiation. In the human body it is mainly found in the oxygen transport and storage proteins haemoglobin (60 - 70%) and myoglobin (10%), in various iron-containing enzymes (2%), as well as in the liver, bone marrow and muscle in the form of the storage proteins ferritin (Ferr) and hemosiderin (20 - 30%) (Fontecave 1993, Halliwell 1999). Only a minor quantity (0.1 - 0.2%) of total iron, mostly bound to the iron-transport protein transferrin, circulates in the plasma and other extracellular fluids (Fontecave 1993, Crichton 2003). The mechanisms by which iron homeostasis is normally maintained is presented in **Figure 1**.

Besides its essential character, excessive free iron could adversely affect the human body, by augmenting oxidative stress, mainly via the Fenton and Haber-Weiss reactions. As already has been mentioned, ferritin, hemosiderin and transferrin, assist the system to maintain iron balance under tight control by keeping free iron levels low and hence restrain the conversion of hydrogen peroxide to the highly reactive hydroxyl radical (Bacic 2008) that disturbs cellular homeostasis when it is increased at toxic levels.

Iron absorption is the main mechanism through which iron balance is maintained. Nevertheless, iron losses may occur at multiple organs, such as the gastrointestinal tract (Nachtigall 1996, Stewart 1984, de Oliveira 2009), the skin (Brune 1986, DeRuisseau 2002), the urinary tract (Nachtigall 1996), and additionally due to several physiological conditions such as the menstrual cycle in women (Harvey 2005, Lyle 1992). To compensate for these losses, as well as for satisfying the body's demands during growth and pregnancy, iron is absorbed from the diet. The percentage of food-iron that is absorbed from the intestine is approximately 10%, with heme iron being absorbed in greater amounts compared with non-heme-iron (Hurrell 2010, Anschuetz 2010, Herbert 1987). Thus, from a typical daily diet of 2000 kcal that contains adequate quantities of meat, 1.8 mg of iron per day are absorbed (Herbert

1987). In general, daily iron turnover (absorption and excretion) is approximately 1-2 mg per day (Fontecave 1993, Crichton 2003, Brune 1986).

There is a strong body of evidence suggesting that exercise affects iron status (Koehler 2012, Reinke 2012, Malczewska 2001, Nielsen 1998), although other studies do not support this association (Malczewska 1997, Malczewska 2000, Magnusson 1984). Iron plays a critical role in oxygen transport and critical for aerobic capacity. Iron is also needed for the optimal function of many oxidative enzymes affecting the intracellular metabolism (i.e., the electron transport chain and oxidative phosphorylation pathway in mitochondria) (Beard 2001). Not only prolonged aerobic exercise but, to some extent, short duration activities (i.e. sprints), may influence the above mechanisms (Rowland 2012). Consequently, a compromised iron status would negatively affect physical performance. On the other hand, iron deficiency is frequently attributed to exercise (Koehler 2012, Reinke 2012, Malczewska 2001). Therefore, iron supplementation is commonly used to avoid exercise-induced perturbations of iron homeostasis and maintain the required iron stores that are necessary to address exercise needs or enhance physical performance. The importance of iron homeostasis in exercise is presented in details in section 1.5.1., whereas the mechanisms by which iron homeostasis can be compromised due to exercise are discussed in section 1.5.2.

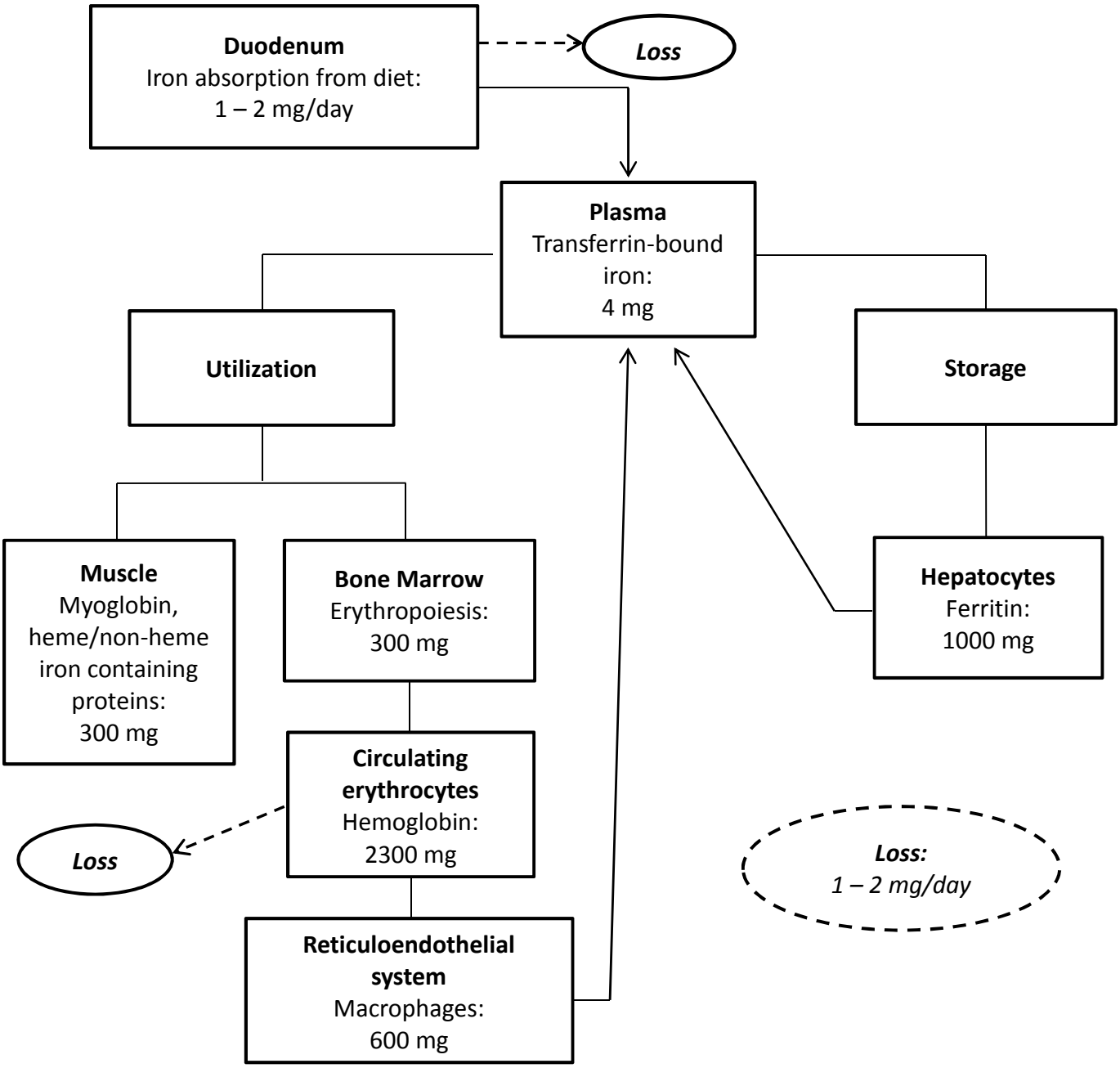

Figure 1. Iron homeostasis in the human body. Transferrin supplies iron to cells that require it for cell-division and/or production of iron-proteins, and erythroblasts in the bone marrow (BM). Most of the iron is used for haemoglobin (Hb) synthesis. Iron enters plasma from the intestinal mucosa and the macrophage system. Normally this iron is rapidly bound to transferrin. Non transferrin-bound iron is found in plasma when transferrin iron-binding capacity is saturated (in conditions of iron overload). Effete red blood cells are destroyed by macrophages of the reticuloendothelial system. Iron is subsequently released into the plasma from these cells as ferritin and haemoglobin, as well as in the form of low-molecular complexes. Within cells iron first enters a labile iron pool which is in equilibrium with the storage pool of iron in ferritin and also supplies iron for the production of essential iron-containing proteins. Iron is lost from the body together with exfoliating cells or during blood loss.

1.5.1. The importance of iron homeostasis during exercise

Exercise and/or physical activity is characterized by a substantial increase in oxygen needs. Iron is an indispensable factor for the formation of HGB, the protein responsible for oxygen transport from the respiratory organs to the peripheral tissues. Lack of adequate amounts of iron for the formation of HGB due to iron deficiency, can strongly affect physical work capacity, by reducing oxygen conveyance to the exercising muscles (Beard 2001). Iron is also a vital component for the formation of myoglobin, the iron-storage protein within the muscle that regulates the diffusion of oxygen from the erythrocytes to the cytoplasm and on to the mitochondria where it is used as the final acceptor of electrons processed by the respiratory chains producing water and forming energy in the process (Dallman 1978, Hood 1992). The concentration of myoglobin in skeletal muscle is drastically reduced (40 - 60%) following iron deficiency, thus limiting the rate of oxygen diffusion from erythrocytes to mitochondria (Beaton 1989) which ultimately compromises the muscle's oxidative capacity.

Apart from oxygen transport and storage, iron is also needed for the optimal function of many oxidative enzymes and proteins regulating the intracellular metabolism (Beard 2001, Hood 1992, Beard 2000). The mitochondrial content of oxidative enzymes and proteins is an important factor regarding the muscle's capacity for work, as there is a strong association between the ability to maintain prolonged submaximal exercise and the activity of iron-dependent oxidative enzymes (Beard 2000). Iron deficiency negatively affects mitochondrial respiration mainly through the decline in heme iron-containing respiratory chain proteins cytochrome c and cytochrome c oxidase, as well as non-heme iron-containing enzymes succinate dehydrogenase and NADH dehydrogenase, but also the non-heme iron-sulfur protein content (Hood 1992). Furthermore, iron is an essential co-factor in the antioxidant enzyme catalase (Powers 2004). Catalase is located in both the cytosol and the mitochondria and is responsible for removing hydrogen peroxide from cells. An iron deficiency would not only impair oxygen transport in the body, but would also compromise the body's antioxidant capacity by lowering catalase activity in cells (Halliwell and Gutteridge, 1999). Therefore, iron deficiency may have

detrimental effects, especially on endurance performance which is susceptible to, and negatively affected by disturbances in skeletal muscle's iron concentrations (Hood 1992).

Besides athletes' training at sea level, iron deficiency could also affect athletes training at altitude. Staying at high altitude causes an increase in erythropoiesis in the bone marrow, stimulated by hypoxia. This increase in erythropoiesis is followed by an elevation in red blood cells volume and concentration (Friedmann 1999, Chapman 1998). Iron deficiency could negatively affect the above mechanism by limiting the rate of erythropoiesis and consequently aerobic performance. It has been demonstrated that athletes with low ferritin levels do not increase total red blood cell volume after 4 weeks at altitude, despite an acute increase in erythropoietin (Stray-Gundersen 1992). In contrast, a significant increase in erythropoietin but also in reticulocytes occurred in non-iron-deficient athletes during training at moderate altitude (Friedmann 1999). Such data suggests that iron sufficiency is critical for the favorable response of the athletes training to altitude, in an attempt to enhance their performance.

1.5.2. Potential mechanisms for iron balance disturbances due to exercise

Iron absorption mainly, and to a lesser extent iron nutrition, are the two critical mechanisms by which iron balance is maintained since there is no other physiological process for iron excretion. The repletion of iron due to increased losses as well as the body's need during growth and pregnancy are covered by dietary iron intake. Consequently, a low dietary iron intake, could lead to compromised iron status (Harvey 2005). According to values reported by the Institute of Medicine, Food & Nutrition Board (2001), the recommended dietary intake (RDI) for total iron is 8 mg/day for adult men and 18 mg/day for menstruating women. Usually, male, but not female athletes achieve the RDI for iron (Koehler 2012, Nielsen 1998, Powell 1991). The mechanism of iron absorption by the intestine is regulated by iron bioavailability in diet and by individual's iron status. Iron bioavailability has been found to be affected by the type of the diet and by the type of dietary iron (Hurrell

2010). Hence, mixed diet and heme iron provide greater bioavailability and absorption as compared to a vegetarian diet and non-heme iron, (Hurrell 2010, Anschuetz 2010, Herbert 1987). Furthermore, iron deficiency augments iron absorption.

Besides iron absorption and intake, several other mechanisms have been proposed to account for iron loss and iron balance disturbances, and ultimately the prevalence of iron deficiency in athletes. These mechanisms include increased gastrointestinal blood loss, hematuria, hemolysis (Stewart 1984, de Oliveira 2009, Nielsen 1998, Siegel 1979, Choi 2001, Gaudin 1990, Mccabe 1986), increased iron loss in sweat (Brune 1986, DeRuisseau 2002), as well as menstruation in women (Harvey 2005, Lyle 1992, Hallberg 1991).

In athletes, gastrointestinal bleeding usually accompanied by occult blood, is a well-established phenomenon, mostly seen in distance runners (de Oliveira 2009, Nickerson 1989). Gastrointestinal blood loss has shown to be the main contributor to the negative iron balance, as the excretion of Fe in sweat and urine appears to be negligible compared to fecal excretion of 3 - 5 mg/day (Nachtigall 1996). Running a marathon was associated with a gastrointestinal blood loss (Mccabe 1986), and positive occult heme stools were found in runners after intensive training or competitive running (Nachtigall 1996, Stewart 1984, Nickerson 1989, Mccabe 1986). The origin of running-related intestinal bleeding has still to be clarified, but endoscopic examination has revealed bleeding lesions in the stomach and colon (Choi 2001, Gaudin 1990). Gastrointestinal bleeding is partly attributed to ischemic injury, and running has been shown to reduce visceral blood flow by up to 43% of pre-exercise levels (Qamar 1987) due to the diversion of blood flow from the splanchnic viscera to the working muscles. Exercise intensity, seems to play a significant role in the development of gastric ischemia (Otte 2001), which increases mucosa permeability and enhances occult blood loss (de Oliveira 2009).

Increased iron loss through sweat has also been proposed as a mechanism related to the compromise of iron status as a result of increased sweat rates during exercise in

athletes, or increased temperature in individuals living and exercising in hot climates. The daily loss of iron from the skin has been reported to be 0.24 mg/d (Green 1968) or 0.33 mg/d (Jacob 1981). The reported 0.183 mg of iron loss during prolonged exercise at 50% of VO, represented the 55% - 76% of the estimated daily iron loss from the skin, and the 23% for men and 10% for women of the estimated total daily iron loss (DeRuisseau 2002). It has to be mentioned that although the sweat rate increases during the 1st 2max hour of exercise and remains constant thereafter, and males have higher sweat rates than females, the iron loss in males and females remains comparable. Additionally, the sweat iron loss declines in both genders during the 2nd hour of exercise (DeRuisseau 2002), or after the first 30 min in a hot environment (Brune 1986). This reduction could be attributed to the initial sweat containing iron present in cellular debris (Brune 1986), to the increased sweat rates while the total iron loss remains constant, or to a conservation mechanism that may prevent excessive iron loss during exercise (DeRuisseau 2002). Still, iron loss in sweat remains insignificant compared to that of the gastrointestinal tract.

Another explanation for compromised iron status in athletes is the shift of iron return to hepatocytes, rather than the RES, as a consequence of the increased intravascular hemolysis occurring mostly in weight-bearing activities, such as running. In these activities, hemolysis is due to the impact forces generated by the foot strike (Sureira 2012, Telford 2003). Increased intravascular hemolysis has been reported in runners (Magnusson 1984) and female artistic gymnasts (Sureira 2012). However, foot strike cannot totally explain the exercise-induced hemolysis since hypohaptoglobinemia, a situation that reveals the presence of hemolysis, has also been observed in swimmers (Selby 1986). In non-weight-bearing activities hemolysis may result from the compression of the blood vessels caused by the vigorous contraction of the involved muscles (Selby 1986).

Female athletes seem to be more prone to the development of iron deficiency (Koehler 2012, Malczewska 2001) and blood loss during menstruation may further explain this greater prevalence. Although menstrual blood loss in a single woman is very constant during menarche and throughout the fertile life, there is a large

variation in blood loss among women (Hallberg 1991). Thus, in a mean cycle length of 28 days, menstrual blood loss may vary by as much as 26 - 44 ml, with a corresponding daily iron loss of about 0.5 - 0.7 mg (Harvey 2005, Hallberg 1966). This great variation in blood and iron loss reported by these two studies could be associated with an extensive use of oral contraceptives which are known to reduce the amount of blood loss during menstruation (Larsson 1992). Finally, menstrual iron loss in women has been shown to negatively correlate with serum Ferr, and iron status to significantly correlate with the duration and intensity of the menses in endurance athletes (Malczewska 2000). Taking into consideration the iron loss during menstruation along with the relative failure to achieve the daily RDI for iron the greater frequency of iron deficiency in female athletes can be justified.

1.5.3. The pro-oxidant character of iron

On the opposite side of antioxidants, pro-oxidants are substances characterized as capable of disturbing the redox balance and lead to increased production of RONS that are involved in critical biological processes such as gene expression, signal transduction and enzyme activity. The term pro-oxidant refers to any endobiotic or xenobiotic substances capable of disturbing the redox balance and induce oxidative stress either by generating RONS, or by inhibiting antioxidant systems (Rahal 2014); it can include all reactive and free radical-containing molecules in cells or tissues. Iron is characterized as a pro-oxidant, meaning that excessive free iron could disturb the redox balance due to its capacity to facilitate the production of the very reactive $OH^{\bullet}$, and lead to lipid peroxidation, protein oxidation, or DNA damage (Halliwell 2007). Iron is mainly found in two forms, the trivalent (Fe^{3+}, ferric iron) and the bivalent (Fe^{2+}, ferrous iron), with the latter being the active form of iron. It is the ferrous iron that contributes to the formation of hydroxyl radicals through Fenton reaction (eq. 2) or through Haber-Weiss reaction (eq. 3) (Prousek 2007). Additionally, as already has been mentioned, iron-containing haem and haem proteins haemoglobin and myoglobin can occasionally result in the release of $O_2^{\bullet-}$. Consequently, ferrous iron by facilitating the formation of free radicals, can adversely affect the human body, augmenting oxidative stress. Indeed, several

indices of iron status have been correlated with various pathological conditions such as hypertension (Galan 2006), cardiovascular diseases (de Valk & Marx 1999, Jomova and Valko 2011, Berdoukas 2015), cancer (Valko 2007) haemoglobin-associated diseases (Coates 2014), obesity, serum triglycerides, HDL cholesterol and inflammation (Williams 2002), situations that are directly or indirectly associated with enhanced free radicals production.

1.5.4. Iron-induced oxidative stress during eccentric exercise: potent mechanisms

Considering its pro-oxidant potentiality, iron could affect the magnitude of RONS production and alter the usual responses following muscle-damaging exercise. The potential of iron as a pro-oxidant in eccentric exercise rests in its ability to interact with intermediate RONS, such as $O_2^{\bullet -}$, HOCl and H_2O_2. These intermediates are produced by neutrophils and macrophages, through activation of NADPH oxidases and cytokines, as a physiological response of the human body to the occurring injury. The mechanisms by which these RONS are produced during eccentric exercise have already been described in section 1.2.2.

1.6. Current approach to exercise-induced muscle damage and oxidative stress

Research so far has focused on the effort to diminish oxidative stress during and/or after exercise-induced muscle damage. The typical approach was to provide antioxidants to minimize the production of RONS with the skeptical that decreased RONS production during exercise would provoke less damage, ameliorate the intensity of the injury symptoms, and sustain muscle performance. Nevertheless, the effectiveness of such an approach is still under debate, as the results between the studies are divergent. In fact, there is a body of evidence supporting the beneficial role of antioxidant supplementation on muscle performance and reduction of oxidative stress (Goldfarb 2005), but there is also studies that either state no alterations of redox adaptations (Theodorou 2011), or even more, enhanced oxidative stress due to supplementation (Childs 2001) and a slowing-down of the adaptive response of the muscle (Khassaf 2003).

Considering its pro-oxidant potentiality, iron could affect the magnitude of RONS generation and alter the usual responses following muscle-damaging exercise. Nevertheless, the role of iron, that could modify redox responses and change muscle function and performance after muscle-damaging exercise, has not yet been examined. Moreover, despite the extended research conducted in adults, very few studies have addressed the temporal changes in exercise-induced muscle damage symptoms in children (Marginson 2005, Gorianovas 2013, Soares 1996). In brief, it seems that children sustain less severe muscle damage compared to adults, indicated by the attenuated symptoms of muscle injury following exercise.

1.7. Aim of the study

The main aims of the present study ware the following:

1) To investigate the effects of iron supplementation for three weeks in male adults and children at rest on:
   a) iron status indices
   b) blood redox status indices
   c) muscle damage and performance indices

2) To investigate the effects of iron supplementation for three weeks prior to and another 96h after an acute bout of eccentric exercise in male adults and children on:
   a) iron status indices
   b) blood redox status indices
   c) muscle damage and performance indices

1.8. Study hypotheses

The main hypotheses of the present doctoral dissertation were the following:

a) Iron supplementation for three weeks will provoke changes on iron status, blood redox status, and muscle damage and performance
b) Acute eccentric exercise will provoke changes on iron status, blood redox status, and muscle damage and performance
c) The responses on the above indices will be different in the iron supplemented group compared to the placebo group
d) The responses will be different in children compared to adults

1.9. Study limitations

The limitations of the present dissertation were the following:

a) Only males participated in the study
b) Fewer blood drawings have been conducted in children compared to adults, and thus it is possible that some of the responses of the estimated biochemical parameters have not been detected in children.

1.10. Study eligibility criteria

The eligibility criteria for the present study were the following:

a) Healthy adults and children participated in the study, capable of giving their consent for participation. Additionally, regarding children, only after the written consent of their parents they could participate in the study
b) Participation in the study was voluntarily, without any financial or material reward
c) Participants were of normal iron status, without being allergic in iron salts
d) Only individuals with normal BMI participated in the study
e) Only individuals that did not consume any dietary supplement for at least 3 months prior to the beginning of the investigation participated in the study
f) Participants abstained from eccentric exercise for at least 6 months prior to, and through the entire study
g) All measurements were conducted by the same investigator, under stable conditions (room, temperature, humidity, day-time).

2. LITERATURE REVIEW

2.1. Evaluation of iron status: reference values of the most commonly used parameters

Due to the significant role of iron in optimal physical performance and health, the evaluation of iron status in athletes is of great importance in order to prevent iron deficiency. According to the World Health Organization (Worwood 2004), iron deficiency progresses in three stages: in the first stage iron stores in bone marrow, liver, and spleen are depleted (serum Ferr concentrations <12μg/L); in the second stage, erythropoiesis decreases as iron supply to erythroid marrow is reduced (TS <16%); in the final stage production falls drastically (concentration <12g/L) resulting in anemia.

Iron status evaluation is not a single-parameter estimation. Day-to-day or acute phase response variations occur in several indices of iron status. Therefore, in order to make a valuable and more accurate assessment, the estimation of iron status indices and several hematological parameters is needed. The most commonly used hematological and biochemical indexes are HGB and HCT reflecting red cell mass and plasma volume (Lynch 2004); serum iron concentration, total iron binding capacity and transferrin saturation indicating plasma iron status (Tietz 1995, Beard 2004); serum ferritin and hemosiderin representing body iron stores (Lynch 2004, Tietz 1995); erythrocyte protoporphyrin (EP) or zinc protoporphyrin (ZPP), and the soluble transferrin receptor (sTfR) reflecting the adequacy or inadequacy of iron for erythropoiesis into the bone marrow and tissues (Labbe 1999, Labbe 2004); also, haptoglobin is used for the estimation of hemolysis (Tietz 1995). Reference range for the main indicators of iron status, as well as reported values in athletes is presented in **Table 1**.

Table 1. The main iron status indicators: reference values for non-athletes and reported values in athletes

		Hb g/dL	HCT %	RBC x 10^6 c/μL	Ferr μg/L	Iron μg/dL	TIBC μg/dL	TS %	sTfR mg/L	HP mg/dL	EP μg EP/dL
Reference values for non-athletes adults*											
	Males	13.2-17.3	39-49	4.3-5.7	20-300	65-175	250-425	20-50	1.15-2.75	15-200	< 40-50
	Females	11.7-15.5	35-45	3.8-5.1	10-120	50-170	250-425	15-50	1.15-2.75	15-200	< 40-50
Reported iron status indicators in athletes (M±SD)											
Koehler et al. (2012) [v]	Males	14.7±1.1	42.3±2.6		55.4±36.7						
	Females	13.2±0.9	38.6±2.4		35.4±22.0						
Della Valle & Haas, (2012) [v]	Females(N)	13.1±0.7	40.1±2.1		43.0±20.3				6.4±2.5		
	(D)	13.0±0.7	40.2±2.1		13.9±5.1				6.4±2.1		
Reinke et al. (2012) [↑]	Males (CS)	12.2-17.1			6-127			10.7-85.4			
	(R)	11.6-17.2			20-133			9.0-54.2			
	(P)	12.4-16.5			15-148			13.9-43.2			
Schumacher et al. (2002) [v]	Males (EA)	15.7±1.0	46.6±3.3	5.24±0.52	125.6±91.5	125.0±50.2				73.0±38.9	
	(PA)	16.4±1.0	47.4±3.1	5.47±0.36	82.3±61	105.5±38.6				55.2±30.7	
Malczewska et al. (2001) [v]	Males (N)	16.38±1.1	0.49±0.0[¥]	5.26±0.36	65.9±1.83	104.3±28.2	310±36.7		1.78±1.3	1.29±0.58	
	(D)	15.92±1.1	0.47±0.0[¥]	5.12±0.32	19.5±2.14	72.8±20.5	348±41.2		3.15±1.2	1.16±0.54	
	Females(N)	14.5±0.9	0.42±0.0[¥]	4.5±0.37	40.6±1.76	105.3±32.9	315±42.6		1.72±1.2	1.29±0.89	
	(D)	13.8±0.8	0.42±0.0[¥]	4.6±0.33	20.1±1.54	81.7±20.4	344±49.2		4.36±2.5	1.43±0.60	
Rowland et al. (1987)	Males	14.7±1.0			29.4±17.8						
	Females	13.3±0.4			26.6±11.4						
Magnuson et al. (1984)	Males	14.6±0.9			64.3±47.8	19.1±7.3[ǂ]		31.8±11.6		52.0±32	27±18

Hb: Haemoglobin; HCT: Hematocrit; RBC: Red blood cells; Ferr: Ferritin; TIBC: Total iron binding capacity; TS: Transferrin saturation; sTfR: soluble transferrin receptor; HP: Haptoglobin; EP: Erythrocyte protoporphyrin;

(√) Values reported in Mean±SD, ↑ the observed ranges, ¥Values reported in L/L, ╬Values reported in μmol/L

N: normal iron status, D: iron deficiency, CS: Competitive season, R: Recovery, P: Preparation, EA: Endurance trained athletes, PA: Power trained athletes

* WHO, Assessing the Iron Status of populations (2004); Suominen et al., 1998; Tietz, 1995; Lynch, 2004; Beard, 2004; Labbe et al., 1999.

2.2. Exercise-induced alterations of iron status

Chronic exercise

There is a great body of evidence indicating that several hematological and iron status parameters often appear altered as a result of chronic exercise giving the impression that athletes may be iron-deficient (Koehler 2012, Reinke 2012, Malczewska 2001, Nielsen 1998, Rowland 2012, Schumacher 2002). **Table 2** summarizes the effects of iron supplementation on several indices of iron status. Several hematological variables in strength-trained athletes have been reported to be similarly low or even lower than that of endurance athletes (Koehler 2012, Spodaryk 1993). Nevertheless, it is mostly the endurance type of training that has been linked to lower values of several hematological indices (Brigham 1993, Schumacher 2002). Actually, although within normal values, lower levels of RBC have been reported in endurance and/or power athletes compared to sedentary individuals, while HGB and HCT were significantly lower in endurance athletes only when compared with power athletes (Schumacher 2002). These lower levels in endurance athletes have been attributed to reticulocytosis and expansion of plasma volume associated with chronic aerobic training (Brigham 1993, Schumacher 2002, Schumacher 2002). However, abnormal HGB concentration (< 13 g/dL) was not only reported in endurance athletes, but in male athletes of combat sports as well (Koehler 2012). What is more, even levels below 12 g/dL, defining iron deficiency anemia, has been reported for female rowers, indicating that abnormal decrements in concentration can be found in athletes other than runners (DellaValle 2011).

While in the general population a serum Ferr concentration below 12μg/L is used for the identification of first stage iron deficiency, wider serum Ferr cut-offs values (ranging from 12 to 40 μg/L) have been adopted for the identification of diminished iron stores and iron deficiency in athletes (Koehler 2012, Reinke 2012, Rowland 1988, Peeling 2007, Klingshirn 1992, DellaValle 2011). Additionally, iron deficiency has also been distinguished to absolute, when serum Ferr is below 30μg/L, or functional, when serum Ferr is within 30 - 90μg/L or serum Ferr is within 100 - 299μg/L and TS is below 20% (Reinke 2012). Although within normal range, athletes demonstrated lower values of serum Ferr but similar transferrin and Hp values compared with sedentary controls (Schumacher 2002). Similarly, in the study of

Spodaryk (1993) significantly lower Ferr concentration in endurance athletes is reported compared with strength-trained athletes and controls, and Ferr levels below 50μg/L in 18% of endurance athletes as compared to 12% in controls. Decreased serum Ferr values (< 35 μg/L) were recorded to one third of elite athletes (Koehler 2012). These results are in agreement with those of Nachtigall et al. (1996) where decreased serum Ferr values (< 35μg/L) and low, instead of normal, hepatic iron stores were also reported in male distance runners, indicating a true prelatent iron deficiency. The lower cut-off point of < 20 μg/L for Ferr adopted by DellaValle et al. (2011), identified 30% of the rowers as being non-anemic iron-depleted at the beginning of a pre-training period, while another 10% were identified as anemic according to values of less than 12.0 g/dl. A very intense training or competitive period may lead to absolute (Ferr < 30μg/L) and functional (Ferr within 30 - 90μg/L or Ferr within 100 - 299 μg/L + TS <20%) iron deficiency in professional male soccer players (Reinke 2012, Escanero 1997), elite rowers (Reinke 2012) and female swimmers (Brigham 1993). In some cases, the allowed recovery period before the next training phase may not be sufficient for the replenishment of the depleted iron stores (Reinke 2012), and this point definitely needs closer attention.

Based on data of several investigations, iron status disturbances are more frequent in female than in male athletes. Female athletes were about twice as likely to exhibit reduced Ferr levels (Koehler 2012). In that study, 58.8% of females had Ferr below 35 μg/L, whereas the corresponding percentage of their male counterparts was 31.2%. In another study (Rowland 1987), 45% of the female cross country runners became iron-deficient at the end of the competitive season, while in males only 17% of them were characterized as iron-deficient. Similarly, the prevalence of iron deficiency was greater in female athletes of several events, as compared to male athletes. Determination of the transferrin receptor-ferritin index (sTfR/logFerr) revealed values of 2.62±0.94 and 3.33±1.71 for iron-deficient male and female athletes, respectively (Malczewska 2001). Additionally, critically low Ferr levels below 15μg/L or even below 12μg/L have also been reported for female runners (Rowland 1987, Gropper 2006, Nickerson 1989).

The cause of reduced levels in Ferr or serum iron in athletes is not fully understood. Exercise induced hemolysis, as documented by the reduced HP values, may offer a plausible explanation. In the study of Magnusson et al. (1984), although no differences were observed in iron concentration, Hct, Ferr, TS, and bone marrow hemosiderin were lower in athletes compared to controls. However, no true iron deficiency was established based on the normal mean cell volume (MCV) and EP values, as well as on the normal sideroblast count in bone marrow smears of all athletes, confirming an adequate supply of iron to normoblasts. The lower Hct and Ferr values in athletes could be explained by the simultaneous marked decline of HP levels, indicating a shift of iron to the hepatocytes as a result of increased intravascular hemolysis.

Acute exercise

Not only chronic exercise, but also acute strenuous physical activity may alter several indices of iron status. A significant reduction in serum iron levels of 12.2 μmol/L was reported after a triathlon completion (Rogers 1986). The authors proposed that heavy sweating or a prelatent iron deficiency may explain the observed severe reduction of serum iron. However, sweat iron concentration does not correlate with the increased whole body sweat rates (DeRuisseau 2002). A slight increase in sTfR, although within the normal range, has also been recorded after incremental running to exhaustion, but not after 45 min of submaximal exercise or after 3 consecutive days of aerobic training in highly trained endurance cyclists (Schumacher 2002). After the incremental running, an increase in Ferr, as well as in Packed Cell Volume (PCV) was also observed. This increase was mainly attributed to the concurrent haemoconcentration, as evidenced by the pronounced fall in plasma volume.

Regardless the acute or chronic character of exercise where most studies report variable responses in iron status, there are also studies that do not support significant differences in iron status between trained and untrained individuals. Indeed, similar incidence of iron deficiency between male endurance young athletes and non-athletes involved in several

sport disciplines has been reported (Malczewska 1997). High physical activity of athletes did not affect iron stores, as it was found to be higher than in control subjects. It has to be mentioned though, that athletes had higher iron intake from the diet than controls, and that 18% of those that were iron-sufficient reported consumption of iron supplements. In a more recent study of the same institute (Malczewska 2000) that involved female endurance athletes, lower incidence of iron deficiency was reported in athletes as compared to controls. These studies may lead to the assumption that the increased iron dietary intake and dietary factors involved in iron metabolism compensated for the augmented, exercise-induced losses of iron in young athletes. Regarding iron deficiency in athletes whose iron intake was sufficient, the authors attributed its prevalence in its diminished absorption for the male, and its leak to the blood due to menstrual cycle for the female athletes.

Taken together, these studies that attempted to evaluate the effects of exercise on iron status of athletes suggest that high volume training during a competitive season may compromise iron homeostasis. One determining factor that could help explain the reported discrepancies in iron status due to acute or chronic exercise is diet. Unfortunately, not many studies report athletes' daily dietary intake of iron, and since iron intake or absorption are determining factors for iron balance future studies need to address this issue.

2.3. Iron supplementation and exercise-induced alterations of iron status

Numerous studies have attempted to clarify the effectiveness of enhanced iron intake, either through diet or through supplement consumption, to restore iron status or to enhance physical performance. Yet, no valid conclusions have been drawn. The results of these studies are contradictory as some of them produced positive effects (Friedmann 2001, Rowland 1988) whereas others dispute such effects (Tsalis 2004). An important factor in iron absorption seems to be the previous iron status of the individual. This means that, several iron parameters are seen to be ameliorated following iron supplementation in situations of iron deficiency, whereas this is not always the case for individuals with normal iron status. **Table 3** summarizes the effects of iron supplementation on iron status and physical performance.

Iron supplementation in iron-deficient individuals

Whether the increased uptake of iron through diet or supplements improves iron status in athletes is still under debate. This is mainly due to the great divergence of iron doses, intervention period, population, and exercise regimens used between studies. In situations of iron deficiency a proposed minimum therapeutic requirement corresponds to 100 mg/day of elemental iron, for a period of 12 weeks (Nielsen 1988). However, in several studies, much lower quantities of 20 - 50 mg/day of elemental iron for 12 weeks (Lyle 1992, Brigham 1993) or smaller duration (of even two weeks) of iron supplementation have also been used and reported to be adequate to restore iron status to normal (Schoene 1983). A treatment with 100 mg/day of ferrous iron for 3 months significantly increased the values of serum Ferr (from 34±11 to 54±18 μg/L) and liver iron (from 105±42 to 227±67 μg/g liver) (Nachtigall 1996). In this study, 23 out of 45 athletes showed decreased baseline serum values (<35μg/L), and the typical iron deficiency in runners was confirmed in a subgroup of eight athletes in which iron metabolism was studied in detail using radio-iron labelling and liver iron quantification. These eight athletes showed up-regulated ^{59}Fe absorption and a decreased liver iron concentration as compared to a control group. The results of the eight athletes confirm that in cases of true iron deficiency, iron absorption is greater. A moderate dose of 39 mg/day of elemental iron for 5 weeks effectively prevented the negative changes

of iron status over the course of a competitive season in female collegiate swimmers (Brigham 1993). Absence of iron supplementation resulted in decreased levels despite mean dietary iron intakes of 16.3 mg/day. The ingestion of 105 mg/day of elemental iron combined with 500 mg of Vitamin C for 60 days resulted in the amelioration of iron status of previously iron-depleted, non-anemic elite female athletes (Pitsis 2004). The improved iron stores were reflected by the increase of Ferr in conjunction with the decrease of transferrin, sTfR and sTfR/log ferritin index. Taken together the aforementioned results suggest that the initial stage of either iron sufficiency or iron deficiency, combined with the amount of iron ingested, plays a critical role in the absorption of iron from diet or supplementation.

Iron supplementation in individuals with normal iron status

Supplementation of iron is commonly used, not only in iron-deficient athletes, but also in athletes with normal iron status. The rationale behind this practice dictates that supplementation will preserve or enhance their performance. This concept is probably based on the catalytic role of iron on the oxygen transport and optimal function of oxidative enzymes and proteins during exercise. The hypothesis could be that with increased consumption of iron, the above mechanisms would be reinforced and exercise performance would be improved. Nevertheless, unlike the numerous studies addressing iron-deficient individuals, only few (Tsalis 2004, Escanero 1997, Powell 1991) have focused in iron-sufficient athletes. The response of iron stores during a sports season was assessed in professional football players with normal iron stores at the beginning of the season (Escanero 1997). The players consumed 50 mg/day of elemental iron over two periods during the training season. Supplementation took part for 15 days prior to the beginning of the season and 15 days during the middle season. Blood was collected three times during the season, one following the first supplementation period, another following the second supplementation period and a third time at the end of the season, where no iron supplementation had occurred. Ferritin, as well as calculated iron stores, showed a significant reduction at the end of the season which coincided with the absence of iron supplementation. In contrast, Ferr and iron store levels remained stable following supplementation regardless of the intensive training. In another study, non-anemic, non-

iron-deficient adolescent male and female swimmers aged 12-17 years old were either supplemented with 47 mg of elemental iron daily or consumed a diet rich in iron (Tsalis 2004). Both approaches failed to affect the athletes' iron status. In that study, despite the significant fluctuations during the six months of training, iron levels, TS and Ferr levels were similar at the end of the study as compared to baseline values. The authors attributed the failure of high iron intake to affect iron status to homeostatic mechanisms such as iron absorption. It could also be suggested that the quantity of elemental iron was not enough to improve iron status and that higher doses of iron are needed to achieve a favorable change in iron status. The younger age and the possible higher demands in the study of Tsalis et al. (2004) compared with that of Escanero et al. 1997, may have influenced the absorption of iron that resulted in different responses in these two studies.

Table 2. The effect of exercise on iron status

Study	Study protocol	Subjects	Estimated indices	Results
		Compromised iron status		
Chronic exercise				
Kohler et al. (2012)	Retrospective estimation of iron status in athletes from 25 different events	96 males 16.1±2.3yo and 97 females 16.3±3yo elite athletes	Nutrition, Ferr, Fe, hematological parameters, CK, VO_{2peak}	**Dietary iron:** 81% of male and 39% of female athletes reached the RDA; iron density was lower in males; 57% of the females and 31% of the males had Ferr<35μg/L; similar VO_{2peak} in athletes with low and normal iron status
Della Valle & Haas (2012)	Determination of the impact of iron depletion on performance at the beginning of a training season	165 female rowers (19.7±1.2yo)	Hb, Ferr, sTfR, 2-km TT	30% of the athletes were iron deficient (**Ferr** < 20μg/L) 10% of the athletes had iron deficiency anemia (**Hb** < 12 g/dL)
Reinke et al. (2012)	Assessment of iron status after 3 seasons: championship, recovery, pre-season training	10 professional male soccer players 20-36yo, 20 elite rowers 21-35yo	Hematological indices, Ferr, TS	27% of the athletes had iron deficiency after championship season which persisted in all time points in 14% of the athletes **Ferr:** no significant increase during recovery **sTransf:** ↑ in the recovery period, and ↓in the pre-season training **Hb:** 10% of the athletes had apparent or border line anemia in all time points
Schumacher et al. (2002a)	Epidimiological study, estimation of hematological and iron status in endurance, mixed of power athletes	747 male athletes (24.2±8yo), 104 controls (29.9±6.9yo)	RBC,Hb,Hct, Fe, Ferr, Tf, , VO_{2peak}	**Hb, Hct, RBC**: ↓in athletes than controls; ↓in endurance athletes **Ferr:** ↓in athletes than controls **Fe, Tf, :** no differences **VO_{2peak}:** ↑in endurance athletes
Malczewska et al. (2001)	Assessment of frequency of iron deficiency in athletes	131 males, 121 females of several events 16-36 years old	sTfR, Fe, TIBC, Ferr, TfR/log Ferr index	Latent iron deficiency in 29% of female and 11% of male athletes; higher sTfR and TIBC, lower Ferr levels in iron deficient compared with normal only in females
Nachtigall et al. (1996)	Estimation of iron status and iron metabolism throughout a	45 male distance runners	Ferr, ^{59}Fe absorption	Ferr values <35μg in 51% of the athletes; up-regulated ^{59}Fe absorption, decreased liver iron concentration

	training period			
Spodaryk et al. (1993)	Estimation of hematological and iron status in endurance (E), strength-trained (S) athletes and controls (C)	39 male athletes from the 1988 Polish Olympic team (20.4 ± 2.2yo)	Hb, PCV, RBC, Ret, Fe, Ferr, Tf, , TS	**Hb, PCV, RBC, TS:** ↓in E compared with C **Ferr:** ↓in E compared with S and C **Ret, GOT:** ↓in E compared with S
Brigham et al. (1993)	Estimation of iron status during a competitive season	25 female varsity collegiate swimmers	Hb, Ferr	At baseline 17 athletes were iron depleted and 5 athletes were anemic. After 5 wk Hb decreased (≥6 g/L) in 44%, and Ferr (≥5 μg/L) in 24% of the athletes
Nickerson et al. (1989)	Estimation of stage II iron deficiency (Ferr<12ng/ml and TS<16%) during the running session; iron supplementation or iron-rich diet or controls	41 female and 25 male cross-country runners and controls (15-18yo)	Ferr, TS, blood losses	**Iron deficiency:** 34% of females and 8% of males became iron deficient by the 45th or 75th day of running **Blood losses:** 14 stools in females and 1 stool in males with >4mg Hb/g of stool
Rowland et al. (1987)	Estimation of iron status during a competitive season; supplementation of iron in the iron	30 male and 20 female cross country runners (14.3-18.6yo)	Ferr, Hb, RBC parameters	45% of females and 17% of males identified as iron deficient during the season
Magnusson et al. (1984b)	Hematological and iron status comparison between distance runners	43 runners (19-46yo) and 119 controls (19-37yo)	Hb,Hct, MCV, MCHC, EP, Fe, TS, Ferr, BMHem, Sideroblasts,	**Hb:** no differences between groups **Hct, Fe, Ferr, TS, BMHem:** ↓in athletes **MCV, EP, MCHC:** no iron deficiency
Acute exercise				
Rogers et al (1986)	Evaluation pre, post, and 30 min, 24h, and 48h after a 160-km triathlon (canoeing, cycling, and running)	18 triathlon athletes	Fe, TIBC, lactoferrin, Ferr, cortisol, WCC, CRP, various enzymes	Post: ↑ in cortisol, WCC, lactoferrin and ↓ in Fe an TS; 24h: ↑by 300% in CRP, ↓ in
Schumacher et al. (2002b)	Estimation of exercise on sTfR and other variables after an incremental running test till exhaustion (IRTTE), 45min submaximal running, 3d aerobic cycling	39 athletes (33 men and 6 women) (19.6-32.5y)	Hb, Hct, BV, PV, RCV, sTfR, Ferr, Fe	**Hb, Hct:** ↑ aft r exercise **PV, BV:** ↑after incremental and submaximal rur ning ↓after 3d aerobic cycling **sTfR:** ↑after the incremental running **Ferr:** ↑after incremental and submaximal running

		No alterations in iron status		
Malczewska et al (1997)	Estimation of iron status in endurance athletes	178 male athletes, 52 male controls 18-20 years old	Hb, PCV, RBC, WBC, MCHC, MCV, MCH, Fe, TS, TIBC, Ferr,	Similar iron depletion in athletes (19%) and controls (20%); ↓ Ferr, Fe and ↑TIBC in the iron depleted subgroups
Malczewska et al (2000)	Estimation of iron status in endurance athletes	126 female athletes, 52 female controls (16-20y)	Hb, Hct, RBC, WBC, MCHC, MCV, MCH, Fe, TIBC, TS, Ferr,	Lower iron deficiency (26%) in athletes than controls (50%); ↓ Ferr, Fe and ↑TIBC in the iron deficient subgroups

VO_{2max}: Maximum Oxygen Consumption; TT: Time Trial; CK: Creatine Kinase; Ferr: Ferritin; Fe: Iron; Tf: Transferrin; TS: Transferrin Saturation; sTfR: Soluble Transferrin Receptor; TIBC: Total Iron Binding Capacity; Hb: Haemoglobin; Hct: Hematocrit; PCV: Packed Cell Volume; Ret: Reticulocytes; WCC: White Cells Count; RBC: Red Blood Cells; RCV: Red Cells Volume; WBC: White Blood Cells; MCH: Mean corpuscular Haemoglobin; MCHC: Mean Corpuscular Haemoglobin Concentration; MCV: Mean Corpuscular Volume; BMHem: Bone Marrow Hemosiderin; EP: Erythrocyte Protoporphyrin; Hp: Haptoglobin; PV: Plasma Volume; BV: Blood Volume; GOT: Glutamic Oxaloacetic Transaminase; CRP: C Reactive Protein.

2.4. Iron supplementation and physical performance

Iron supplementation in iron-deficient individuals

There is no doubt that iron-deficiency anemia, which amongst other indicators (e.g. Ferr<12μg/L, TS<16%), is characterized by a decline in blood concentration, clearly impairs physical performance by limiting oxygen transport to exercising muscles (Rowland 2012). However, the need for iron supplementation in cases of depleted iron stores without observed anemia for optimal physical performance is still under debate (Table 3). Some studies have shown that iron supplementation improved physical performance (Friedmann 2001, Rowland 1988), whereas others report no alterations following iron supplementation (Tsalis 2004, Klingshirn 1992, Powell 1991). The improvement of iron status due to iron supplementation has been accompanied by an improvement in endurance capacity (Friedmann 2001, Rowland 1988). In young elite athletes with normal concentrations, the return of low Ferr to normal values following supplementation of 200 mg/day of elemental iron for 12 weeks, even in the absence of increased erythropoiesis, has been shown to improve maximal aerobic capacity (Friedmann 2001). Iron supplementation also prevented the decline in performance that was associated with the progressive reduction of serum Ferr levels (Rowland 1988). Iron deficient cross-country female runners were treated with 975 mg/d of ferrous sulfate or placebo for 4 weeks. Iron supplementation resulted in an increase in ferritin levels which was accompanied by an improvement of physical performance. Subjects not receiving iron therapy exhibited a decline in their performance (Rowland 1988).

Besides the aforementioned positive results in exercise performance there are studies reporting no beneficial effects due to iron supplementation (Tsalis 2004, Klingshirn 1992, Powell 1991). In the study of Powell et al. (1991), no significant improvement of iron status or metabolic parameters related to running performance was found after 2 weeks of 130 mg elemental iron supplementation in non-anemic, iron-deficient female cross-country runners. Likewise, Klingshirn et al. (1992), report that 8 weeks of iron supplementation in iron-depleted, non-anemic female distance runners, resulted in similar improvement of the endurance capacity in the

supplemented and the placebo group, despite the improved iron status in the iron-supplemented group. In another study, the injection of 2 mL of Ferrum H (100mg of elemental iron) five times daily for 10 days did not result in any beneficial outcomes and time to fatigue in non-anemic, iron-deficient female runners (Peeling 2007). This study, failed to demonstrate any beneficial effect of iron supplementation on aerobic capacity, despite a significant rise in serum Ferr levels (from 19 to 65μg/L).

Iron supplementation in individuals with normal iron status

In one of the very few studies that used healthy, non-iron-depleted and non-anemic adolescent swimmers, the enhanced iron intake either through supplement or diet ranging from one to five times the RDA, did not change iron status or result in favorable changes of physical performance (Tsalis 2004). The authors attributed the observed fluctuations over the training period of six months to the different demands of each training phase irrespective of iron treatment. These observations strengthen the notion that the initial levels of iron status are of critical importance in the improvement of physical performance as a result of iron supplementation. In (Brigham 1993), the mean dietary intake of 16.3 mg/day was not adequate to prevent the disturbance of iron status in female collegiate swimmers. Haemoglobin levels decreased at about 6 g/L in 44% of the athletes given placebo treatment, whereas the corresponding decrement in plasma Ferr was 5μg/L in 24% of the swimmers given the iron supplement. Consequently, the reductions in Ferr levels were lower in the athletes that were under iron supplementation.

Table 3. The effect of dietary or supplemented iron on exercise-induced changes of iron status and physical performance

Studies	Study protocol	Subjects	Estimated Indices	Results
		Improvement in iron status		
Nachtigal et al. (1996)	100mg/d of elemental iron for 3 months, radio-iron labeling (^{59}Fe) in 8 iron deficient athletes	45 runners (23 out of 45 were iron deficient, Ferr<35μg/L), and controls	Ferr, iron absorption, liver iron	**Ferr:** ↑ from 34±11 to 54±18 μg/L **liver Fe:** ↑from 105 ±42 to 227±67 μg/g liver
Brigham et al. (1993)	39mg/d of elemental iron (IG) or placebo (PG) for 5 wks	25 female, iron depleted, varsity collegiate swimmers	Hb, Ferr	**Hb**:↑ in 24% of the subjects in the IG and in 12% in PG **Ferr**: ↑ in 68% of the subjects in the IG and in 4% in PG
Pitsis et al. (2004)	105mg/d elemental iron + 500mg/d Vit C for 60 days	36 elite iron-depleted, non-anemic female athletes of several disciplines (13-26y)	Red cell & reticulocyte parameters, Fe, Ferr, Tf, TS, sTfR	**Ferr:** ↑ **Tf:** ↓ **sTfR:** ↓ **sTfR/ log Ferr:** ↓ **Red cell and reticulocyte parameters:** no changes
Rowland et al. (1987)	Estimation of iron status during a competitive season; supplementation of iron in the iron deficient athletes (IG) in the midpoint of the season (the dose is not reported)	30 male and 20 female cross country runners (14.3-18.6y)	Ferr, Hb, RBC, MCV, RBCDW,	**Ferr:** ↑in IG and ↓in untreated athletes at the end of the season **Hb, RBC, MCV, RBCDW:** no changes
Escanero et al. (1997)	Variation of iron metabolism through a season; 50mg of iron/day for the last 15 days at the beginning (A) and the middle of (B), but not at the end (C) the season	9 soccer players at the 1st division (24±2.1y)	RBC, MCV, MCH, MCHC, Fe, Tf, Ferr, TIBC, TS	**Ferr, Iron stores:** ↓at the end of the season (no iron supplement); remained stable at the beginning and the middle of the season (iron supplementation)

Improvement of performance				
Friedmann et al. (2001)	2 x 100mg/d elemental iron (IG) or placebo (PG) for 12 wks; the usual training	40 iron depleted endurance athletes (13.6-21.1y, Ferr < 20 ng/mL); IG: 20 males and females, PG: 20 females and 8 males (14.5 -17.5y)	Hematological indices, Fe, Ferr, Trf, TS, BV, PV, VO_2, VCO_2, VE, MAOD, LA	IG: **VO_{2max}, VO_2, TTE**: ↑ **Ferr:** ↑ 20.1μg/ L **Tf:** ↓ PG: no changes
Rowland et al. (1988)	975mg/day ferrous sulfate (IG) or placebo (PG) for 4 wks (time C), after a 4wk control period (time B)	14 iron-deficient (Ferr < 20ng /mL) female cross-country runners (high-school age)	TTE, HR, VO_{2max}, VE, Hb, MCV, RBCDW, Ferr	**TTE:** ↑in both groups at time B, ↑ in IG and ↓in PG at time C **Ferr:** ↓at me B in both groups, ↑at time C in I **Hb, MCV, RBCDW:** No changes in both groups
No changes in iron status or performance				
Peeling et al. (2007)	Intramuscular iron injections (5 x 2mL Ferrum H/day) (IG) or placebo for 20 days	16 iron depleted, female distance runners and controls	VO_{2max}, HR, LA, run-TTE, 10min submaximal economy test, Ferr	**Ferr:** ↑in IG **HR, LA, TTE:** no differences between IG and PG
Tsalis et al. (2004)	47mg/d (IG) or dietary plan rich in iron (DIG) or regular diet for 6 months (endurance training: 3m; power training: 2m; tapering: 1m)	21 males and 21 females, 12-17y, non-anemic, non-iron-deficient	Hematological indices; Fe, TIBC, TS, Ferr; swimming tests: 2000m, 800m, 200m and 25sprint	**Fe, TIBC, TS, Ferr:** fluctuations within the phases **At m6:** no differences from baseline **RBC, Hb and PVC**: ↑at m6 **performance tests**: similar ↑in all groups
Klingshirn et al. (1992)	8wks of iron supplementation (IG), or placebo (PG)	18 iron depleted, female distance runners and controls, (22-39y)	VO_{2max}, endurance run-TTE, LA, iron status	**Ferr, TIBC:** ↑ in IG compared with PG at wk 8 **Endurance performance, LA:** similar ↑ both groups

Powell & Tucker (1991)	130mg of elemental iron /day (IG) or placebo (PG), for 2wks; single blind design	10 female cross-country runners (20.2±1.3y) with normal iron status	VO_{2max}, CO2, RER, VE,Fe, TIBC, Ferr, Hp, Hb, Hct, MCHC, MCV, WCC, LA	**Hematological & iron status parameters:** no significant changes **Metabolic parameters:** no changes

VO2max: Maximal Oxygen Consumption; VCO2: Exhaled Carbon Dioxide; VE: Ventilation; MAOD: Maximal Accumulated Oxygen Deficit; TTE: Time to Exhaustion; HR: Heart Rate; CK: Creatine Kinase; LA: Lactate; Ferr: Ferritin; Fe: Iron; Tf: Transferrin; TS: Transferrin Saturation, sTfR: Soluble Transferrin Receptor, TIBC: Total Iron Binding Capacity, Hb: Haemoglobin, Hct: Hematocrit, PCV: Packed Cell Volume, MCHC: Mean Corpuscular Haemoglobin Concentration, BV: Blood volume, PV: Plasma Volume, MCV: Mean Corpuscular Volumes, RBC: Red Blood Cells Count, RBCDW: Red Blood Cell Distribution Width, WCC: White Cells Count, MAOD: Maximal Accumulated Oxygen Deficit.

2.5. Eccentric exercise and muscle damage

Eccentric muscle lengthening contractions are an essential component of human daily activities, and almost all of the athletic actions. During this type of contraction the length of the muscle is increasing whilst the muscle itself attempts to contract. It is well documented that unaccustomed eccentric exercise can lead to muscle damage which is accompanied by several physiological, hematological, and biochemical responses that consist the symptoms of exercise-induced muscle injury. Alongside with morphological and ultra-structural changes to muscle architecture (Friden 1984), muscle damage is manifested by muscle soreness, force and power output decline (Eston 2003, Clarkson 1992), deterioration of range of motion (ROM) (Cleak 1992), deterioration in running economy (Paschalis 2005, Chen 2009), alterations in position sense and reaction angle (Paschalis 2007, Paschalis 2010), connective tissue damage (Tofas 2008, Brown 1997b), muscle swelling (Howell 1993), increased flux of muscle proteins into the circulation (Jamurtas 2000), migration of monocytes and neutrophils into the injured area (Al-Majid, Tidball 2010, Stavropoulos-Kalinoglou 2014, Fehrenbach 2006, Peake 2005, Cantini 2002, Smith 2008), and increased oxidative stress in blood and muscle (Paschalis 2007, Theodorou 2011).

Initial injury: In exercise-induced muscle damage, the initial injury is thought to occur either via mechanical or metabolic stress (Kendal 2002). Particularly in eccentric exercise, the contribution of mechanical stress is greater due to relatively low metabolic demand (Kendal 2002). The primary sequence of events leading to exercise-induced muscle trauma is believed to involve initial mechanical disruption of sarcomeres (Friden 1992) followed by impaired excitation-contraction coupling and calcium (Ca^{2+}) signaling, and finally, activation of calcium-sensitive degradation pathways (Peake 2005a). The initial myofibril disruption has been attributed to high forces (Tiidus and Ianuzzo 1983), high tensile stresses (McCully and Faulkner 1985), or an imbalance in adjacent sarcomere tension (Friden and Lieber 1992). During eccentric contractions, fewer fibers are activated to exert a given amount of force as compared to concentric or isometric contractions; therefore, larger forces per muscle fiber are developed, resulting in greater damage. Furthermore, during an

eccentric contraction, some sarcomeres in muscle fibers are more resistant to stretching than others, forcing weaker sarcomeres to absorb more stretch. With repeated eccentric contractions, the weaker sarcomeres first and then the stronger sarcomeres are overstretched. If the latter fail to withstand the stretching force during the relaxation phase, damage may occur (Jamurtas 2012). Histological evidence indicates that following an intense bout of 300 maximal voluntary eccentric contractions of the knee extensors, approximately one third of the muscle fibers show intense myofibrillar disruptions, myofillament disorganization and loss of Z line integrity (Armstrong 1984). Especially concerning Z-bands of sarcomeres, they seem to be the most affected region of the myofibrillar and may be broadened, smeared, or even totally disrupted (Friden 1992).

Secondary injury: After the initial trauma, progressive myofibril degeneration is observed in some fibers suggesting a secondary sequence of events, with release of intracellular proteins and infiltration of the tissue by neutrophils and macrophages (Round 1987). This secondary injury occurs as a result of the disruption of the membrane of the sarcoplasmic reticulum or sarcolemma and disturbance of Ca^{2+} homeostasis (Raj 1998). Increases in intracellular Ca^{2+} concentration lead to additional degradation of muscle fibers due to activation of calcium-dependent proteolytic enzymes (Raj 1998, Proske & Allen 2005), such as the calpain mediated proteases (Raj 1998). These processes are accompanied by inflammatory responses and associate with fiber breakdown and repair (Proske & Allen 2005). Neutrophils invade the injured tissue within several hours following the initial insult, while macrophages and proinflammatory cytokines are produced in the muscle within 24 hours and can be present for several days following eccentric exercise (Beaton 2002). In addition, reactive oxygen and nitrogen species (RONS) that are produced in excess by neutrophils and macrophages, consequently leading to oxidative stress, contribute to the initial damage (Close 2005), but they also facilitate the restoration of the injured muscle by degrading the damaged tissue, and probably by enhancing the repair processes (Tidball 2005; 2010).

2.5.1. Effects of eccentric exercise on muscle function and performance

Exercise-induced muscle damage is manifested by a number of classic physiological symptoms such as DOMS, deterioration in ROM, reduced muscle contractility and force production, disproportionate loss of force at low frequencies of stimulation compared to higher frequencies, but also leakage of muscle proteins and muscle enzymes into the circulation, as well as elevated markers of connective tissue break down in blood and urine (Howell 1993, Paschalis 2010, Paschalis 2007, Nikolaidis 2007, Lee 2002, Tofas 2008, in Eston 2003, Clarkson 1992, Cleak &Eston 1992, Edwards 1977). The effect of muscle-damaging exercise on muscle damage, and muscle function and performance indices are summarized in **Table 4.**

Force decline

Prolonged strength loss after eccentric exercise is considered to be one of the most valid and reliable indirect measures of muscle damage in humans (Warren 1999). Estimation of isometric peak torque (IPT) has been the most widely used method of determining muscle function after eccentric exercise, but concentric and eccentric peak torque have also been used. Isometric force declines immediately after eccentric exercise and recovery is gradual and elongated (Howell 1993, Paschalis 2010, Paschalis 2007, Paschalis 2005, Brown 1997b, Nikolaidis 2007, Lee 2002). Concentric and eccentric peak torque generally follows the same pattern of deterioration and recovery (Close 2004, Paschalis 2005, Eston 1996, Tofas 2008).

Upper extremities seem to suffer greater symptoms of muscle damage compared to lower extremities (Jamurtas 2005). Typically, there is a 50-60% reduction in isometric force of the elbow flexors immediately after maximal eccentric exercise compared to baseline that recovers gradually in the next two weeks (Clarkson 1992, Cleak &Eston 1992). The corresponding force decline for knee and ankle extensors is around 35%, and the recovery is achieved faster than that observed for elbow flexor, that is 4-7 days (Eston 2003, Paschalis 2010, Paschalis 2007, Nikolaidis 2007). The greater severity of muscle damage in the elbow flexors probably accounts for the greater force reduction compared to ankle and knee extensors.

The loss of strength after eccentric exercise, is disproportionate at short versus optimal or long muscle lengths (Philippou 2003, McHugh & Tetro 2003, Saxton & Donnelly, 1996) and there is a shift to the right of the optimal angle for torque generation, indicating a shift in the length-tension relationship towards longer muscle lengths for maximal force generation (McHugh & Tetro 2003; Whitehead 1998). The "popping sarcomere hypothesis" of Proske & Morgan (2001), could explain this phenomenon. According to this hypothesis, lengthening of active muscle does not occur by uniform lengthening of all sarcomeres, with some sarcomeres rapidly over-extending ("popping") beyond myofilament overlap and failing to re-interdigitate upon relaxation. Such over-extended sarcomeres would result in the remaining functional sarcomeres adopting a shorter length to compensate and a shift in the length-tension relationship towards longer muscle lengths. Deterioration of the excitation-contraction coupling process (Ingalls 1999) can further explain the disproportionate loss of strength at short muscle lengths after a damaging exercise protocol (Jones 1996).

The type of eccentric exercise, as well as the extent of muscle damage affects muscle performance in a different way after eccentric exercise. High intensity isokinetic eccentric exercise provoked greater decrements in eccentric (EPT) and isometric peak torque (IPT) compared to low intensity exercise of the same volume, although muscle damage was similar after both high and low intensity eccentric bouts (Paschalis 2005). On the other hand, an intense acute bout of plyometric exercise (Tofas 2008), although increased muscle damage, DOMS and collagen breakdown, did not result in decreased muscle functional capacity as both EPT and concentric peak torque but also ROM, did not alter after exercise. This was probably due to the fact that the provoked muscle damage after plyometric exercise was not great enough compared to high intensity eccentric exercise used in the study of Paschalis et al. (2005) to decrease muscle performance.

Except for force decline, deterioration of power output (Sargeant and Dolan 1987) vertical jump (Horita 1999, Fatouros 2010), sprinting ability (Semark 1999, Fatouros 2010) but also endurance performance, has been reported after eccentric exercise-induced muscle damage.

Delayed Onset of Muscle Soreness

The development of delayed onset of muscle soreness (DOMS) is one of the most prevalent symptoms of exercise-induced muscle injury. Soreness is experienced as a dull, aching pain felt after eccentric exercise, and its sensation is elicited when mechanical stimuli such as pressure, stretching, or contraction are imposed on the affected muscles (Nosaka 2008). Sore muscles are often described as being “stiff” or “tender” (Howel 1993). The inflammatory responses that accompany eccentric exercise-induced muscle injury lead to prostaglandin and leukotriene synthesis (Connolly 2003). Prostaglandin E_2 directly causes the sensation of pain by synthesizing type III and IV pain afferents to the effects of chemical stimuli, whereas leukotrienes increase vascular permeability and attract neutrophils to the site of damage. Swelling resulting from the movement of cells and fluid from the blood stream into the interstitial spaces can further contribute to the sensation of pain (Connolly 2003). Muscle soreness appears many hours after performance of damage-inducing exercise (Maughan 1989), peaks 24-72 h post exercise, and then subsides and disappears 5-7 days post-exercise (Connolly 2003, Armstrong 1984, Nosaka 2008, Paschalis 2010, Paschalis 2007, Eston 1996, Nikolaidis 2007, Lee 2002, Close 2004, Maughan 1989), although the persistence of DOMS for more than 7 days has also been reported (Brown 1997b). DOMS is accompanied by reduced mobility or flexibility, and the muscles are sensitive, particularly upon palpation or movement. What is interesting though is the fact that the magnitude of DOMS resulting from exercise-induced muscle damage, does not necessarily represents the magnitude of muscle damage, and the time-course of DOMS does not represent the time-course of changes in indicators of muscle damage (Nosaka 2008). In the study of Jamurtas et al. (2005), eccentric exercise, performed with both arms and legs, resulted in similar DOMS, although the arm eccentric exercise induced larger decreases and slower recovery of strength, as well as larger increases in blood markers of muscle damage (CK, myoglobin), than the leg exercise. Nevertheless, similarly with greater force reduction, maximal eccentric contractions of the elbow flexors, also produces greater DOMS than isokinetic eccentric exercise of the knee extensors or downhill running (Clarkson 2002).

Range of movement (ROM)

Range of movement is defined as the arc over which a joint may operate and this constrains the muscle length range (Warren 1999). ROM is determined by skin, subcutaneous tissue, tendon, articular capsule and bone properties. Pain free ROM, that is the segment's movement from 0^{o} extension to flexion where the subject feels any discomfort, is usually used as a complementary tool to define changes in muscle function. ROM is disturbed as a result of eccentric exercise. Disturbance of ROM is evident immediately after eccentric exercise, usually reaching a nadir at 48h, and returns to resting values several days after exercise (Jamurtas 2005, Paschalis 2010, Paschalis 2005, Tofas 2008, Lee 2002).

Leakage of muscle proteins and enzymes into the circulation

The presence of muscle proteins and muscle enzymes into the circulation, is used as an indirect marker of muscle damage in terms of muscle membrane permeability and as evidence of lethal injury to muscle fibers (Friden 1989). Creatine kinase (CK) is the most frequently used marker, but other enzymes and proteins such as LDH, myoglobin (Mb) and glutamic oxaloacetic transaminase (GOT), has also been engaged.

Changes in CK activity in adults has been shown to occur up to 7days after muscle damaging exercise (Brown 1997a), usually peaking between 2d and 4d (Jamurtas 2005, Child 1999, Childs 2001, Hellsten 17, Brown 1997b, Gleeson 1995, Paschalis 2010, Paschalis 2007, Paschalis 2005, Eston 1996, Theodorou 2011, Lee 2002, Close 2004, Maughan 1989) while reaching values as high as 150-fold (Hellsten 1997) the pre-exercise levels. LDH (Brown 1997b, Maughan 1989, Tiidus 1992, Tofas 2008, Childs 2001), Mb (Jamurtas 2005, Peake 2005, Childs 2001), and GOT (Tiidus 1992) were also elevated in blood after eccentric exercise.

The intensity, as well as the duration of eccentric exercise, that may reflect muscle fatigue and damage, has been shown to play a role on muscle membrane permeability (Brown 1997a, Tiidus 1992). Brown et al. (1997a) demonstrated elevated CK concentration after 30 and 50, but not after 10 maximal isokinetic

eccentric voluntary contractions of the knee extensors, although force loss was evident in all of the above 3 exercise protocols. Similarly, under constant total work, Tiidus et al. (1992) reported higher CK, LDH and GOT concentration, as well as higher DOMS after high intensity, short-duration exercise (80% 10RM), compared to low intensity, long-duration exercise (30% 10RM).

Except for muscle enzymes and muscle proteins, collagen constituents into the blood or in urine, are used as markers of connective tissue breakdown. Hydroxyproline and hydroxylysine are reported to increase at several time points following eccentric exercise (Tofas 2008, Brown 1997b), remaining raised up to 9 days post exercise (Brown 1997b). It seems that high forces generated during eccentric muscle contractions are also capable of increasing the susceptibility of connective tissue structures to exercise-induced injury (Stauber 1989).

2.5.2. Muscle damage and muscle performance after eccentric exercise in adults compared to children

The well-established symptoms of muscle injury are coming by research that has mostly been conducted in adults. In contrast, there is limited data regarding exercise-induced muscle damage responses in children and even less comparing these responses with those of adults (Marginson 2005, Gorianovas 2013, Soares 1996, Marginson 2001). Nevertheless, it seems that children are more resistant in exercise-induced muscle damage, as they sustain milder symptoms of muscle injury, and also, they recover faster compared to adults.

In the study of Marginson et al. (2005), DOMS and isometric strength reduction that appeared 30 min after 8 sets of 10 plyometric jumps, remained up to 72h in men while only up to 24h in children. In another study (Soares 1996), participants performed bench press at 80% of their individual 1RM until subjective exhaustion. DOMS, although was evident in both men and boys the days after exercise, it was more pronounced in men compared to boys 2 and 3 days after exercise. Isometric force significantly reduced up to 6 days after exercise in men, while no changes

occurred in boys. The response of CK activity was also different, with men demonstrating higher values 48h and up to 1 week after exercise while no elevations occurred in boys. In a more recent study (Gorianovas 2013), maximal isometric voluntary contraction was lower and muscle soreness and CK higher in both adults and children after 100 intermittent drop jumps, yet, these changes were more expressed in adult males than in boys. An interesting finding in the study of Marginson & Eston (2001) was the shift to the right in the length tension curve in children compared with adults, such that children's maximal voluntary isometric force of the quadriceps measured in the sitting position on an isokinetic dynamometer was lower at 20^{o}, 40^{o} and 60^{o}, and higher at 90^{o} and 100^{o} (0^{o} = full extension) compared to adults. This, along with other possible reasons that are debated later in the discussion section, could in part explain the apparent decreased susceptibility in muscle damage of children.

Table 4. The effect of eccentric exercise on muscle damage, and muscle function and performance

Authors	Study protocol	Subjects	Estimated indices	Results
		Adults		
Jamurtas et al. (2005)	6 sets of 12 isokinetic eccentric contractions with both ARMS (elbow flexors) & LEGS (knee extensors); intensity: 75% of EPT_{max}	11 healthy untrained males (21.2 ±1.0 y)	EPT, IPT, ROM, DOMS, CK, LDH, Mb	**EPT:** ↓ in ARMS at 3 and 4d post ex, Greater ↓ in ARMS vs LEGS at 2, 3, 4d post ex **IPT:** ↓in ARMS 1up to 4d; ↓in LEGS at 1 and 2d, Greater ↓ in ARMS vs LEGS **ROM:** ↓ in ARMS & LEGS 1up to 4d **DOMS:** ↑ in ARMS & LEGS 2 up to 4d post ex **CK:** ↑ in ARMS & LEGS at 1d and peaked at 4d, Greater ↓ in ARMS vs LEGS **LDH:** ↑ in ARMS: 1up to 4d **Mb:** ↑ in ARMS & LEGS 3d and 4d post ex
Paschalis et al. (2010)	5 sets of 15 isokinetic eccentric MVC with both ARMS (elbow flexors) & LEGS (knee extensors)	12 healthy active males (23.1 ±1.0 y)	IPT, ROM, DOMS, PS, KJRA, CK	**IPT:** ↓ in ARMS post and up to 4d and↓ in LEGS post and up to 3d, Greater ↓ in ARMS vs LEGS at 1 up to 3d **ROM:** ↓ in ARMS post and up to 4d and ↓ in LEGS post and up to 3d, Greater ↓ in ARMS vs LEGS at 1 up to 3d post ex **DOMS:** ↑ in ARMS post and up to 4d, ↑ in LEGS at 1d and up to 3d, Greater ↑ in ARMS vs LEGS at 1up to 3d post ex **CK:** ↑ in ARMS & LEGS at 2d, peaked at 3d and remained up to 4d, Greater ↓ in ARMS vs LEGS at 2d and 4d post ex **Proprioception:** ↓ PS and ↑ KJRA in ARMS & LEGS at several time points, Greater ↓ in ARMS vs LEGS
Paschalis et al. (2007)	5 sets of 15 isokinetic eccentric MVC of the knee extensors	12 healthy untrained females (20.0±1.0y)	IPT, ROM, DOMS, PS, KJRA, CK	**IPT:** ↓ post and up to 48 h post ex **DOMS:** ↑ post and up to 72h post ex **CK:** ↑24h and peaked at 72h post ex **Proprioception:** ↓ PS post ex; ↑ KJRA post and up to 72h post ex
Brown et al. (1997a)	G10: 10, G30: 30, G50: 50 isokinetic eccentric MVC of the knee extensors	24 untrained subjects (G10: 20.5±1.1y, G30: 20.1±0.8y,	MVC, ROM, DOMS, CK, MTF(100Hz), MTF(20Hz)	**MVC:** ↓ in all groups, Greater ↓ in G50 vs G30 and G50 at 3, 7, 9d post ex **CK:** G10, no changes; ↑ in G30 2d post ex; ↑ in G50: 2 and up to 7d post ex

		G50: 22.1±2.4y)		
Paschalis et al. (2005)	Comparison of high (HIG) and low (LIG) isokinetic eccentric ex of equal volume	12 healthy men (21.0±1.0)	IPT, EPT, ROM, DOMS, CK	**IPT & EPT**:↓ at 24 post ex in HIG, Greater force reduction in HIG vs LIG **ROM:** ↓ at 1 and 2d in HIG, ↓at 1d in LIG **DOMS:** at 1d up to 4d in HIG & LIG, **CK:** ↑at 1d up to 4d in HIG & LIG
Brown et al. (1997b)	50 isokinetic eccentric MVC of the knee extensors	2 males & 6 females (22±2y)	IPT, DOMS, 20:100Hz MTF, CK, LDH, ALP, PYD:Cr, HP:Cr, HL:Cr	**IPT:** ↓post and up to 3d **20:100Hz MTF:** ↓ post and up to 1d **DOMS:** at 1d and up to 6d **CK:** ↑at 3 and 7d **LDH:** ↑ at 3d **ALP:** no changes **HP:Cr:** ↑at 2d **HL:Cr:** ↑at 2, 5 and 9d **PYD:Cr:** no changes
Eston et al. (1996)	100 eccentric MVC of the knee extensors	10 healthy males CG: (21.8 ±2.9y) EG: (23.2 ± 2.y)	Muscle tenderness (MT), CK, EPT, CPT,	**MT:** ↑at 2d and 4d **EPT & CPT:** ↓post and up to 2d **CK:** ↑at 2d and 4d
Tiidus et al. (1992)	INTG: 150 reps at 35%, 70%, and 90% of 10RM; DURG: 100, 200, and 300 reps at 70% 10RM; CTWG: constant work, HI-short duration, LI-long duration; knee extensors	21 untrained male and female (20-45 y)	CK, GOT, LDH, DOMS	**CK, GOT, LDH:** ↑at 8h up to 1d, Greater ↑at HI, short-duration vs LI, long-duration ex; Greater ↑ after 300reps vs 100 and 200 reps at 70% 10RM **DOMS:** ↑at 1d and 2d; Greater ↑at HI vs LI ex; Greater ↑at long vs short duration ex
Tofas et al. (2008)	8 sets of 12 jumps over 50-cm hurdles repetitions and 8 sets of 12 jumps onto a 50-cm plyometric box	18 untrained healthy men; CG: (21.3±1.3y) EG: (22.1±1.8y)	CPT, EPT, ROM, DOMS, CK, LDH, HP, HL, LA	**CPT, EPT, ROM:** no changes **DOMS:** ↑at 48h **CK:** ↑at 48 & 72h **LDH:**↑at 24, 48, 72h **HP:** ↑at 24 up to 72h **HL:** ↑at 48h; LA: ↑post ex
Peake et al. (2005)	45 min downhill run at 60% VO2max	10 well-trained male runner and	TLC, Neutrophils, CK, Mb, IL-6, IL-8	**TLC, Neutrophils, IL-6:** ↑post up to 1h **CK, Mb:** ↑post up to 1d

		triathletes (28 ± 3 y)		
Gleeson et al. (1995)	Bench stepping	8 healthy untrained adults (28.0±8.0 y)	CK, DOMS, WBC, CRP, Zinc, Fe, IgG, IgM, albumin	**DOMS, CK:** ↑at 1d up to 4d **CRP:** ↑at 1d; **WBC:** ↑post up to 4h and ↓ at 2 and 3d **Fe, Zinc:** ↓1 and 2d **Albumin, IgG, IgM:** ↓at 1d up to 7d
		Children vs Adults		
Marginson et al. (2005)	8 sets of 10 plyometric jumps	10 boys (9.9±0.3y) 10 men (22.2±2.7y)	Power output, DOMS, IPT, SJ, CMJ Torque-joint angle	**Power output:** ↑ in men; **DOMS: Boys:**↑ post up to 24h; **Men:** ↑ post up to 3d; Greater ↑in men vs boys **IPT: Boys:** ↓post up to 24h; **Men:** ↓ post up to 3d; Greater ↓in men vs boys **SJ: Boys:** ↓post; **Men:** ↓ post up to 3d; Greater ↓in men vs boys; **Torque-joint angle:** Relative torque was higher in men vs boys at 20, 40, and 60°, but higher in boys, at 100°.
Soares et al. (1996)	5 sets of bench press at 80% of 1RM until subjective exhaustion	10 boys (12.1 + 0.2 y) 10 men (28.3 ±3.5y)	MIF, DOMS, CK	**MIF: Boys:** no changes; **Men:** ↓ post up to 72h; Greater↓ in men vs boys DOMS: **Boys:** ↑ post up to 72h; **Men:** ↑ post up to 72h; Greater↑ in men vs boys **CK: Boys:** no changes; Men: ↑at 72h up to 1 wk; Greater ↑ in men vs boys
Gorianovas et al. (2013)	100 intermittent drop jumps (DJs) from 0.50 m	11 boys (11.8±0.9y) 11 adults (20.8±1.9 y) 11 elderly males (63.2±3.6 y)	CK, IPT, MTF 20Hz, MTF 100 Hz, MVC, DJs height	**CK: Boys, Adults, Elderly:** ↑at 24h up to 2d; Greater ↑in adults vs boys and elderly **DOMS:** ↑ at 24h in adults and elderly; no changes in boys **MVC: Boys, Adults, Elderly:** ↓ post ex up to 2d; Greater ↓ in adults vs boys and elderly **DJs height: Boys, Adults, Elderly:** ↓ post ex up to 2d; Greater ↓ in adults vs boys and elderly
Marginson & Eston (2001)	3 s of isometric MVC with knee extensors at 20^{o},	8 boys (9.3±0.9 y) 8 men (21±2.1 y)	Torque-joint angle	**IPT: Men:** IPT attained at 80^{o}; **Boys:** IPT attained at 80^{o} and 90^{o}; **Relative IPT:** Greater at 20^{o}, 40^{o}, 60^{o} in men vs boys; Greater at 100^{o}

40^{o}, 60^{o}, 80^{o}, 90^{o}, 100^{o}	in boys vs men.

MVC: Muscular voluntary contractions; G: Group; HIG: High intensity group; LIG: Low intensity group; DJs: Drop jumps; INTG: Intensty group; DURG: Duration group; CTWG: Constant total work group; CG: Control group; EG: Experimental group; MVC: Maximum voluntary contractions; IPT: Isokinetic peak torque; CPT: Concentric peak torque; EPT: Eccentric peak torque; MTF: Mean tetanic force; MIF: Maximum isometric force; ROM: Range of movement; DOMS: Delayed onset of muscle soreness; PS: Position sense; KJRA: Knee joint reaction angle; SJ: Squat jump; CMJ: Countermovement jump; CK: Creatine kinase; LDH: Lactate dehydrogenase; Mb: Myoglobin; ALP; Alkaline phosphatase; PYD: Pyridinoline; HP: Hydroxyproline; HL: Hydroxylisine; Cr: Creatinine; GOT: Glutamic oxaloacetic transaminase; LA: Lactic acid; IL-6: Interleukin-6; IL-8: Interleukin-8; WBC: White blood cells; CRP: C-reaction protein; Fe: Iron; IgG: Immunoglobulin G; IgM: Immunoglobulin M;

2.6. Redox status responses to eccentric exercise

As stated in the previous sections, oxidative stress occurs when the production of free radicals and RONS overwhelms the antioxidant capacity of the human body, disturbing that way the redox balance. In blood, oxidative stress is estimated through the changes in concentration of several indirect indices, mainly byproducts of the oxidation of important biomolecules, whose increase indicates lipid peroxidation, protein or DNA oxidation. Malondialdehyde (MDA), TBARS, lipid hydroperoxides, conjugated dienes and isoprostanes are widely used as markers of lipid peroxidation, while protein carbonyls (PC) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) are frequently used as markers of damaged proteins and DNA, respectively (Nikolidis 2008). In addition, changes in concentration of antioxidants also indicate oxidative stress; antioxidants may either be up-regulated or diminished, both resulting from the human body's effort to combat the excessive free radicals production through its defense mechanisms. The main enzymatic antioxidant compounds SOD, GPx and CAT, and nonenzymatic compounds vitamin E, vitamin C and GSH as well as total antioxidant capacity (TAC), are mostly being investigated. Total antioxidant capacity represents the ability of a tissue (plasma or serum in most of the times) to neutralize ROS and NO derivatives (Nikolidis 2008).

Indeed, increased generation of RONS, as well as changes in antioxidants that persist for, or appear several days after the termination of eccentric or eccentrically biased exercise, has been reported by a great number of studies (Theodorou 2011, Goldfarb 2011, Lee 2002, Paschalis 2007, Close 2004, Maughan 1989, Sackeck 2003, Hellsten 1997, Goldfarb 2005, Childs 2001). These changes in oxidative stress indices are usually in accordance with the changes in markers of muscle damage. Nevertheless, there are also studies that report no changes in redox status indices after muscle-damaging exercise (Lee 2002, Saxton 1994, Child 1999). The effects of muscle-damaging exercise on blood redox status are summarized in **Table 5**.

Lipid peroxidation

Not all of the studies report similar responses on the above indices after muscle-damaging exercise. In fact, there are studies that present evidence of lipid peroxidation in at least one time point after the end of exercise (Sackeck 2003, Goldfarb 2005, Maughan 1989, Close 2004, Paschalis 2007), whereas others do not support such results (Hellsten 1997, Saxton 1994, Child 1999). These inconsistencies may be due in part to variations in duration, intensity, and muscle damage potential of the exercise examined.

Childs et al. (2001) reported increased levels of isoprostanes 3 and 4 days after eccentric contractions of the elbow flexors muscles in healthy untrained males, while lipid hydroperoxides elevated at 2 days and persisted for up to 4 days post exercise. Similarly, Goldfarb et al. (2005), reported higher MDA concentration 2 days following the same type of eccentric exercise in healthy women. Eccentric maximal voluntary contractions of the knee extensors muscles resulted in elevated concentration of TBARS 2 and 3 days (Paschalis 2007), but also 2 up to 4 days (Nikolaidis 2007) after the end of exercise. Downhill running increased MDA concentration post exercsise (Sackek 2003), but also 3 days (Close 2004) after exercise, while isoprostanes rose above the pre exercise levels 1 day and remained elevated up to 3 days after the end of exercise (Sackeck 2003).

Usually, lipid peroxidation is evidenced many hours after exercise and muscle damage does not seem to affect lipid peroxidation shortly after exercise but after the first 24h. However, in the study of Maughan et al. (1989) a relatively early effect of exercise on lipid peroxidation is indicated as TBARS increased after only 6h post exercise. In the same study, those subjects with the greatest increases in CK also demonstrated higher lipid peroxidation levels. This relationship between muscle-enzyme release and oxidative damage might have resulted from an increase in membrane permeability due to lipid peroxidation and/or increased membrane permeability rendering polyunsaturated fatty acids of membranes more susceptible to peroxidation (Nikolaidis 2008).

Except for the studies reporting changes in redox status parameters that indicate the occurrence of lipid peroxidation after muscle-damaging exercise, there are also studies that do not support such an effect, although CK, DOMS and force production capacity are disturbed for several days after, as a result of the provoked muscle damage. Actually, in the study of Hellsten and colleagues (1997), maximal eccentric exercise of the knee extensors, although increased xanthine oxidase levels in the muscle, did not alter MDA levels. Similarly, in the study of Saxton et al (1994), neither arms', nor legs' eccentric maximum voluntary contractions (MVC) resulted in significant changes of lipid peroxidation indices. More specific, plasma TBARS after eccentric contractions of the arm flexors, as well as muscle MDA after eccentric contractions of the knee extensors' did not change. Childs et al. (1999) also report no changes in MDA after eccentric MVC of the knee extensors.

Protein oxidation

Protein carbonyls are formed through a variety of pathways, which are not always dependent on the direct oxidative modification of the protein (Davies 1999). Additionally, PC assays detect a range of products that are not completely characterized (Davies 1999). However, since carbonyls are usually formed by oxidative mechanisms, it would appear that assessment of this protein modification is likely to provide a reasonable marker of oxidative stress status (Nikolaidis 2008).

Muscle-damaging exercise has been reported to induce significant and high (60-107%) increases in protein carbonyls mainly 1d post exercise, persisting as long as 4d after exercise (Goldfarb 2005, Lee 2002, Paschalis 2007, Nikolaidis 2007). Eccentric MVC of the elbow flexors resulted in marked elevations of PC 1 and 2 d following exercise (Goldfarb 2005, Lee 2002). The time course and magnitude of PC changes after arms' eccentric contractions seems to be similar to those of legs' eccentric contractions. Indeed, in the study of Paschalis et al. (2007), PC significantly increased 1 d, and persisted for up to 3 d, while in the study of Nikolaidis et al. (2007), PC significantly increased 1 d, and persisted for up to 4 d after eccentric MVC of the knee extensors.

DNA oxidation

Exercise has been associated with increases in DNA damage and strand breakage (Okamura 1997). Urinary 8-OHdG excretion, with concomitant plasma TBARS, LDH, CK, CK-MB, and myoglobin elevations, have been reported after daily distance running of 30 km during 8-day training camp (Okamura 1997). In contrast, in the study of Sackeck et al. (2003), no significant changes were demonstrated in 8-OHdG concentration in leucocytes before and 24 hours post-downhill running in humans. The different response of 8-OHdG between the two studies could be attributed to the greater duration and exercise volume in Okamura's study compared to the study of Sackeck, probably resulting in greater muscle damage. Yet, although DNA was adversely affected in Okamura's study, this effect was not accumulated by consecutive exercise, although it was sustained as long as the exercise was repeated. Additionally in the study of Sackeck et al. (2003) measurements were conducted up to 24h after exercise; thus, the absence of changes in 8-OHdG in the first 24h does not exclude the possibility that DNA damage may occurred in a subsequent time point after 24 hours, particularly since this is when the greatest changes in TAC and lipid peroxidation were observed.

DNA damage has been significantly correlated with the increase in plasma CK activity in untrained and trained human subjects (Nikolaidis 2008), but also after intensive exercise in humans (Hartmann 1994). These correlations indicate that exercise-induced DNA damage is associated with exercise-induced muscle damage.

Antioxidants

Both GSH and GSSG concentration, as well as GSH:GSSG ratio have been reported to change after muscle-damaging exercise. Typically, GSH and GSH:GSSG decline, whilst GSSG increases, and these changes are sustained for several days after exercise (Paschalis 2007, Theodorou 2011, Nikolaidis 2007). Only Goldfarb et al. (2005), refer elevation in GSSG immediately after and up to only 2h after, but not at 6, 24 and 48 h post eccentric exercise of the elbow flexors. On the other hand, Lee at al. (2002) refers no significant changes in GSSG and GSSG:TGSH after eccentric exercise,

whereas Close et al. (2005), report non-significant decline of total GSH after downhill running.

The activity of the antioxidant enzymes SOD, CAT, and GPx, but also the concentration of uric acid, ceruloplasmin, and TAC has been investigated after muscle-damaging exercise. Superoxide dismutase increases 3 d (Childs 2001), while CAT 2 d after eccentric exercise, and remains in higher levels compared to pre-exercise, up to 3 d. Ceruloplasmin and Oxygen Radical Absorbance Capacity (ORAC) decreases 3 days after downhill running (Sackeck 2003). Uric acid and TAC increases at several time-points following eccentric exercise, remaining in higher than pre-exercise levels up to 7 d (Childs 2001, Paschalis 2007).

Except for significant effects, there are also studies that do not report any significant modifications of antioxidants as a result of muscle-damaging exercise. In the study of Hellsten et al. (1997), no significant changes have been demonstrated in uric acid and TAC after eccentric MVC of the knee extensors. As uric acid mainly accounts for plasma antioxidant capacity (Halliwell 2007), the similar responses between these two markers in this study, can be justified.

2.6.1. Redox status after eccentric exercise in adults compared to children

There is limited data in children (Nikolaidis 2007) and adolescents (Zalavras 2015) regarding exercise-induced redox alterations. What is more, no study so far has examined redox status alterations as a result of eccentric exercise-induced muscle damage in children. Nevertheless, contrary to our results, Nikolaidis et al. (2007) reported significant increases in TBARS, PC, CAT, TAC, and oxidized glutathione (GSSG) concentration, as well as significant decreases in GSH and GSH:GSSG, after 12 bouts of 50 m swimming, performed at 70% - 75% of 50 m maximum velocity in children. The results from a recent study from our laboratory (Zalavras 2015) indicate a greater rise in PC, TBARS and TAC and greater GSH decline in adults compared to adolescents following an aerobic exercise bout. Uric acid, CAT and bilirubin were similar amongst adults and adolescents immediately after and 1 h

following the aerobic trial. The different exercise modes and experimental designs probably account for the divergent results between the studies.

Table 5. The effect of exercise-induced muscle damage on redox status

Authors	Study protocol	Subjects	Estimated indices	Results
		Adults		
Childs et al. (2001)	Eccentric exercise with the elbow flexors at 80% of 1 RM	14 healthy untrained males (24.4 ±3.6 y)	BDI, MPO, IL-6, TAC, 8-LOOH, Iso-PGF_{2a}, GPx, SOD,CK, LDH, Mb	**PLG: BDI, LOOH, CK, LDH, Mb:**↑at 2 up to 4d **TAC:** ↑at 2 up to 7d **MPO, IL-6:** ↑at 2d **Iso-PGF_{2a}:** ↑at 3 and 4d **SOD:** ↑at 3d
Theodorou et al. (2011)	Maximal eccentric exercise with the knee extensors	28 healthy active males (25.6 ±1.2 y)	IPT, ROM, DOMS, CK, GSH, GSSG, TBARS, PC, CAT, TAC	**IPT, ROM:** ↓ at 2d up to 4d **DOMS:** ↑1 up to 4d **CK:** ↑at 2 up to 4d **GSH, GSH/GSSG:** ↓ at 2 up to 4d **GSSG:** ↑ at 2d up to 4d
Goldfarb et al. (2005)	Eccentric exercise with the elbow flexors	18 women (19-31 y)	PC, MDA, TGSH, GSSG, GSSG/TGSH	**PC:** ↑at 1 and 2d **MDA:** ↑at 2d **GSH:** ↓ post up to 2h **GSSG, GSSG/TGSH:** ↑post up to 2h
Lee et al. (2002)	High intensity eccentric ex of the elbow flexors	8 healthy men (26.5 ± 1.5 y)	MIF, ROM, DOMS, CK, PC, GSH, GSSG, GSSG/TGSH	**MIF:** ↓ post up to 4d **DOMS:** ↑1 up to 4d **ROM:** ↓ post up to 3d **CK:** ↑at 48 up to 96h **PC:** ↑at 1d (83%) and 2d (62%) **GSH, GSSG, GSSG/TGSH:** no changes
Hellsten et al. (1997)	Maximal eccentric exercise with the knee extensors	7 sedentary men (20-28 y)	IL-1β, IL-6, CK, XO, HX, MDA, TAC, Uric acid, MVC, DOMS	**MVC:** ↓90 min up to 4d **XO, CK:** ↑at 1d up to 4d **DOMS:** at 1 and 2d **IL-6L:** ↑at 45min up to 4 **HX:** ↑post ex **IL-1β, MDA, TAC, Uric acid:** no changes

Sackeck et al. (2003)	Supplementation of vit E for 12wks prior to downhill run	16 young (26.4±3.3 y); healthy men	ORAC, Ceruloplasmin, MDA, iPF_{2a}, 8-OHdG, LA, CK	**CK:** ↑at 1d **Ceruloplasmin, ORAC :** ↓at 3d **iPF_{2a}:** ↑ at 1d up to 3d **MDA:** ↑post ex **LA:** ↑post ex, Greater ↑in older group **8-OHdG:** No changes
Maughan et al. (1989)	45 min downhill treadmill run	16 healthy young males (22±1 y)	TBARS, CK, LDH, AST, DOMS,	**CK:** ↑at 6h up to 3d **TBARS, AST:** ↑at 6h up to 2d **LDH:** ↑at 6h up to 1d **DOMS:** ↑at 1 up to 3d
Close et al. (2004)	30 min downhill or flat run	8 physically active male (24.9±3.0 y)	ESR, MDA, CK, WBC, LYM, Neutrophils, DOMS	**DWH run:** **ESR, MDA:** ↑at 3d **CK:** ↑at 1 and 2d **DOMS:** ↑at 2d and 3d **CPT, EPT:** ↓at 1 up to 3d **WBC, LYM, Neutrophils:** ↑post ex **Flat run:** No changes
Paschalis et al. (2007)	Maximal eccentric exercise with the knee extensors	10 untrained healthy females (21 ± 3 y)	IPT, DOMS, CK, TBARS, PC, CAT , BIL, Uric acid, TAC, GSH, GSSG	**IPT:** ↓ post up to 1d **DOMS, CK:** ↑post up to 3d **GSH, GSH/GSSG:** ↓at 24 up to 3d **GSSG, TAC:** ↑at 2d **PC, Uric acid:** ↑at 1 up to 3d **TBARS, CAT, BIL:** ↑at 2 up to 3d
Saxton et al. (1994)	Maximum concentric vs eccentric ex; ARMS and LEGS	14 males (19-39 y)	**ARMS:** CK, TBARS, DCC, ANG, DOMS, IPT **LEGS:** MDA, PCD,	**ARMS ECC ex: CK:** ↑at 2d up to 7d **DOMS, ANG:** ↑at 1 up to 4d **IPT:** ↓at 1 up to 3d **plasma TBARS, DCC:** No changes **ARMS CON ex:** No changes **LEG ECC ex: muscle MDA, PCD:** No changes **LEG CON ex: PCD:** ↑post ex
Child et al.	Maximal eccentric	Physically active males	IPT, DOMS, 20:100Hz	**IPT, 20:100Hz:** ↓post ex

(1999)	exercise with the knee extensors	andfemales (21-31 y)	force ratio, CK, Urate, TAC, MDA, G6PDH, β-Glucuronidase (β-G)	**DOMS:** ↑at 2 up to 6d **Blood: CK:**↑at 3 up to 5d **TAC, β-G, Urate, MDA:** No changes **Muscle: G6PDH, β-G, TAC:** ↑at 7d
Nikolaidis et al. (2007a)	Maximal eccentric exercise with the knee extensors	12 healthy females (23±2 y)	IPT, ROM, DOMS, CK, TBARS, PC, CAT, UA, BIL, TAC, GSH, GSSG	**IPT:** ↓post up to 4d **ROM:** ↓ post up to 3d **DO:MS:** ↑post up to 4d **CK:** ↑at 3 and 4 d **GSH, GSH/GSSG:** ↓at 2 and 3d **GSSG:** ↑ at 2 and 3d **TBARS, PC, CAT, BIL:** ↑at 2 up to 4d **UA:** ↑ at 2 and 3d
Okamura et al. (1997)	8-day training camp	10 well trained long distance runners	8-OHdG, TBARS, LDH, CK, CK-MB, Mb, β-carotene α-tocopherol	**8-OHdG:** ↑ during the camp; no changes after the camp **TBARS, LDH, CK, CK-MB, Mb:** ↑ aft r the camp **α-tocopherol:** ↑ after the camp
Children & adolescents				
Zalavras et al. (2015)	11-month (September through July), two-peaked, training macrocycle	30: adolescents [13 trained (TAD) (14.1±1.1 y), 11 controls (UAD) (14.8±0.9 y)] 30: adults [13 trained (TA) (25.2±6.8 y), 11 controls (UA) (27±6 y)]	TBARS, PC CAT, GSH, TAC UA, BIL	**PC:** ↑post and 1h; Higher resting PC in adults vs adolescents **TBARS:** Similar resting TBARS in adults and adolescents **TAC:** ↑post and 1h; Higher resting PC in adults vs adolescents **GSH:** ↓post ex and 1h; Higher resting GSH in adults vs adolescents; greater decline in adults vs adolescents **CAT, UA, BIL:** ↑post ex and 1h; similar changes in adults and adolescents
Nikolaidis et al. (2007b)	12 x 50m swimming at 70%–75% of 50 m	11 boys and 11 girls (aged 9-11 y)	TBARS, PC CAT, GSH, GSSG, TAC	**GSH, GSH/GSSG:** ↓ post ex **GSSG:** ↑ post ex

maximum velocity	**TBARS, PC, CAT, TAC:** ↑ post ex

PLG: placebo group; SupplG: Supplementation group; IPT: Isometric peak torque; ROM: Range of movement; DOMS: Delayed onset of muscle soreness; MIF: Maximum isometric force; MVC: Maximal voluntary contractions; BDI: Bleomycin detectable iron; MPO: Myeloperoxidase; IL-6: Interleukin-6; IL-1β: Interleukin-1β; TAS: Total antioxidant capacity; LOOH: lipid hydroperoxides; Iso-PGF2a: Isoprostanes; GPx: Glutathione peroxidase; SOD: Superoxide dismutase; CK: Creatine kinase; LDH: Lactate dehydrogenase; Mb: Myoglobin; 8-OHdG: 8-hydroxy-deoxyguanosine; GSH: Reduced glutathione; GSSG: Oxidized glutathione; TGSH: Total glutathione; TBARS: Theobarbituric acid reactive substances; PC: Protein carbonyls; CAT: Catalase; MDA: malonaldehyde; ORAC: Oxygen Radical Absorbance Capacity; 8-OHdG: 8-hydroxy-2'deoxyguanosine; DCC: Diene conjugated compounds; PCD: Protein carbonyl derivatives; XO: Xanthine oxidase; HX: Hypoxanthine; LA: Lactic acid; AST: Aspartate transaminase; ESR: electron spin resonance; WBC: White blood cells; LYM: Lymphocytes; DCC: Diene conjucated compounds; PCD: Protein carbonyls derivatives; G6PDH: Glucose-6-phosphate dehydrogenase;

3. METHODOLOGY

3.1. Participants

Power analysis determined that a sample size of 10 subjects was required to detect a statistically meaningful treatment effect between consecutive measurements after acute eccentric dynamometry with a level of 0.90. Eighteen healthy recreationally active adults (men, 18-45yo) and eleven children (boys, 10-12yo) participated in the present study. Both adults and children were participating in several health-promoting physical activities without being systematically-trained. At the first cycle, one adult dropped the study after the first post exercise evaluation at 24 h, due to inability of concluding all of the other measurements. Another two adults did not repeat the second cycle; hence, fifteen adults and eleven boys finished the study. The anthropometric characteristics of the participants are presented in Table 1. Participation criteria included a normal BMI, the absence of any injury or a surgery in their legs, no drug/supplement consumption, and absence of any allergy in iron salts. Additionally, they did not undertake any scheduled eccentric exercise or other activities with large eccentric component for at least 6 months before the study, and they did not spend more than 3h weekly on sport activities. Written informed consent to participate in the study was provided by all men and boys after the volunteers were informed about all risks, discomforts, and benefits involved in the study. Moreover, boys' parents approved the participation of their children in the study. The procedures were in accordance with the 1975 Declaration of Helsinki, as revised in 2000, and approval was received from the institutional review board.

3.2. Supplementation

In a double blind, randomized crossover study that was conducted in two cycles, participants received daily either the iron supplement [37mg of elemental iron, Resoferon c.tab 125(37) mg/tab, Novartis Hellas] or the placebo (lactose) for three weeks prior to, and up to 96 hours after an acute eccentric exercise bout. The participants were advised to consume the supplement in the morning on an empty

stomach or at least 2 hours after breakfast and 2 hours before lunch, for better absorption. Each individual received the supplement pre-packed in daily doses labeled with the day of consumption, and was asked to return the package with any capsules left, so that the compliance could be calculated.

3.3. Experimental design of the study

The experimental approach of the study is presented in Figure 2. During their first visit, participants' body mass was measured to the nearest 0.5 kg (Beam Balance 710; Seca, Birmingham, United Kingdom) while the subjects were lightly dressed and barefoot. Standing height was measured to the nearest 0.5 cm (Stadiometer 208; Seca). Percentage body fat was calculated from 7 skinfold-thickness measures (average of 2 measurements of each site) by using a Harpenden caliper (John Bull, St Albans, United Kingdom). The Siri skinfold-thickness equation was used to calculate body fat. Biological age of children was assessed through self-estimation of the participants of Tanner's sexual maturation stages (Morris 1980). Maximum isometric, concentric and eccentric torque of the knee extensor muscles were measured on an isokinetic dynamometer (Cybex, Ronkonkoma, NY). Subjects performed the exercise during the first cycle with one leg, which was followed by a four-week washout period, before performing the exercise during the second cycle with the contralateral leg. Physiological parameters were estimated in both age groups at baseline, after 3 weeks of supplementation (pre-exercise), immediately after exercise, 24, 48, 72, and 96 hours after exercise. Blood samples in adults were collected at baseline, pre-exercise, 24, 48, 72, and 96 hours post-exercise, while in children at baseline, pre-exercise, and 72 hours following exercise.

3.4. Eccentric exercise

Subjects were placed on the dynamometer in a seated position. The axis of rotation of the dynamometer was aligned with the knee joint axis of rotation with the line that passes through the lateral femoral condyle, with the knee flexed at 90o. The

resistance pad was placed to the ankle joint proximally to the lateral malleolus (Perrin 1993). The range of motion was determined with the initial point at 0o of full extension and 120o for flexion to prevent hyperextension and hyperflexion. Gravity correction was made to account for the effect of limb weight on torque measurements and valid data to be obtained (Baltzopoulos 1989). The position of each subject was recorded and used in follow-up measurements. At the first cycle, each participant performed 5 sets of 15 maximal eccentric voluntary contractions of knee extensors of their one leg at an angular velocity of 60o.sec-1 in the seated position and the same procedures were followed during the second cycle using this time the contralateral leg. A two min rest interval was incorporated between sets. Before the exercise bout, a warm-up consisting of 8 min of cycling (80-100 rpm and 50 W) was performed on a Monark cycle ergometer (Monark, Vansbro, Sweden) that was followed by 5 min of stretching exercises of the major muscle groups of the lower limbs. This protocol of eccentric exercise has been used in several studies by our laboratory and is capable of inducing severe muscle damage and oxidative stress (Theodorou 2011, Paschalis 2007, Nikolaidis 2007).

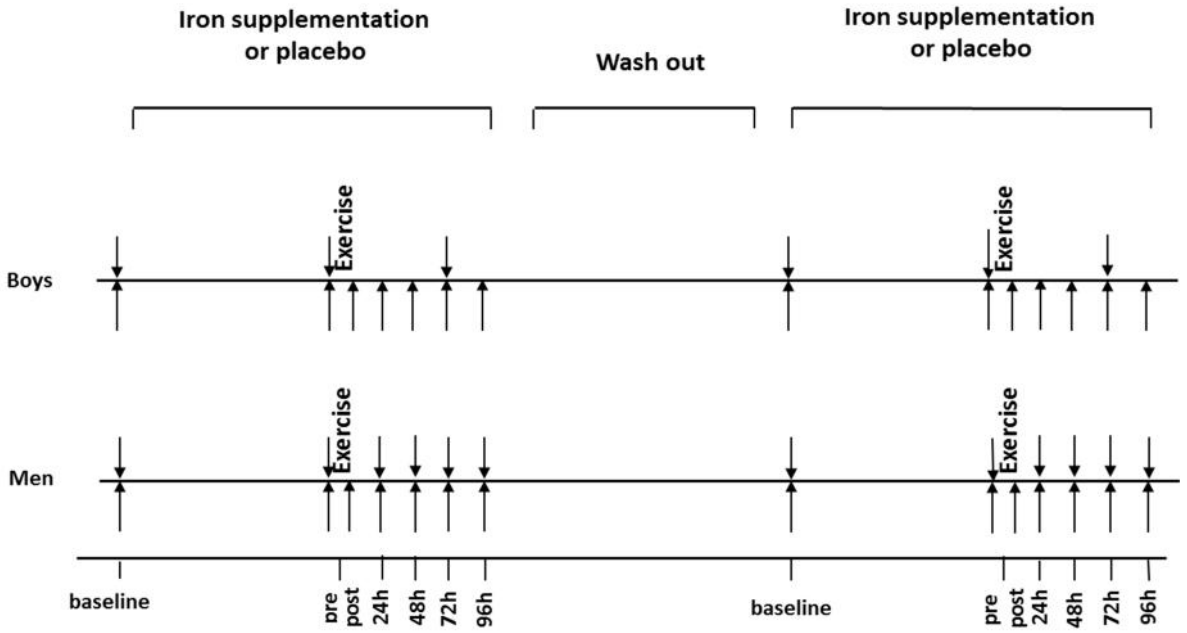

Figure 2. Experimental design of the study. Upward arrows indicate physiological measurements, and downward arrows indicate blood drawings.

3.5. Muscle function and muscle performance indices

The isokinetic dynamometer was also used for the measurement of isometric knee extensor peak torque at 90o knee flexion, as well as concentric and eccentric peak torque at an angular velocity of 60o.sec-1. The best of 3 maximal, and the best of 5 maximal voluntary contractions were recorded for isometric and concentric or eccentric peak torque, respectively. To ensure that the subjects provided their maximal effort, the measurements were repeated if the difference between the lower and the higher torque values exceeded 10%. A two min rest interval was incorporated between the efforts. The assessment of pain-free ROM was performed manually. The investigator moved the calf at a very low angular velocity from 0o knee extension to the position where the subject felt any discomfort. Each participant assessed delayed onset of muscle soreness (DOMS) during walking and squat movement (90o knee flexion), and perceived soreness was rated on a scale ranging from 1 (normal) to 10 (very sore). All volunteers were instructed to stay away from strenuous physical activity for 2 days preceding and 2 days following the experiment.

3.6. Blood collection and handling

Blood samples (20 mL) were drawn from a forearm vein with subjects in a seated position. All blood samples were drawn in the morning after an overnight fast and abstinence from caffeine and alcohol for 3 d before sampling. A blood aliquot (1.5 mL) was collected into ethylenediamine tetraacetic acid (EDTA) tubes for the estimation of hematological parameters. For plasma, blood was collected into EDTA-containing tubes and centrifuged immediately at 1370 x g for 10 min at 4o C, and the plasma was collected. The packed erythrocytes were lysed with 1:1 (v/v) distilled water, inverted vigorously, and centrifuged at 4000 x g for 15 min at 4o C, for the collection of red blood cells lysate. For serum, blood was collected into tubes containing coagulation agent and after staying for 20 min to clot, it was centrifuged at 1370 x g for 10 min at 4o C, and serum was collected. Plasma, erythrocytes lysate

and serum, were stored in multiple aliquots at –80o C and thawed only once before the analyses.

3.7. Assays

3.7.1. Iron status indices

Iron concentration and Total Iron Binding Capacity: Iron concentration and Total Iron Binding Capacity (TIBC) were measured in a Clinical Chemistry Analyzer Z1145 (Zafiropoulos Diagnostica, Athens, Greece) with commercially available kits (Zafiropoulos, Athens, Greece). Each sample was analyzed in duplicates.

Transferrin saturation: Transferrin saturation (TS) was calculated through the ratio of iron concentration and TIBC (TS = iron concentration/TIBC x 100).

Ferritin: For the determination of serum ferritin, an immunoenzymometric assay kit based on sandwich ELISA was used (Accubind, Monobind Inc., USA®). According to this method, 25μL of the appropriate serum reference and specimen was added into the assigned streptavidin coated well. Then 100 μL of biotinylated monoclonal anti-ferritin antibody were pipetted into each well. After swirling the mixture gently for 30 sec and incubating for 30 min at room temperature, the contents of the microplate were discarded and three washes of the microplate with 350 μL of wash buffer were performed. Then 100 μL of ferritin enzyme conjugate were added to each well and after the incubation of the complex at room temperature for 30 min, the contents of the microplate were discarded and another three washes of the microplate with 300 μL of wash buffer were followed. 100 μL of substrate solution were then added into each well and after 15 min of incubation at room temperature, 50 μL of stop solution were added and a gentle mixing of the complex for 20 sec was performed. The absorbance in each well was read at 450 nm in a microplate reader (Biochrom Asys Expert 96, UK) within 30 min of adding the stop solution. For the calculation of the concentration of ferritin, a dose response curve was used and the absorbance for each serum reference was plotted versus the corresponding ferritin concentration in ng/ml on linear graph paper and the best-fit curve through the

plotted points was drawn. The concentration of ferritin of each sample was determined by locating the absorbance for each sample on the vertical axis of the graph and after finding the intersecting point on the curve, the concentration was read from the horizontal axis (in ng/ml). Assays were performed in duplicate. Inter- and intra-assay coefficients of variation for all blood parameters ranged from 2.1 to 6.5% and from 2.9 to 7.1%, respectively.

3.7.2. Redox status indices

Thiobarbituric acid reactive substances (TBARS): For TBARS determination, a slightly modified assay of Keles et al. (2001) was used. According to this method, 100 µL of plasma was mixed with 500 µL of 35% TCA and 500µlL of trishydroxymethylaminomethane hydrochloride (Tris–HCl) (200 mM, pH 7.4) and incubated for 10 min at room temperature. One milliliter of 2 M Na2SO4 and 55 mM thiobarbituric acid solution was added and the samples were incubated at 95 C for 45 min. The samples were cooled on ice for 5 min and were vortexed after adding 1 mL of 70% TCA. The samples were centrifuged at 15,000g for 3 min and the absorbance of the supernatant was read at 530 nm. A baseline absorbance was taken into account by running a blank along with all samples during the measurement. Calculation of TBARS concentration was based on the molar extinction coefficient of malondialdehyde. The intra- and inter-assay CV for TBARS were 3.9% and 5.9%, respectively.

Protein carbonyls (PC): Protein carbonyls were determined based on the method of Patsoukis et al. (2004). In this assay, 50 lL of 20% TCA was added to 50 lL of plasma and this mixture was incubated in an ice bath for 15 min and centrifuged at 15,000g for 5 min at 4o C. The supernatant was discarded and 500 lL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) [in 2.5 N hydrochloride (HCl)] for the sample, or 500 lL of 2.5 N HCl for the blank, was added in the pellet. The samples were incubated in the dark at room temperature for 1 h, with intermittent vortexing every 15 min and were centrifuged at 15,000g for 5 min at 4o C. The supernatant was discarded and 1 mL of 10% TCA was added, vortexed and centrifuged at 15,000g for 5 min at 4o C.

The supernatant was discarded and 1 mL of ethanol–ethyl acetate (1:1 v/v) was added, vortexed and centrifuged at 15,000g for 5 min at 4o C. This washing step was repeated twice. The supernatant was discarded and 1 mL of 5 M urea (pH 2.3) was added, vortexed and incubated at 37o C for 15 min. The samples were centrifuged at 15,000g for 3 min at 4^{o} C and the absorbance was read at 375 nm. Calculation of protein carbonyl concentration was based on the molar extinction coefficient of DNPH. The intra- and inter-assay CV for protein carbonyls were 4.3% and 7.0%, respectively.

Reduced glutathione (GSH): GSH was measured according to Reddy et al. (Reddy 2004). The intra- and inter-assay CV for GSH were 3.1% and 4.5%, respectively.

Catalase: Catalase activity was determined using the method of Aebi et al. (1984). The intra- and inter-assay CV for catalase were 6.2% and 10.0%, respectively.

Total antioxidant capacity (TAC): The determination of TAC was based on the method of Janaszewska and Bartosz (2002). Briefly, 20 lL of plasma were added to 480 lL of 10 mM sodium potassium phosphate (pH 7.4) and 500 lL of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and the samples were incubated in the dark for 30 min at room temperature. The samples were centrifuged for 3 min at 20,000g and the absorbance was read at 520 nm. TAC is presented as mmol of DPPHreduced to 2,2-diphenyl-1-picrylhydrazine (DPPH:H) by the antioxidants of plasma. The intra- and inter-assay CV for TAC were 2.9% and 5.4%, respectively.

Uric acid and Bilirubin: Uric acid and Bilirubin were estimated in a Clinical Chemistry Analyzer Z 1145 (Zafiropoulos Diagnostica, Athens, Greece) with commercially available kits (Zafiropoulos, Athens, Greece). Each sample was analyzed in duplicates. The intra- and inter-assay CV for Uric acid and Bilirubin were 4.5% and 3.4%, respectively.

3.8. Dietary analysis

Participants' food intake was analyzed prior to the commencement of the study to verify that obtained adequate amounts of iron (above recommended RDA). Participants were asked to maintain their usual eating habits and completed daily dietary recall forms that were analyzed with ScienceFit Diet 200A (Science Technologies, Athens, Greece) during the experimental period. Participants were given detailed guidelines on how to monitor and record food and drink intake.

4. Statistical analyses

The normality of the sample distribution was examined with a Kolmogorov-Smirnov test. To estimate possible differences as a result of 3 weeks of iron supplementation in iron status, and redox status in adults compared to children, a three way ANOVA [time (baseline-3wks) x condition (placebo-iron supplement) x age (adults-children)] with repeated measures on time was used.

Because of fewer blood drawings performed in children following the acute bout of exercise, the statistical analyses of blood related parameters (iron status, redox status and CK were done separately in adults and children. Therefore, in adults, possible differences as a result of eccentric exercise and iron supplementation were assessed through a two way ANOVA [time (pre exercise-24h-48h-72h-96h after exercise) x condition (placebo-iron supplement)] with repeated measures on time. In children, a two way ANOVA [time (pre exercise-72h after exercise) x condition (placebo-iron supplement)] with repeated measures on time was performed.

To estimate possible differences in physiological indices of muscle damage and muscle performance in adults compared to children, as a result of eccentric exercise and iron supplementation, a three way ANOVA [time (pre exercise-post exercise-24h-48h-72h-96h after exercise) x condition (placebo-iron supplement) x age (adults-children)] with repeated measures on time was applied.

When significant main effects or interactions occurred, Sidak pairwise comparisons were performed. The level of statistical significance was set at $p < 0.05$. For all statistical analyses SPSS, version PASW 18.0 (SPSS Inc., Chicago, Ill.) was used.

5. RESULTS

5.1. Anthropometric characteristics and dietary intake

Significant differences between adults (AD) and children (CHI) came up for all of the anthropometric and physiological characteristics. Regarding height, weight, BMI, and body fat AD presented higher values compared to CHI at **(Table 6)**. Concerning isokinetic strength, AD presented higher absolute values at isometric, concentric and eccentric strength; in contrast, when body weight was taken into consideration, relative isokinetic strength was higher in children compared to adults (Table 6).

No significant differences were found in daily energy and macronutrient intakes between adults and children or between the iron-supplemented (FE) and placebo (PL) groups before the supplementation except for energy intake in the iron-supplemented compared to placebo group **(Table 7)**.

Table 6. Anthropometric and physiological characteristics of adults and children at baseline[1]

Variable	Adults (n = 15)	Children (n = 11)
Age (y)	35.7 ± 2.08	11 ± 0.23
Height (cm)	180 ± 0.02***	154 ± 0.01
Weight (kg)	83.5 ± 3.31***	42.4 ± 2.1
BMI (kg/m^2)	25.7 ± 0.87***	17.8 ± 0.65
Body fat (%)	19.5 ± 1.7 ***	7.6 ± 1.22
Tanner Stage		2.36 ± 0.15
Isometric torque (N·m)	216 ± 8.3***	124 ± 4.8
Concentric torque (N·m)	205 ± 6.2***	93 ± 5.0
Eccentric torque (N·m)	264 ± 10.2***	137 ± 6.0

[1]All values are means ± SEMs. Significant differences between adults and children existed for age, height, weight, body fat, isometric, concentric, and eccentric torque (unpaired student's t-test). N·m, Newton meter. *** p=0.000.

Table 7. Analysis of daily energy intake in adults and children and in iron-supplemented and placebo groups at baseline[1]

Variable	Adults (n = 15)	Children (n = 11)	Iron group (n = 26)	Placebo group (n = 26)
Energy (kcal)	1940 ± 133.3	1857 ± 120.3	2105 ± 140.9 *	1698 ± 99.2
Carbohydrate (% of energy)	51.4 ± 2.3	56.3 ± 1.6	55.3 ± 1.8	52.5 ± 2.1
Fat (% of energy)	25.7 ± 1.7	23.3 ± 1.2	23.9 ± 1.4	25.1 ± 1.4
Protein (% of energy)	22.9± 1.5	20.4 ± 1.0	20.8 ± 0.9	22.4 ± 1.6
Iron (mg)	12.1 ± 1.1	10.7 ± 0.7	12.4 ± 1.0	10.4 ± 0.8
Vitamin C (mg)	133.6 ± 29.0	87.1 ± 13.4	102.2 ± 24.8	117.4 ± 20.6

[1]All values are means 6 SEMs. There were no significant differences between adults and children, neither between iron-supplemented and placebo groups, except for energy intake between iron-supplemented and placebo groups (unpaired Student's t test). *Significantly different from the placebo group, $p<.05$.

5.2. The effect of 3 weeks of iron supplementation on iron status, redox status and muscle damage and performance at rest

5.2.1. Iron status

Main effect of age came up for FE concentration, total iron binding capacity (TIBC), transferrin saturation (TS) and ferritin (FERR) (**Table 8**). Adults had lower TIBC and higher FE, TS and FERR compared to CHI.

Time by condition interaction came up for FE concentration and TS. Iron supplementation for 3 weeks increased FE concentration and TS **(Figure 3)**.

Time x condition x age interaction came up for TIBC with AD of the PL group showing lower TIBC after 3 weeks of iron supplementation. TIBC was not altered in CHI or in FE groups (Figure 3).

Table 8. Iron status in adults and children after 3 weeks of supplementation[1]

Variable	Adults		Children	
	Baseline	3 weeks	Baseline	3 weeks
FE (mg/dL)	95.2 ± 5.4 ^{A} *	95.2 ± 6.3 ^{A} *	68.5 ± 6.3	87.7 ± 7.3
TIBC (μmol/L)	379 ± 15.0 ^{A} *	372 ± 13.8 ^{A} *	427 ± 17.5	417 ± 16.1
TS (%)	26.5 ± 1.8 ^{A} *	30.0 ± 3.1 ^{A} *	17.7 ± 2.2	21.8 ± 3.8
FERR (g/mL)	93.1 ± 10.9 ^{A} ***	91.6 ± 11.0 ^{A} ***	22.2 ± 13.1	22.2 ± 13.2

[1]All values are means (± SEM). Three way ANOVA (supplement x age x time) with repeated measures on time and post hoc Sidak pairwise comparisons were applied. A, main effect of age. ***p<.001; *p<.05. FE, iron concentration; FERR, ferritin; TIBC, total iron binding capacity; TS, transferrin saturation.

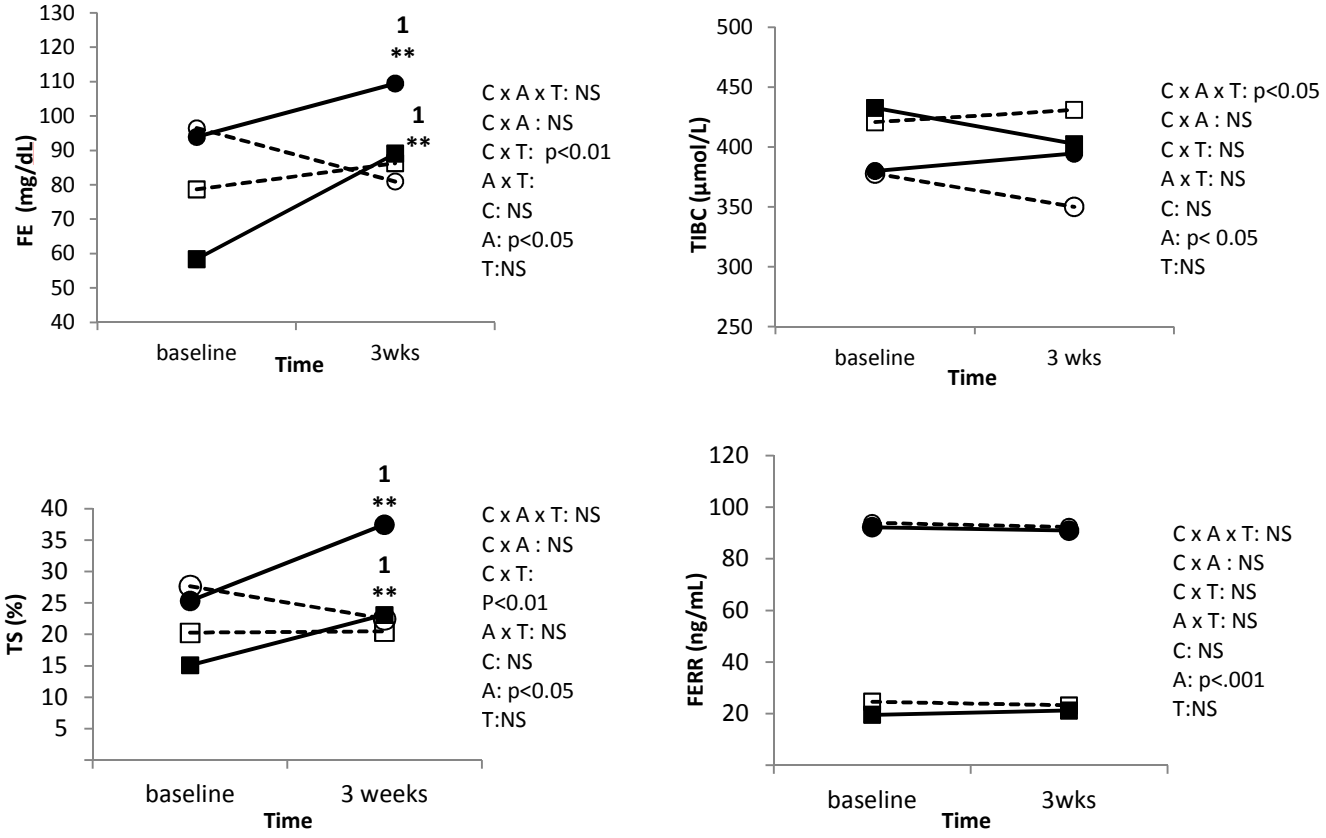

Figure 3. Iron status after 3 weeks of supplementation. Mean (± SEM) FE concentration (A), TIBC (B), TS (C) and ferritin (D) after 3 weeks of supplementation in adults of the iron-supplemented (●) and placebo (○) groups, and in children of the iron-supplemented (■) and placebo groups (□). Three way ANOVAs with repeated measures on time and post hoc Sidak pairwise comparisons were applied. C, main effect of condition; A, main effect of age; T, main effect of time. Interactions are shown. [1]Significantly different from baseline in the same group.

5.2.2. Blood redox status

Main effect of age came up for reduced glutathione (GSH), catalase (CAT), uric acid (UA) and bilirubin (BIL). Children had higher GSH and CAT, while lower UA and BILL compared to adults **(Table 9).**

Main effect of time came up for thiobarbituric acid reactive substances (TBARS). TBARS increased after 3 weeks of supplementation and the increase was greater in iron compared to the placebo group (**Figure 4**).

Table 9. Redox status indices in adults and children after 3 weeks of supplementation[1]

	Adults		Children	
Variable	**Baseline**	**3 weeks**	**Baseline**	**3 weeks**
GSH (μmol/g HGB)	5.3 ± 0.3 ^{A} *	5.3 ± 0.3 ^{A} *	6.5 ± 0.4	6.7 ± 0.4
CAT (U/mg HGB)	202.7 ± 8.0 ^{A} **	206.6 ± 8.3 ^{A} **	236.9 ± 8.9	246.1 ± 9.7
TAC (μmol DPPH)	0.91 ± 0.02	0.93 ± 0.02	0.87 ± 0.02	0.87 ± 0.02
UA (mg/dL)	5.6 ± 0.2 ^{A} ***	5.6 ± 0.2 ^{A} ***	3.8 ± 0.3	3.9 ± 0.3
BIL (μM)	0.8 ± 0.05 ^{A} **	0.8 ± 0.05 ^{A} **	0.6 ± 0.06	0.6 ± 0.05
TBARS (μmol/L)	6.3 ± 0.4 ^{T} **	6.5 ± 0.4 ^{T} **	6.4 ± 0.5	6.9 ± 0.4
PC (nmol/mL)	19.3 ± 0.8	18.5 ± 0.9	19.1 ± 1.0	18.2 ± 1.1

[1]All values are means (± SEM). Three way ANOVA (supplement x age x time) with repeated measurements on time and post hoc Sidak pairwise comparisons were applied. A, main effect of age. T, main effect of time. ***p<.001; **p<0.01; *p<.05. BIL, bilirubin; CAT, catalase; GSH, reduced glutathione; PC, protein carbonyls; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances; UA, uric acid.

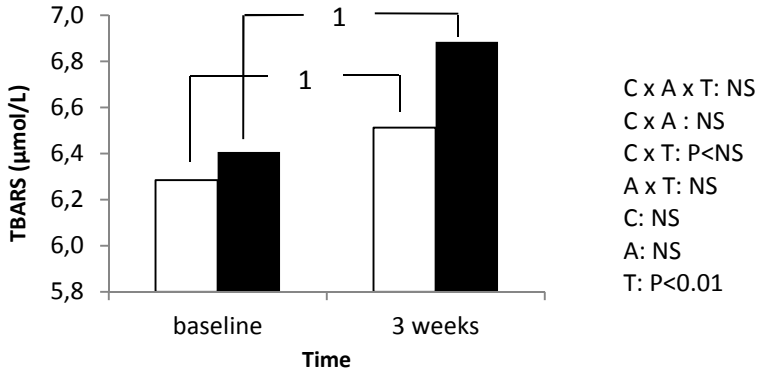

Figure 4. TBARS after 3 weeks of supplementation. Mean (± SEM) thiobarbituric acid reactive substances (TBARS) after 3 weeks of supplementation in iron (■) and placebo (□) groups. Three way ANOVAs with repeated measures on time were applied. C, main effect of condition; A, main effect of age; T, main effect of time. [1]Significantly different from baseline in the same group.

5.2.3. Muscle damage and performance

Main effect of time came up for eccentric torque. Eccentric torque increased after 3 weeks, whereas no other muscle performance or muscle damage index was changed. Main effect of age came up for isometric, concentric and eccentric torque, with AD presenting higher values than CHI in both baseline and after 3 weeks. Nevertheless, when body weight was taken into consideration, the main effect of age still existed for concentric and eccentric peak torque, but CHI demonstrated greater relative values compared to AD. No differences between the two age groups came up for relative isometric peak torque (Table 10).

Iron supplementation had no effect on muscle damage and performance.

Table 10. Absolute and relative to body weight isokinetic force at baseline and after 3 weeks in adults and children[1].

	Adults		Children	
	Baseline	**3 weeks**	**Baseline**	**3 weeks**
Isometric torque (N·m)	216± 6.9^{A***}	218 ± 7.5^{A***}	123 ± 8.1	126 ± 8.8
Relative to BW isometric torque (N·m/kg)	276 ± 10.3	278 ± 10.2	296 ± 12.0	300 ± 11.9
Concentric torque (N·m)	205 ± 5.6^{A***}	207 ± 6.3^{A***}	93 ± 6.5	96 ± 7.3
Relative to BW concentric torque (N·m/kg)	261 ± 6.5^{A**}	263 ± 6.7^{A**}	219 ± 7.6	226 ± 7.8
Eccentric torque (N·m/kg)	246 ± 8.6^{T,A**}	276 ± 8.6^{A**}	137 ± 10.0^{T**}	147 ± 10.0
Relative to BW eccentric torque (N·m/kg)	336 ± 9.1^{T**}	351 ± 9.9	324 ± 10.6^{T**}	347 ± 11.6

[1]All values are means (± SEM). Three way ANOVA (supplement x age x time) with repeated measurements on time and post hoc Sidak pairwise comparisons were applied. A, main effect of age. T, main effect of time. ***p<.001; **p<0.01.

5.3. The effect of eccentric exercise and iron supplementation on iron status, redox status, and muscle damage and performance

5.3.1. Iron status

Adults: In adults, time x condition interaction came up for FE concentration. In iron group, FE concentration decreased at 24h and up to 72h compared to pre ex levels, while no changes occurred for PL group (**Figure 5**). Time main effect came up for TIBC which increased at 48h after eccentric exercise with a similar way in both PL and FE group and remained higher up to 96h compared to pre exercise levels (Figure 4). No significant differences came up for TS and FERR after eccentric exercise as a result of iron supplementation. Although both parameters exhibited fluctuations in several time points after eccentric exercise, these fluctuations were not significant (Figure 5).

Children: In children, no significant changes occurred for any of the estimated iron status parameters as a result of eccentric exercise or iron supplementation (Figure 5).

5.3.2. Blood redox status

Adults: In adults, time main effect came up for total antioxidant capacity (TAC) **(Figure 6)** and protein carbonyls (PC) **(Figure 7)**. Both TAC and PC increased 24h after eccentric exercise compared to pre exercise levels, and returned to pre exercise levels thereafter.

Children: In children, no significant main effects or interactions came up for any of the measured antioxidant substances (Figure 6) or indices of oxidation (Figure 7). Multiple comparisons showed a trend for lower GSH at 72h after eccentric exercise in both the iron-supplemented and placebo groups (Figure 6).

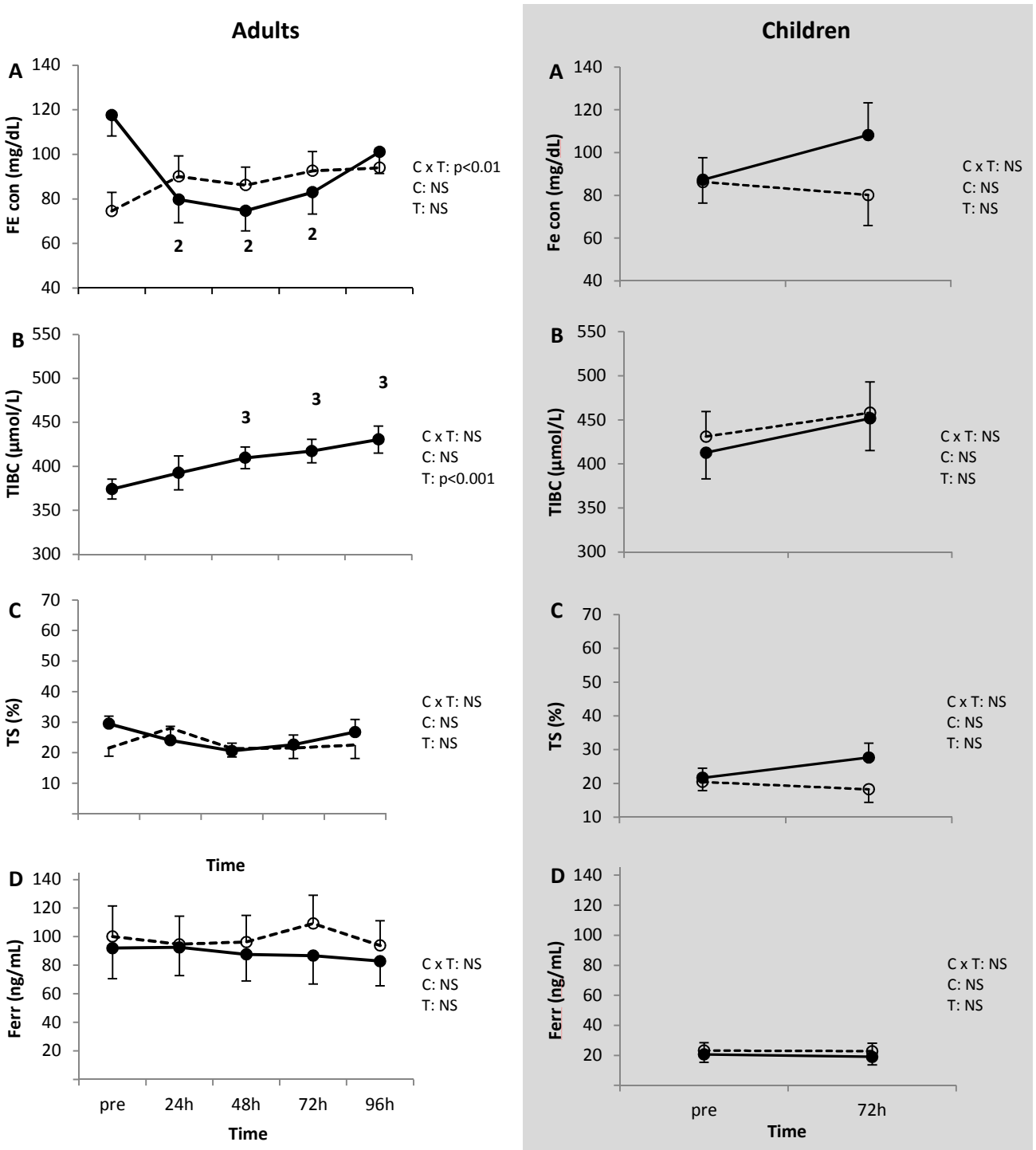

Figure 5. Iron status after eccentric exercise in adults and children. Mean (± SEM) for iron (FE) (A), total iron binding capacity (TIBC) (B), transferrin saturation (ST) (C) and ferritin (FERR) (D) after muscle-damaging eccentric exercise in adults and in children of the iron-supplemented (●) and placebo (○) group. Separate two way ANOVAs (condition x time) with repeated measures on time and post hoc Sidak pairwise comparisons were applied. C, main effect of condition; T, main effect of time. Interactions are shown. [2]Significantly different from pre exercise in the same group. [3]Significantly different from pre exercise independently of condition.

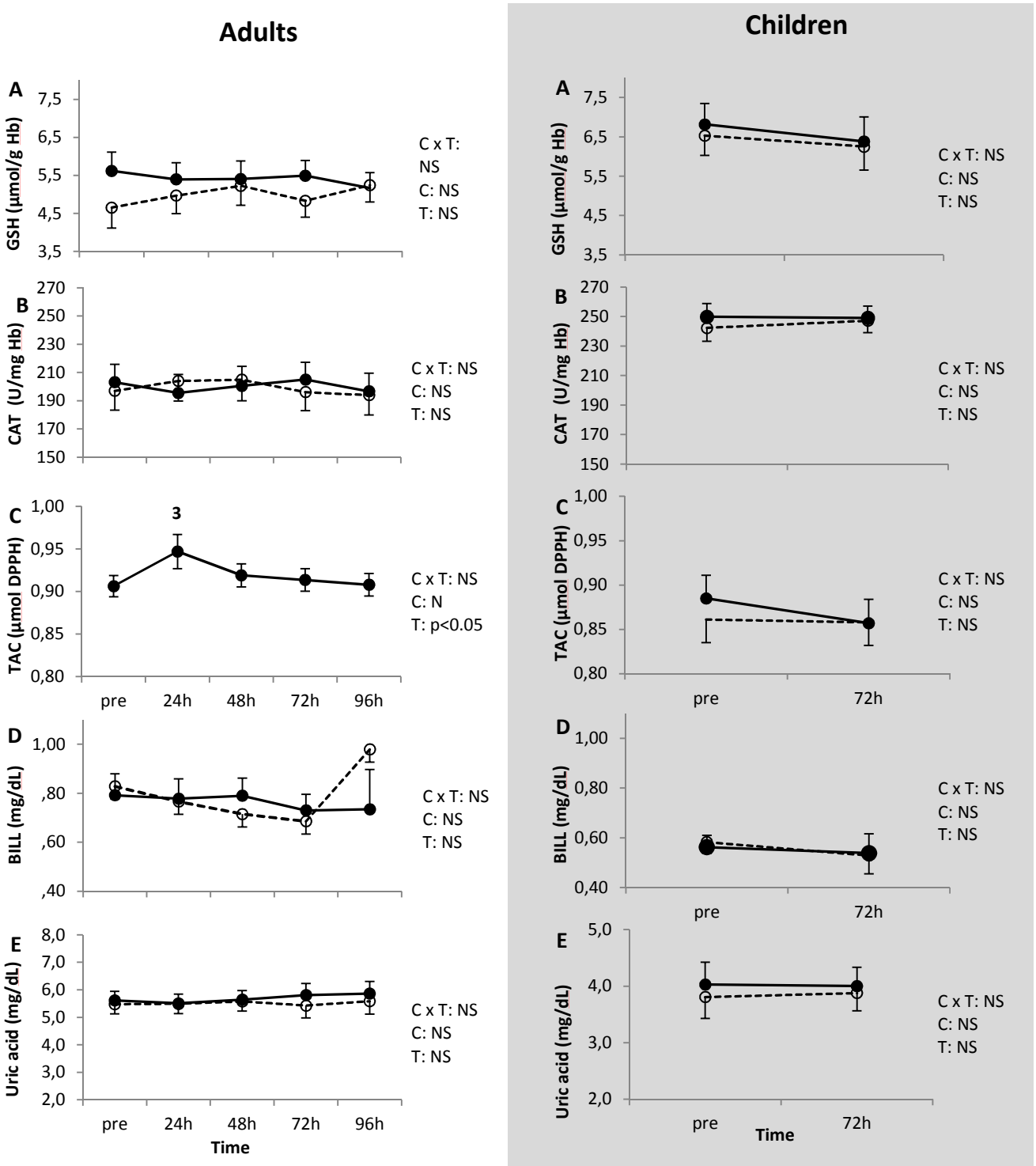

Figure 6. Antioxidants after eccentric exercise in adults and children. Mean (± SEM) reduced glutathione (GSH) (A), catalase (CAT) (B), total antioxidant capacity (TAC) (C), reduced glutathione (GSH) (C), bilirubin (BIL) (D) and uric acid (UA) (E) after muscle-damaging eccentric exercise in adults and children of the iron-supplemented (●) and placebo (○) group. Two way ANOVAs (condition x time) with repeated measures on time and post hoc Sidak pairwise comparisons were applied. C, main effect of condition; T, main effect of time. Interactions are shown. [3]Significantly different from pre exercise independently of condition.

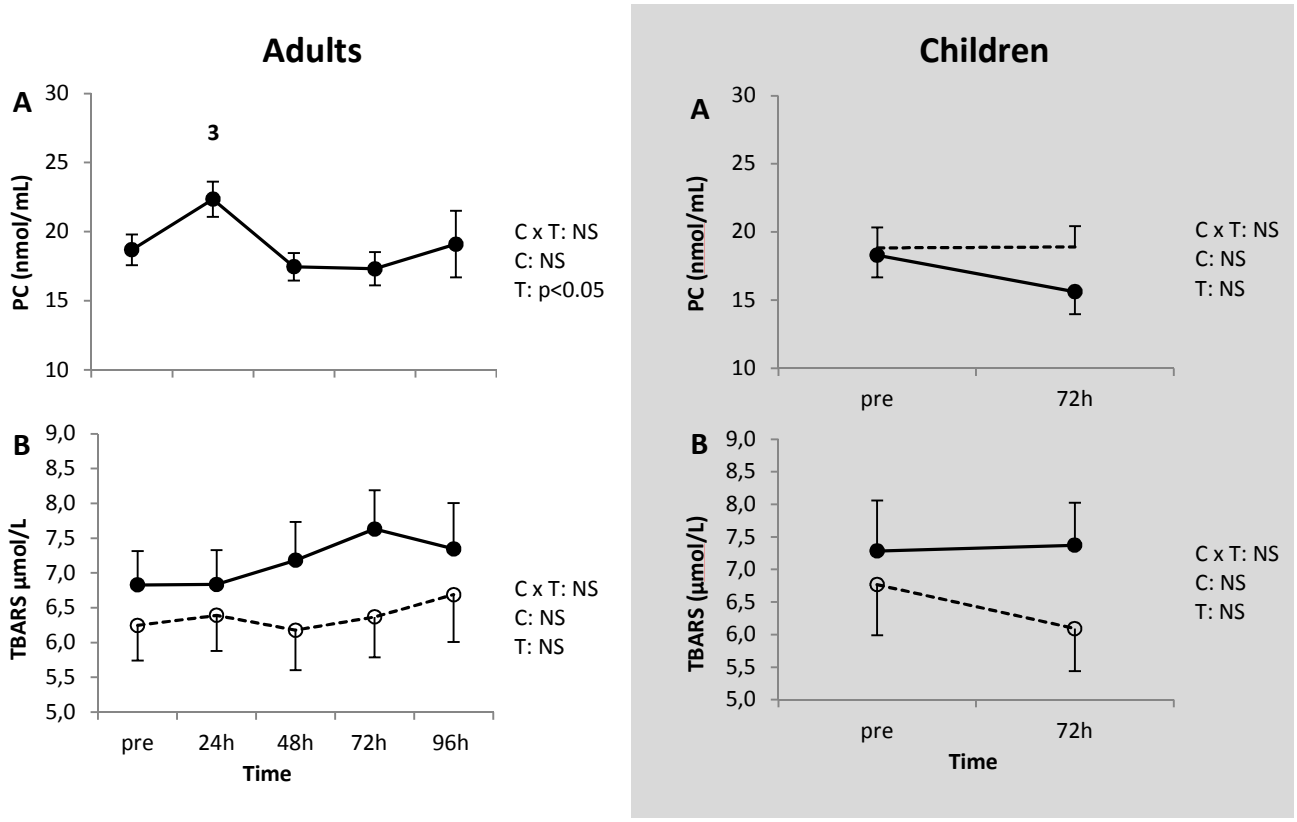

Figure 7. Lipid peroxidation and protein oxidation after eccentric exercise in adults and children. Mean (± SEM) protein carbonyls (PC) (A) and thiobarbituric reactive substances (TBARS) (B) after muscle-damaging eccentric exercise in adults and children of the iron-supplemented (●) and placebo (○) group. Two way ANOVAs (condition x time) with repeated measures on time and post Sidak pairwise comparisons were applied separately in adults and children. C, main effect of condition; T, main effect of time. Interactions are shown. [3]Significantly different from pre exercise independently of condition.

5.3.3 Muscle damage and performance

Main effect of age came up for DOMS at walking and DOMS at squat, isometric, concentric and eccentric force (**Figure 8**), as well as relative to body weight isometric concentric and eccentric force (**Figure 9**). Adults also demonstrated greater DOMS in both walking and squat compared to children. Concerning isokinetic strength, AD presented higher absolute values in isometric, concentric and eccentric torque compared to children, in the entire study regardless of condition (Figure 8). When body weight was taken into consideration, relative isometric and eccentric peak torque was greater in CHI compared to AD, while concentric peak torque was similar between the two age groups (Figure 9).

Main effect of time and came up for DOMS at walking and squat, ROM, isometric, concentric and eccentric torque (Figure 8), and also relative to body weight isometric, concentric and eccentric torque (Figure 9).

Time by age interaction came up for DOMS at walking and squat, ROM, isometric, concentric and eccentric peak torque (Figure 8), and also relative to body weight concentric and eccentric peak torque (Figure 9). ROM in adults declined post and up to 72h after the eccentric exercise, whereas in children the deterioration in ROM remained only up to 24h compared to pre exercise levels (Figure 8). DOMS at walking was greater post and up to 96h after the eccentric exercise in adults, whereas in children DOMS at walking was greater post and up to 24h. DOMS at squat was greater post and up to 96h after the eccentric exercise in adults, whereas in children DOMS at squat increased and remained greater than pre exercise levels up to 48h. Additionally, adults had significantly greater DOMS at squat than children at 48h, 72h and 96h after the eccentric exercise (Figure 8). Isometric force decreased in adults post and up to 72h after the eccentric exercise, whereas in children isometric force declined only post exercise compared to pre exercise levels. Concentric force decreased in adults post and up to 72h after the eccentric exercise, whereas in children no significant changes occurred. Eccentric torque declined in adults post and up to 48h after the eccentric exercise, whereas in children only post exercise compared to pre exercise levels (Figure 8). Regarding relative to body

weight isokinetic force, in AD concentric peak torque declined post exercise and up to 48h, whereas in CHI only post exercise. Eccentric peak torque declined in AD post and 24h after exercise, while in CHI only post exercise; additionally, CHI had greater relative to body weight eccentric peak torque than AD at 24h, 48h, 72h and 96h after exercise (Figure 9).

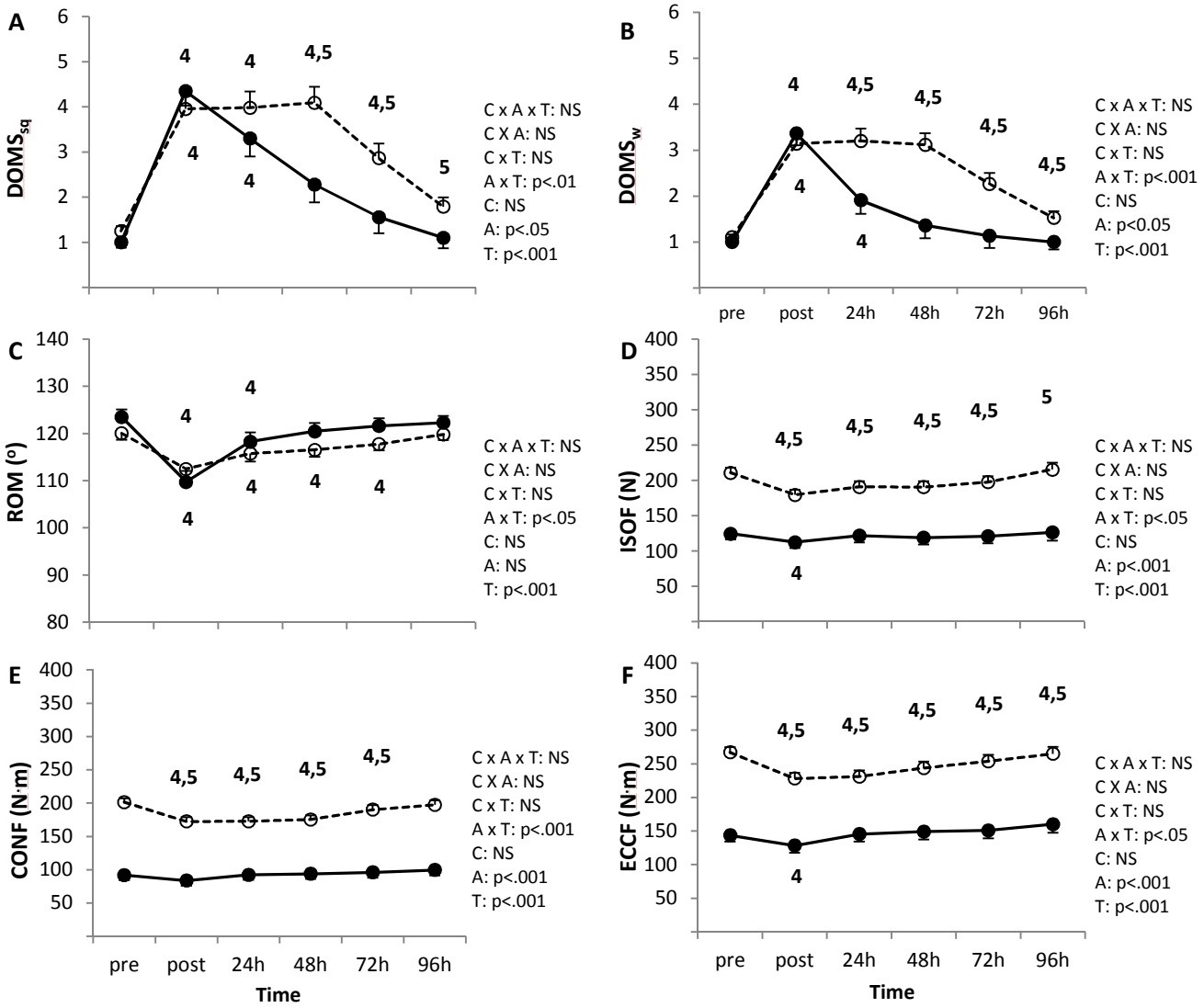

Figure 8. Muscle damage and performance after eccentric exercise in adults and children. Mean (± SEM) delayed on set of muscle soreness at squat (DOMSsq) (A) and at walking (DOMSw) (B), range of motion (ROM) (C), isometric force (ISOF) (D), concentric force (CONF) (E), and eccentric force (ECCF) (F) after muscle-damaging eccentric exercise in adults (○) compared to children (●). Three way ANOVAs (condition x age x time) with repeated measures on time and post hoc Sidak pairwise comparisons were applied. C, main effect of condition; A, main effect of age; T, main effect of time. Interactions are shown. [4]Significantly different from pre exercise in the same age group. [5]Significantly different between adults and children at the same time point.

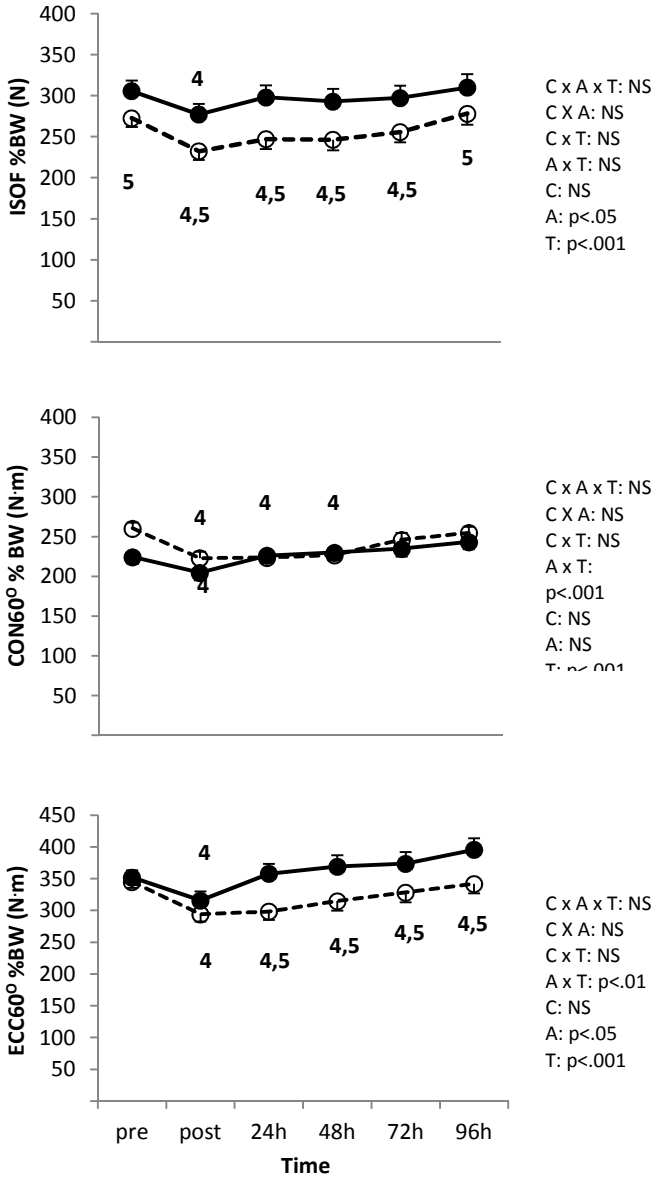

Figure 9. Relative isokinetic force after eccentric exercise in adults and children. Mean (± SEM) relative to body weight isometric force (ISOF % BW), concentric force (CONF %BW), and eccentric force (ECCF %BW) after muscle-damaging eccentric exercise in adults (○) compared to children (●). Three way ANOVAs (condition x age x time) with repea measures on time and post hoc Sidak pairwise comparisons were applied. C, main effect of condition; A, main effect of age; T, main effect of time. Interactions are shown. [4]Significantly different from pre exercise in the same age group. [5]Significantly different between adults and children at the same time point.

Regarding CK, separate ANOVAs in adults and children were performed. In adults, main effect of time came up, and CK increased as a result of eccentric exercise in the same way, in both PL and FE group, 24h and up to 96h compared to pre exercise levels **(Figure 10).** In children, no significant changes occurred in CK (Figure 10).

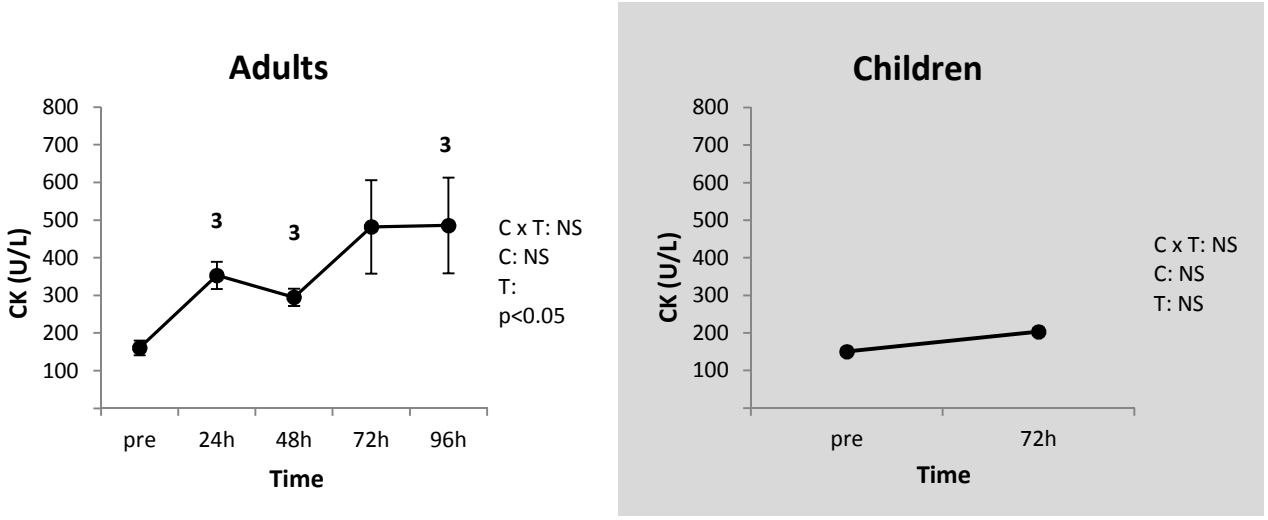

Figure 10. CK after eccentric exercise in adults and children. Mean (± SEM) CK in adults and in children. Two way ANOVAs (condition x time) with repeated measures on time and post hoc Sidak pairwise comparisons were applied separately for adults and children. C, main effect of condition; T, main effect of time. Interactions are shown. [3]Significantly different from pre exercise independently of condition.

6. DISCUSSION

The present study investigated the effects of iron supplementation on possible alterations of indices of muscle damage, performance, iron and redox status, after muscle-damaging eccentric exercise in adults and children. The current results failed to support any effect of iron supplementation on muscle damage, muscle performance and blood redox status, as eccentric exercise provoked similar modifications in both the iron-supplemented and placebo groups. On the other hand, iron status was differently modified in adults but not in children of the supplemented group, the days after eccentric exercise. Furthermore, in the present study it was apparent the critical role of age on exercise-induced muscle damage as children practically sustained minimum damage compared to adults, no changes in redox status, and only few modifications in iron status parameters.

Supplementation effects at rest

Iron status

Supplementation effects: Three weeks of iron supplementation improved iron status in both age groups, since iron concentration and TS increased. Additionally, in the iron-supplemented groups TIBC was not significantly modified in contrast to the observed decline in the placebo group. These results confirm previous reports, using low-to moderate daily doses of elemental iron and similar duration of supplementation (Nielsen, Schoene 1983, Lyle 1992), reporting favorable effects on overall iron homeostasis in healthy individuals.

Age effects: In the present study, age did not affect the supplementation outcome, as iron supplementation favorably affected iron status in both adults and children. Independently of supplementation, iron status parameters were different in adults compared to children, such that FE, TS and FERR were higher and TIBC lower in adults compared to children. Lower ferritin values have previously been reported in children compared to adults (Worwood 2004). The increased utilization of iron in

children due to growth can explain the lower iron status levels compared to adults (Hallberg 2002). Dietary iron intake could also account for the lower iron status in children (Zimmermann 2007); yet, dietary analysis revealed that iron intake was similar between the two age groups. Additionally, all of the participants were healthy and did not have any gastrointestinal disorders that could affect the iron absorption from diet (Zimmermann 2007).

Redox status

Supplementation effects: Our hypothesis that iron supplementation results in elevated basal (independent of exercise) lipid peroxidation was verified since TBARS appeared elevated after 3 weeks of supplementation. The oxidative damage from iron comes mainly from the interaction of Fe2+ with H2O2 to produce hydroxyl radical ($HO^{\bullet}$) through Fenton reaction. $HO^{\bullet}$ is a potent oxidant that can rapidly react with most molecules, including lipids and proteins (Halliwell 2007). The increased amounts of iron due to supplementation may have led to increased lipid peroxidation through the above mechanism. No other redox status marker was significantly modified. The reason for that could be the specific low-to moderate dose, or the rather short duration of supplementation, which although capable of increasing TBARS production, were not sufficient to affect protein oxidation or endogenous antioxidants. Therefore, a higher dose, or a longer duration, could probably lead to different results. The specific dose in the present study was selected due to the very novel approach to the scientific problem, and due to the fact that was close to the proposed minimum «therapeutic» requirement that corresponds to 100 mg of iron preparation daily (Nielsen 1998). Additionally, it has been found that supplementation with quantities of 39 mg - 50 mg per day of elemental iron for 12 weeks (Lyle 1992) or smaller duration (of even two weeks) (Schoene 1983) to adequately improve iron status. Moreover, according to the Food and Nutritional Board, Institute of Medicine (Zimmermann 2007), the suggested tolerable upper intake levels of elemental iron corresponds to 40 mg per day for children aged 9-13 years and to 45 mg per day for individuals aged 14 years and over. Nevertheless, this

response of TBARS after iron supplementation should be considered with caution before any conclusions regarding the pro-oxidant character of iron are made, at least with the present dose and duration of supplementation. More research is needed to establish the pro-oxidant nature of iron supplementation at rest in healthy individuals.

Age effects: Regardless of supplementation, the activity of endogenous antioxidants of different blood components seems to differ between adults and children. More specifically, children's erythrocytes presented greater antioxidant capacity compared to adults as indicated by the higher GSH and CAT activity, while the opposite was observed for plasma antioxidants with adults presenting higher uric acid and bilirubin. The higher GSH and CAT activity in children could be due to the lower overall destruction of senescent erythrocytes by the reticuloendothelial system and a lower overall iron turnover (Finch 1970, Lutz 2013) compared to adults. Erythrocytes, which contain approximately 80% of the body's functional iron (Beard 2001), have an average lifespan of 120 days (Kohgo 2008, Lutz 2013). If the number of erythrocytes destroyed daily by macrophages in children is less compared to adults, then functional iron released from catabolized hemoglobin (Halliwell 2007, Beard 2001, Cooper CE) may also be fewer; this probably results in reduced production of ROS and lower need for the use of endogenous antioxidants, thus leading to higher GSH and CAT levels in children. Additionally, children's erythrocytes are more "fresh" than that of adults' and more rich in antioxidants, thus there is no specific need for significant changes in those indices at rest. Previous research examining children's and/or adolescents' antioxidant activity, and comparing it with that of adult's, is limited. Nevertheless, children's basal GSH and CAT concentrations in the present study are similar with those reported by other studies (Gougoura 2007, abasakalis 2009, Paltoglou 2015) although no comparison with adults was made. Training is another factor that seems to affect GSH concentration in different ages since a recent study that compared GSH in trained adolescents and adults showed significant differences between the two groups, with trained adults presenting higher resting GSH concentration (Zalavras 2015).

The higher uric acid and bilirubin concentrations in adults could be explained as a protective response to increased phagocytosis of senescent erythrocytes and the higher ROS generation compared with children. Indeed, uric acid has been shown to protect erythrocytes against lipid peroxidation at physiological concentrations (Ames 1981). Bilirubin is the end product of heme catabolism, and next to uric acid, one of the most important plasma antioxidants that effectively suppress the oxidation of lipids and lipoproteins (Stocker 1987). Additionally, the higher uric acid in adults in the present study could also be due to increased intake of purine-rich foods (Ames 1981, Shatat 2012, Choi 2005), older age, higher BMI, greater height, and greater weight compared to children (Shatat 2012). More research is warranted before any conclusions are drawn regarding the differences in antioxidant status between adults and children and the possible mechanisms underlying these differences.

Muscle damage and performance

Iron supplementation did not yield any changes in muscle damage or performance indices, except for eccentric torque that appeared to be greater after 3 weeks. This improvement was independent of supplementation and age, and could be attributed rather to the fact that all of the participants were more familiarized with the eccentric type of isokinetic torque production compared to baseline, than an actual effect of iron supplementation. On the other hand, even 2 maximal voluntary isometric contractions have been reported to confer a protective effect on muscle damage induced by maximal isokinetic eccentric contractions of the elbow flexors that lasted up to 4 days (Chen 2013). In our study, baseline maximal voluntary eccentric torque was estimated through 5 maximal voluntary eccentric contractions of the rectus femoris muscles. This short bout of unaccustomed eccentric contractions could possibly has provoked a repeated bout effect (Brown 1997a), at least regarding maximal eccentric torque, on the subsequent estimation 3 weeks later.

Exercise effects

Iron status

Iron homeostasis is of crucial importance for optimal exercise performance. Although changes in iron status (Rogers 1986, Malczewsa 2004, Peeling 2014) and iron supplementation effects (Peeling 2007, De Matos 2013, McClung 2009) have extensively been examined in relation to non-muscle damaging prolonged exercise, only few research attempts have been made using short duration activities (Rowland 2012), and muscle-damaging exercise (Jamurtas 2013, Childs 2001, Gleeson 1995). Hence, there is limited data regarding muscle-damaging related adaptations of iron status parameters and no data regarding the effect of iron supplementation on these parameters. In the present study, the changes in iron status indices seem to be both age- and supplementation-related.

Age effects: Adults' iron status was affected by eccentric exercise. An interesting finding of the present study was the decrease of iron concentration 24 h and up to 96 h after eccentric exercise in adults that was observed only in iron-supplemented group. Possible explanations for this different response in iron status between the supplementation and placebo groups are discussed in the next section. Progressive fall in iron concentration has previously been reported (Gleeson 1995). In that study, despite the fact that no supplementation was prescribed, the fall in iron concentration reached a minimal value at 48 h after muscle-damaging exercise that was similar to our study. On the other hand, increases in iron concentration have been reported after eccentric exercise. High levels of bleomycin-detectable catalytic iron 24 h and up to 96 h after eccentric exercise of elbow flexors have been reported, probably due to inflammatory responses that facilitate the release of catalytic iron from ferritin and myoglobin (Childs 2001).

In children, FE concentration at 72h increased by 20% above the pre exercise levels. At this point, it has to be mentioned that fewer blood drawings conducted in children; the only post exercise blood drawing in children was at 72h whereas in adults, blood drawings were applied 24h and up to 96h after exercise. This comprises a limitation of the study, since possible changes in iron status parameters could have

been occurred in children at the first two days as well as the fourth day after the eccentric exercise. Nevertheless, no data exists in the literature regarding the effect of acute exercise on iron status in children. Further investigation is warranted in order to identify possible differences in iron status between adults and children after eccentric exercise and assess the acute versus the chronic effect of iron supplementation in both age groups.

Supplementation effects: In the present study, iron status was differently affected by eccentric exercise in iron-supplemented compared to placebo groups. The reason for the different response of iron concentration between the iron-supplemented compared to placebo group is not clear. One can assume that the higher pre exercise iron concentration in the iron-supplemented group, caused a greater binding of transferrin to iron (Crichton 2003) resulting in lower iron concentration the days after eccentric exercise. Furthermore, hemolysis occurring after eccentric exercise (Theodorou 2011) could lead to release of iron from erythrocytes and further increase iron concentration into the circulation; this could additionally trigger transferrin binding to iron. This assumption is supported by the fact that the time (48 hrs post-exercise) of the greater decrease (36%) of iron concentration coincided with the higher (43%) TS. Another possible explanation could be the participation of iron in oxidative stress processes that take place after muscle damaging exercise. It is well known that iron can contribute to increased production of RONS (Halliwell 2007) and eccentric exercise also leads to oxidative stress (Childs 2001, Theodorou 2011, Goldfarb 2005). Phagocytosis in the area of the injured muscle and the subsequent increase in oxygen consumption can lead to the production of superoxide anions (O2–) and hydrogen peroxide (H_2O_2) (Halliwell 2007). The higher pre exercise available iron in the iron-supplemented group together with the enhanced RONS production in the injured muscle may have provoked greater iron use compared to the placebo group.

Contrary to our results, Childs et al (Childs 2001) reported higher levels of bleomycin detectable iron 48h and up to 96h after eccentric exercise. What is interesting in that study, is that although an antioxidant combination of vitamin C and NAC was used (in contrast to the potent pro-oxidant in our study), the increase in catalytic serum iron

was not avoided and was even greater than in the placebo group. The authors attributed the increase of catalytic iron to the activation of inflammatory cells and the release of myoglobin due to muscle damage. The authors suggest that supplementation of antioxidants instead of attenuating or preventing the release of catalytic iron, may facilitate the mobilization of iron from its stores, gaining that way a potent pro-oxidant role, at least in cases of exercise-induced muscle damage.

Redox status

Increased generation of RONS for several days after the termination of eccentric or eccentrically biased exercise has been reported by a great number of studies (Childs 2001, Theodorou 2011, Goldfarb 2005, Lee 2005).

Age effects: To our knowledge, this is the first attempt to investigate the effects of iron supplementation on blood redox status in adults and children after eccentric exercise. In the present study, exercise resulted in modulation of oxidative stress indices only in adults. Previous research has shown that muscle-damaging exercise affects the concentration of blood antioxidants temporarily and in no constant time-fashion (either at some hours or days after exercise) (Nikolaidis 2008). According to the present results, in adults, PC and TAC increased 24h and returned to pre exercise concentrations thereafter in both the supplemented and the placebo groups. These results suggest that oxidative stress that was induced by eccentric exercise was compensated by an increase in the total antioxidant activity of the blood that may have reduced the effect of oxidative stress the following days. Lee et al (Lee 2002) also report increased PC 2 h and 48 h after eccentric exercise of the elbow flexors. No changes occurred in any of the other redox markers. The modifications of redox status indices that occurred in the present study in adults were either smaller or shorter in duration compared to other studies, where changes were more intense and prolonged (Childs 2001, Theodorou 2011, Goldfarb 2005) or similar to others reporting no changes (Gleeson 1995, Hellsten 1997). The different exercise models, redox markers, and/or different time points, or even the diversity of study population may account for the discrepancy in the results. Additionally,

mitochondria, which greatly contribute to free radicals production, perhaps were not stressed enough by the type of exercise used in the present study and hence, the changes in the examined oxidative stress indices were relatively low. Another possible explanation could be the proposed redox individuality of the participants, as exercise induces reductive stress in some humans and no stress in others (Margaritelis, Stagos 2015), indicating that exercise stimulus is not equally perceived by all individuals.

In children, no changes occurred in redox parameters. Despite the proposed greater ROS release in children and adolescents in response to exercise than in adults due to their faster VO_2 kinetics and the observed robust increases in circulating neutrophils in children after exercise (Cooper 2004), this was not the case in the present study. Neither iron supplementation, nor eccentric exercise alone, were able to provoke significant oxidative stress in children, as indicated by the absence of changes, at least in the redox status indices used in the present study. This was probably due to the lower extent of muscle damage provoked in children that resulted in lower inflammation-mediated ROS production and lower hemoglobin and myoglobin catabolism (Kohgo 2008). Once again, the fewer time points of assessment in children perhaps did not allow pinpointing significant changes as it happened with the adults. There is limited data in children (Nikolaidis 2007) and adolescents (Zalavras 2015) regarding exercise-induced redox alterations. Contrary to our results, Nikolaidis et al. (2007) reported significant increases in TBARS, PC, CAT, TAC, and oxidized glutathione (GSSG) concentration, as well as significant decreases in GSH and GSH:GSSG, after 12 bouts of 50 m swimming, performed at 70% - 75% of 50 m maximum velocity. The results from a recent study from our laboratory (Zalavras 2015) indicate a greater rise in PC, TBARS and TAC and greater GSH decline in adults compared to adolescents following an aerobic exercise bout. Uric acid, CAT and bilirubin were similar amongst adults and adolescents immediately after and 1 h following the aerobic trial. The different exercise modes and experimental designs probably account for the divergent results between the studies. Additionally, the evaluation of the biological maturity is performed through Tanner stages; the present study has also used this method. Although the validity of this method is well

documented (Morris 1980), testosterone levels are not always related to genital stage (Lee 2010). Thus, different testosterone levels of young participants may also account for the inconsistency between studies.

Supplementation effects: iron-supplementation did not provoke altered redox status responses, and did not prove its pro-oxidant character after muscle-damaging exercise. The lack of different responses in blood redox status in the iron-supplemented group could be due to the specific dose of 37mg/day of elemental iron used in the present study. Although this dose was capable of improving iron status parameters after 3 weeks it did not manage to alter redox responses induced by muscle-damaging exercise. It is interesting, however, the fact that the absence of any significant changes in redox status due to supplementation of a potent pro-oxidant in the present study is consistent with the results of other studies involving supplementation of antioxidants that also do not report any different outcome. High doses of vitamin C and E did not manage to alter the redox status of blood and skeletal muscle after acute or chronic eccentric exercise (Theodorou 2011). What is more noteworthy though is the fact that supplementation with vitamin C and NAC instead of reducing, augmented oxidative stress after eccentric exercise and acted as pro-oxidants, by helping the mobilization of catalytic iron from its stores (Childs 2001). Therefore, iron supplementation, at least as administered in the present study, does not adversely affect aseptic muscle trauma. This could be of great importance in clinical conditions which on one hand they are characterized by skeletal muscle injury and inflammation, and on the other hand, iron supplementation is crucial for maintaining iron homeostasis. Nevertheless, more research is warranted before any valid conclusions are drawn.

Muscle damage and performance

Exercise-induced muscle damage is manifested by a number of classic physiological symptoms such as DOMS, deterioration in ROM, reduced muscle contractility and force production, as well as leakage of muscle proteins and muscle enzymes into the circulation (Gleeson 1995, Theodorou 2011, Peeling 2007, Lee 2002). In the present

study, eccentric exercise resulted in muscle damage as evidenced by the deterioration of the above symptoms.

Age effects: Age was a substantial factor on exercise-induced muscle damage, since adults sustained more severe muscle damage compared to children. That was evident through more intense and prolonged changes in physiological symptoms, as well as greater increase in CK activity, the days following the exercise bout. The temporal changes of the above symptoms of muscle damage in adults of the present study are in accordance with those usually being observed after eccentric (Childs 2001, Gleeson 1995, Theodorou 2011, Brown 1997a, Paschalis 2010, Child 1999) or eccentrically biased exercise (Jamurtas 2013, Hellsten 1997, Marginson 2005). Changes in CK activity in adults has been shown to occur up to 7days after muscle damaging exercise (Brown 1997a), usually peaking between 2d and 4d (Childs 2001, Hellsten 1997, Jamurtas 2013, Theodorou 2011) while reaching values as high as 34-fold (Child 1999) the pre-exercise levels. The fewer blood drawings in children compared to adults may account for the discrepancy regarding the responses of CK activity between the two age groups, since different responses in CK activity could have been detected in children, at different time points.

Data regarding the differences in muscle damage symptoms after eccentric exercise between adults and children is scarce. Nevertheless, the results of the present study are in accordance with those of other studies that also report less severe symptoms of muscle damage in children compared to adults after muscle-damaging exercise (Marginson 2005, Gorianovas 2013, Soares 1996). The mechanisms underlying the reduced susceptibility of children to muscle damage are not yet entirely understood. One possible explanation could be the fact that during eccentric exercise, fast twitch muscle fibers seem to be preferentially damaged (Choi 2014, Warren 1994). Muscle fiber composition of children is such that contain fewer fast twitch fibers (Lexell 1992), thus the resulted muscle injury is less severe. Other proposed reasons are the greater muscle compliance in children (Marginson 2005, Marginson 2001) the greater body weight of adults that generates more force per fiber unit during eccentric contractions (Webber 1989), the lower ability of children to produce strength proportional to muscle size (Kanehisa 1994) and the lower power output of

children compared to adults (Falk 2006). Finally, a situation of pre-conditioning may exist in children, as in their everyday life they perform more eccentric actions such as jumping and hopping compared to adults; thus they may be more familiarized with eccentric work resulting in milder symptoms of muscle damage after eccentric exercise. The results of the present study are not in favor of the greater ability of adults to produce muscle force and power output, since the greater isokinetic force of adults disappeared when the results were normalized for body weight. Indeed, children demonstrated similar relative concentric and eccentric peak torque and higher relative isometric peak torque compared to adults immediately pre-eccentric exercise, and greater isokinetic force following eccentric exercise. More research is needed to clarify the mechanisms that are responsible for the lower susceptibility of children to muscle-damaging exercise and draw generalized conclusions regarding age effects on exercise-induced muscle damage.

Supplementation effects: In the present study iron supplementation did not result in different responses following eccentrically-induced muscle damage. Despite the probable pro-oxidant character of iron and its ability in causing greater ROS-mediated muscle injury, iron supplementation did not alter the magnitude or the time course of muscle damage and muscle performance. Both iron-supplemented and placebo groups sustained muscle injury in the same way. Thus, the potent pro-oxidant character of iron was not proven to affect muscle function and performance during the recovery period after muscle-damaging exercise in a different way. Because of lack of data regarding the effect of iron supplementation on muscle damage and performance after eccentric exercise, the present results cannot be compared with the results of other studies. Future research is warranted in order to draw valid conclusions regarding iron supplementation and muscle damage and performance.

7. CONCLUSIONS

This was the first attempt to determine whether a potential pro-oxidant could differently modify muscle damage symptoms and redox status after muscle-damaging exercise in adults and in children. Iron supplementation as prescribed in the present study, did not manage to affect muscle damage, or redox status in a different way than that typically being observed. Symptoms of muscle damage, as well as RONS production were comparable between iron-supplemented and non-supplemented groups. Higher doses of iron supplementation, may be more effective in producing different amounts of reactive species, and thus alter the responses and recovery after eccentric exercise. Furthermore, the results of the present study also indicate that children are less susceptible in muscle damage compared to adults, yet, due to the limited data, more research is warranted in order to verify this susceptibility and clarify the possible underlying mechanisms.

The present study, investigated the effect of chronic iron-supplementation prior to eccentric exercise. Acute effect of iron supplementation starting immediately after exercise-induced muscle damage was not examined, and future studies should address this issue. Additionally, other exercise stimulus and redox status indices should also be incorporated in future research. In the present study, blood drawings in children were fewer compared to adults, and this may have shielded the true responses of CK activity, iron status, or blood redox status in children. Thus, similar time points in adults and children should be incorporated in future studies. Finally, due to the majority of the research focusing on males, all of the above issues should be addressed also in women and girls, to determine possible gender-responsible alterations.

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