Exercise Redox Biology at the Intersection of Health and Performance in Women: The Role of Ovarian Hormones

Introduction

Female participation in sport and exercise increased significantly over the past two decades. This increase spotlighted the paucity of scientific research that focuses specifically on exercise and female physiology. At the center of research interest in exercising women are the ovarian hormones: estrogen and progesterone. Though the ovarian hormones predominant role is in reproductive development and fertility, estrogen and progesterone simultaneously regulate a variety of physiological processes that influence health and performance outcomes (citations). The effects of the ovarian hormones on women's health and performance are systemic. Ovarian hormones influence various conditions including obesity (Leeners et al., 2017), vascular pathologies (Hage & Oprail, 2013), cardiovascular disease (Medina et al., 2003), neurodegenerative diseases (Beltz & Moser, 2020), and cancer (Mørch et al., 2009). During menopause, the production of ovarian hormones decreases significantly coinciding with increased risk for the aforementioned diseases. In the context of performance, the decrease in ovarian hormones following menopause results in decreased musculoskeletal functional capacity, decreased metabolic capacity, and frailty (citations). In pre-menopausal women, the ovarian hormonal mileu fluctuates due to the menstrual cycle or the use of exogenous hormones such as oral contraceptives. This is in contrast to men who display relatively stable concentrations of testosterone, the primary male gonadal hormone, throughout the lifetime (citation). These changes in hormonal profiles throughout the lifetime highlight a significant difference between men and women that has not been adequately addressed in sport and exercise research. Given that exercise is unequivocally beneficial for health and is necessary for optimal sport performance, uncovering how the fluctuating hormonal milieu in women influences exercise responses and adaptations will help provide specific exercise prescriptions for optimizing health and performance in women.

Though it is well established that the ovarian hormones induce diverse physiological processes, the exact mechanisms for many of their health and performance promoting effects are unknown. A leading hypothesis for their health promoting effects is the regulation of redox biology, namely, estrogen's protective role against oxidative stress. Oxidative stress results from the buildup of free radicals which can subsequently damage cellular components when the antioxidant defense system is overwhelmed. The increased resistance to oxidative stress related pathologies in females is frequently attributed to the presence of estrogen, and in the absence of estrogen much of this pathology resistance disappears (citation). The production of free radicals increases with exercise and is associated with muscle fatigue, exercise performance, and exercise adaptations making strategies to monitor and manage exercise induced free radical production critical for performance. As it stands, many of the health and performance promoting effects of exercise appear to be contingent on free radical production and redox controlled signaling pathways (citation). The role of the ovarian hormones in aging and redox related pathologies is a popular research area, however their role in exercise redox biology is understudied (citations). Exercise redox biology is a relatively young field and is inherently difficult to measure due to the short half-life of free radicals and technological limitations (93). However, redox biology underpins several tenets of exercise physiology (citation) and is simultaneously influenced by changes in the ovarian hormones (citations). The lack of exercise redox investigations in women is noteworthy as redox biology provides a mechanistic rationale for sex-specific physiological responses to exercise.

Due to the absence of literature investigating exercise redox outcomes in women, it is not clear if ovarian hormone changes in response to the menstrual cycle or exogenous hormones impact the cellular redox machinery that governs the health promoting effects of exercise. This review aims to present a rationale for more significant investigations into the redox outcomes of exercising females and their connection with cellular and performance outcomes. To achieve this aim a brief summary of the ovarian hormones role in sport and exercise physiology is presented. This section will also address what is known regarding the influence of the menstrual cycle and exogenous hormones on exercise performance and adaptations. The second section will introduce exercise redox biology and the role of redox homeostasis in regulating exercise performance and adaptations. Finally, a comprehensive review of what is currently known of estrogen's redox functions will be discussed including its contentious role as both an antioxidant and pro-oxidant, mechanisms for its redox effects, and what has to date been investigated regarding estrogen, exercise, and redox biology.

Ovarian Hormones in Sport and Exercise

Role in Exercise Physiology

Estrogen is an anabolic agent that modulates skeletal muscle contractile function and muscle mass. High estrogen levels increase the force producing capacity of smaller muscles such as the adductor pollicis and larger muscles involved for locomotor function (153). However, this effect is not observed universally, it appears the ability of estrogen to promote muscle strength is likely modulated by muscle type and is more apparent in type I muscle fibers rather than type II. Though the exact molecular mechanisms via which estrogen performs these actions in skeletal muscle is unknown it is known that when estrogen is absent it reduces the number of strong binding myosin cross bridges able to produce force. Conversely, this effect is reversed when estrogen is present (156). Estrogen also improves myofibrillar ATPase activity and calcium sensitivity in contracting muscles (154). Accumulating evidence suggests that estrogen deficiency may induce apoptosis in skeletal muscle contributing to the higher rates of sarcopenia and frailty in post-menopausal women compared to eumenorrheic women (155). Though the mechanism for this protective effect is not entirely clear, estrogen regulation of mitochondrial sourced free radical production is a leading hypothesis and will be discussed later. Research that specifically examines the role of progesterone effect on skeletal muscle in the absence of estrogen is lacking though is it generally accepted that progesterone is anti-oestrogenic and catabolic in nature (157).

Both estrogen and progesterone impact metabolic responses in a divergent fashion. Metabolically the anti-oestrogenic effects of progesterone are more pronounced (87,88, 92). Insulin sensitivity appears to be related to estrogen levels, while progesterone increases insulin resistance (103). Estrogen increases glycogen storing and glycogen sparing under endurance conditions. Under high intensity short duration activities estrogen promotes glucose uptake into type 1 muscle fibers while progesterone antagonizes this effect (93). Animal studies indicate estrogen may increase lipolysis during exercise and mobilize lipids from adipose tissue to skeletal muscle (91). More research needs to be done to confirm this action in humans. Estrogen also acts to increase free fatty acid (FFA) availability and FFA intramuscular stores. Recent research indicates many of estrogen's effects on metabolism may occur through the activation of AMP activated protein kinase (AMPK), a major regulator of ATP production and aerobic adaptations to exercise (102). The ovarian hormones also influence protein metabolism, more specifically it appears that progesterone increases protein catabolism while estrogen prevents protein catabolism (96). Though further research is needed to clarify estrogen's role in preventing protein catabolism. The potential for the ovarian hormones to impact carbohydrate, fat, and protein metabolism seems to rely largely on the

estrogen:progesterone (E:P) ratio. As this ratio changes throughout the menstrual cycle the effects of the ovarian hormones become more pronounced (103).

The Menstrual Cycle

The menstrual cycle (MC) is a biological pattern in women of reproductive age that is characterized by daily and weekly hormonal fluctuations that function to prepare the endometrium for fertilization and pregnancy. Normal menstrual cycles vary in length between 21-35 days with a typical MC lasting 28 days. The primary phases of the MC are the follicular phase (days 1-14), which can be further broken down into the early follicular (menstruation) phase (days 1-5) and late follicular phase (days 9-14), the ovulation phase (day 15), and the luteal phase (days 16-28). A graphical representation of the expected ovarian hormone fluctuations throughout different MC phases can be seen in ***Fig 1***. The fluctuations in ovarian hormones may modulate various exercise responses and adaptations in exercising women. Though this hypothesis has been explored under a variety of conditions, very few previous investigations utilized valid methodological strategies to verify ovarian hormone concentrations during different MC phases (citations). A previous review reported the large between study variance of ovarian hormone measurement techniques as a significant limitation in assessing the true effect of the MC (citation

Insert Figure 1 Here

Calendar based counting and basal body temperature (BBT) are frequently used indirect methods to predict MC phase in sport and exercise. Though these techniques are inexpensive and noninvasive, they provide no information about actual hormonal concentrations and as such are not recommended in isolation. These methods must be used in combination with direct indicators of hormonal concentrations. The least invasive method being a urinary luteinizing hormone (LH) measurement. A surge of LH precedes ovulation by approximately 14-26 hours in the majority of females (37). This method can more accurately predict an estrogen surge around ovulation but does not measure actual ovarian hormone concentrations. Ovarian hormones are instead measured through salivary or serum analysis. Though saliva is less invasive than serum analysis, salivary estrogen and progesterone levels can fluctuate considerably within a short timeframe, meaning multiple samples must be taken. As such, serum analysis should be used to measure estrogen and progesterone levels and confirm MC phase. A combination of calendar based counting, BBT, urinary LH analysis, and serum analysis is recommended for researchers interested in examining the role of the MC and ovarian hormones in sport and exercise. For a more detailed discussion on methodological strategies to measure the MC in sport and exercise, readers are referred to a preview review (citation).

It is currently unclear whether or not the MC induces a significant quantifiable effect on exercise performance. The hormonal fluctuations throughout the MC are well established factors that influence readiness to train, motivation, and subjective markers of well-being and performance (citations). However, the influence of the MC on objective performance measures is complicated by methodological shortcomings and makes the development of evidence based strategies for managing the MC difficult (citations). A recent systematic review and meta-analysis examined the effect of the MC on exercise performance in eumenorrheic women and found that performance may be trivially reduced during the early follicular phase of the MC compared to all other MC phases (Eliiot Sale et al., 2020). However, this review acknowledged that the large number of low quality studies and variance of methodological

strategies previously implemented means these results should be interpreted with caution. Studies that neglected to measure ovarian hormone concentrations showed no influence of MC phase on performance outcomes (Kishali et al., 2004) or a wingate test (Stefanovsky et al., 2016). In contrast, two studies that did not measure hormone concentrations did find an impact of the MC (citations). Tenan et al., (2016) found decreased performance during the mid-luteal phase on maximal voluntary contraction (MVC) and force variability during an endurance task. Shaklina et al., (2016) found better performance on a cycle ergometer during the postmenstrual (late follicular) and post-ovulatory (mid-luteal) phases. It is important to note that outcomes like MVC more closely align with a motor task or physiological change than exercise performance *per se* and as such make comparison between studies with different outcomes difficult. Studies that utilized superior validation techniques such as serum hormonal concentrations have also failed to clarify the impact of the MC in sport and exercise. Some studies report no impact of MC phase on aerobic exercise (Rael et al., 2021; Vaiksaar et al., 2016) while others report worse prolonged exercise performance during the mid-luteal phase when BBT increases due to a progesterone spike. (Thompson et al., 2012). Forsyth & Reilly (2008) found improvements in blood lactate dynamics during the mid-luteal phase but no improvements in 2000m rowing performance (citation). This would align with the findings of Jurkowski et al. (1981) which showed decreased lactate production during the luteal phase and no change in time to exhaustion during cycling. An ovarian hormone mediated change in bioenergetics supports more recent studies that indicate the MC may alter physiological responses to exercise like immune mobilization (Hashimoto et al., 2014) and inflammatory cytokine release (citation), but does not impact acute exercise performance. This is an important distinction to acknowledge: an absence of a significant effect of the MC on acute exercise performance does not mean the physiological responses to exercise that mediate adaptation are not impacted. In fact, a handful of studies indicate that focused training at specific MC timepoints may be better at inducing strength training adaptations (140, 141). This suggests an ovarian hormone mediated adjustment of physiological responses that mediate exercise adaptation. Further research that investigates the longitudinal influence of focused exercise training during certain MC phases and the results on cellular and performance markers of adaptation are warranted.

Exogenous Ovarian Hormones

The proportion of women who use hormonal contraceptives between the ages of 15-49 in the United States was about 65% from 2015-2017 (Daniels et al., 2017). Within sports performance the proportion of elite female athletes that use hormonal contraceptives is close to 50% (Martin et al., 2018). Hormonal contraceptives are exogenous ovarian hormones that downregulate the endogenous production of ovarian hormones to prevent pregnancy (citation). There are various different forms of hormonal contraceptives but the most studied form in sport and exercise are oral contraceptives (OCs) which come in combined estrogen and progestin (progesterone), and progestin only forms. The varying type and concentration of exogenous ovarian hormones may impact physiological responses to exercise in a manner similar to endogenous hormones.

Similar to findings on MC phase, any impact of OC usage on acute exercise performance appears to be small or trivial. Again, methodological shortcomings are a limitation in assessing the effect of OCs. Only 17% of studies included in a recent systematic review and meta-analysis received a “high” quality grade (Eliot Sale et al., 2020). Specific effects of OC use on acute resistance training performance are unclear (Thompson et al., 2020). The use of OC on adaptations also requires further research. Some studies indicate no effect of OC on resistance training adaptations (citation) and others indicate an effect

of the specific androgenicity of the progestin component (Rechichi et al., 2009). Another study found significantly better muscle hypertrophy only in type I muscle fibers when OC users took 30mg ethinyl-estradiol compared to naturally menstruating women or women taking 20mg ethinyl-estradiol (Dalgaard et al., 2019). This is interesting as it implies the higher concentration of exogenous estrogen plays a specific role in muscle adaptation. This is in contrast to the aforementioned review (Eliot Sale et al., 2020) that concluded the effects of OCs are likely mediated by exogenous regulation of endogenous hormone production rather than exogenous manipulation of physiological responses.

Limitations and Areas of Future Research

In order for future research to clarify the role of ovarian hormones in exercise performance and adaptations, studies must utilize valid methodological strategies to measure actual hormone concentrations. The lack of continuity in measured outcomes is another confounding variable. It is possible that the MC may impact variables like force tremors on an isokinetic dynamometer but not performance on a VO_2 max test. The mechanisms governing fatigue for single limb exercises and maximal running to exhaustion are different, as they are for submaximal and maximal aerobic and resistance exercise. It is difficult to truly ascertain if a difference exists between performance outcomes due to the methodological limitations. The large variance in subject training ages (e.g. sedentary, recreationally active, active, athlete, elite athletes) further complicates interpretation of previous research. Outcomes that may not be statistically significant for sedentary individuals but may be practically significant in the elite athlete. Most research on hormonal contraceptives focuses on OCs and often neglects to consider the withdrawal phase or the type of exogenous hormone that is being consumed. Different synthetic hormones are metabolized differently which may divergently influence health or performance outcomes (citations). A limitation in previous MC sport and exercise research is the focus on only two or three discrete MC timepoints on observable performance outcomes (citation). Though methodologically convenient, this type of analysis is reductionist. The MC is not a phenomenon that occurs at discrete time points but rather a cyclical biological pattern with daily changes in hormonal concentrations that may influence systemic physiological responses and adaptations to exercise. An emphasis on observable performance outcomes without adequate consideration of the downstream consequences of endogenous or exogenous ovarian hormone changes neglects to acknowledge the impact the hormones may have on cellular machinery that governs health and performance adaptations to exercise. Redox biology presents a mechanism through which even small daily ovarian hormone fluctuations may influence basal redox state and subsequently impact the performance, cell signaling, and adaptive response to exercise (citations).

Exercise Redox Biology

Seminal research in the mid 20th century showed that high levels of free radicals induce cell death and excess free radicals are associated with aging and disease. As such, antioxidants were investigated as a means to attenuate cell death, restore functionality in older individuals, and prevent disease. Around this time it also became apparent that exercise increases the production of free radicals and that increased oxidant production is associated with muscle fatigue. Therefore, there was also a belief that antioxidant supplementation could be used to attenuate muscle fatigue and improve exercise performance. Unfortunately, antioxidant supplementation has not shown the promise researchers hoped for. For the most part antioxidants do not appear to be effective at attenuating fatigue during exercise (citation), treating diseases, or preventing aging. This is because the production of oxidants and

subsequent scavenging by antioxidants is a tightly regulated redox network. This homeostasis between oxidants and antioxidants modulates diverse cellular processes in a variety of tissues which can control exercise responses, exercise adaptations, disease development, and the aging process. In the case of exercise performance and adaptations, it is known that exogenous supplementation with certain antioxidants can negatively impact the expected performance outcomes and exercise adaptations by interfering with redox regulated signaling pathways.

Exercise redox biology is plagued by confusing terminology. Oxidative stress was first defined as "a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage" (Sies, 1985) It is now clear that oxidative stress and production of prooxidants is not strictly damaging and is in fact critical for cellular function and signaling. Despite this, many publications still inappropriately use "oxidized" or "reduced" as systemic blanket terms referring to negative or positive outcomes, respectively (citation). Additionally, the plethora of terms used to describe oxidants (ROS, RONS, RNS, free radicals etc.) makes interpretation of results difficult and as a result the global term *reactive species* is recommended and will be used throughout this paper (Forman et al., 2015). Reactive species will be defined as unstable molecules that easily react with other molecules or components within a cell leading to induction of cell signaling pathways or cellular damage. Use of the term antioxidants can also be problematic because it ignores the fact that antioxidant enzymes can be coupled with redox reactions that produce reactive species. However, for simplicity sake, the term *antioxidants* will be used to refer to molecules that scavenge and neutralize reactive species. Due to the immense volume of literature on this topic, the material in this section is condensed. For further reading the reader is directed to other more comprehensive reviews (59, 95, 149, 150, 152).

Redox Signaling and Oxidative Stress

Endogenous production of reactive species occurs constantly while at rest but can increase tenfold during exercise. Reactive species is an umbrella term that encompasses reactive oxygen species (ROS), reactive nitrogen species (RNS), free radicals, non-radical reactive species (H_2O_2), and biologically active oxidized macromolecules (4-HNE). At rest, production of reactive species occurs predominantly within the mitochondria, however during exercise reactive species production shifts to other sources namely NADPH oxidase (NOX) isoforms located in the sarcoplasmic reticulum, t-tubules, and plasma membrane (citations). Reactive species interact with molecules, proteins, and other cellular components that effectively regulate the activity of intracellular signaling pathways, transcription factors, and gene expression (i.e. *Redox Signaling*). While various reactive species are produced in skeletal muscle, hydrogen peroxide (H_2O_2) appears to be the most critical reactive signaling molecule (citation). Increased concentrations in skeletal muscle induces key signaling pathways such as mitogen activated protein kinases p-38 MAPK and JNK, protein tyrosine phosphatases (PTP), peroxisome proliferator-activated receptor gamma (PPAR-γ), nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor kappa B (NF-κB).

Excessive production of reactive species can disrupt redox signaling pathways, damage cellular components (DNA, proteins, and lipids), and result in cytotoxicity (i.e. *oxidative stress)*. In order to prevent excess buildup of reactive species the body maintains an antioxidant defense system. Similarly to reactive species, various enzymatic and nonenzymatic antioxidants exist within skeletal muscle in both localized cellular compartments and within the cytosol. The primary enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidase (Gpx), catalase (Cat), peroxiredoxin (Prx), glutaredoxin (Grx), and thioredoxin reductase (TrxR). Additional non-enzymatic antioxidants exist

(glutathione, bilirubin, ubiquinone, uric acid etc.) and work cooperatively with enzymatic antioxidants to scavenge excess reactive species and protect skeletal muscle from oxidative damage during periods of intense exercise.

Acute Exercise Responses

The dual nature of reactive species as critical cell signaling molecules and deleterious cytotoxic agents is best observed in the context of skeletal muscle contractile function. Reid et al., (151) developed a theoretical model from the collective results of various studies that demonstrate the biphasic response of free radicals on force production ***(Figure 2).*** Previous literature indicates that force production is significantly reduced when unfatigued muscle is exposed to antioxidants or a reducing agent. Low level of exposure to oxidants, namely H_2O_2, increases submaximal force production and muscle exposed to excessive oxidants significantly decreases force production. Indeed, force producing capacity decreases and muscle fatigue increases as oxidant production increases *in vivo* (citations). However, the interesting finding that pre-exercise antioxidant supplementation and antioxidant supplementation in unfatigued muscle inhibits maximal force production demonstrates the critical nature of reactive species. Therefore, these findings suggest an optimal cellular redox state exists for muscle force production and a deviation from the optimal redox state leads to a reduction in force.

Insert Figure 2 Here

Studies using antioxidants demonstrated an optimal redox state exists for additional cellular responses to exercise including glucose transport and blood flow to skeletal muscle. In regards to glucose transport, the presence of RONS appears to be one of the multiple mechanisms that govern exercise induced glucose uptake into skeletal muscle (Henriquez-Olgin et al., 2020; Richter et al., 2013). Increased glucose availability during exercise is critical for exercise performance as glucose depletion is associated with decreased exercise capacity and performance (152). The process of increasing glucose availability in skeletal muscle is regulated by coordinated cellular actions that involve translocation of glucose transport 4 (GLUT4) to deliver glucose to the active muscle. H_2O_2 is known to stimulate GLUT4 translocation in skeletal muscle and antioxidant supplementation inhibits GLUT4 translocation under electrical (60, 62) and mechanical stress (Chambers et al., 2009). Increased ROS production from upregulated NADPH oxidase (NOX) activity near the sarcolemma is likely required for exercise-stimulated GLUT4 translocation and subsequent glucose uptake to active skeletal muscle (Henriquez-Olguin et al., 2020). Redistribution of blood flow to active skeletal muscle is one of the best known cellular responses to exercise and appears largely contingent on RONS production. Generic antioxidant cocktails significantly reduce RONS production in handgrip tasks and simultaneously impair exercise induced vasodilation (Donato et al. 2010, Richardson et al. 2007). However, this effect is not observed in older individuals (Sindler et al., 2009). Instead, it appears that antioxidant supplementation improved blood flow and task success in older populations indicating that under pro-oxidative conditions like aging, a reducing agent can improve vascular function. The collective evidence suggests that under physiological conditions the presence of oxidants is necessary for vasodilation and this should not be disrupted with exogenous antioxidant supplementation (citation).

Longitudinal Exercise Adaptations

The production of free radicals also plays a critical role in the longitudinal adaptations to exercise training. Quintessential exercise adaptations such as mitochondrial biogenesis and skeletal muscle hypertrophy are redox sensitive (citations). In addition, many of the beneficial health effects of exercise are a result of an improved redox homeostasis and upregulated endogenous antioxidant system that demonstrates greater resilience to oxidative insults. The subcellular location of free radical production in addition to the type of free radical produced plays a critical role in the magnitude of these adaptations.

Mitochondrial biogenesis is an archetypal aerobic training adaptation that is controlled by the redox sensitive master regulator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) and inhibited by antioxidant supplementation (Drake et al. 2015). Daily administration of the antioxidant vitamin C attenuated aerobic exercise induced increases in PGC1a activity in rodents and healthy young men engaging in an exercise training program (Gomez-Cabrera et al. 2008; Ristow et al. 2009). Vitamin E and alpha-lipoic acid supplementation blunted PGC1a signaling in rats following treadmill running (Strobel et al., 2011) and combo supplementation of vitamin C and E dampened PGC1a signaling and decreased PGC1a protein content compared to exercise alone in humans (Paulsen et al., 2014). Unfortunately, the mechanisms underpinning RONS regulation of mitochondrial biogenesis remain elusive. A lack of exercise specific mechanistic studies means there is significant speculation regarding how various reactive species activate PGC1a and induce mitochondrial biogenesis.

On the other end of the exercise spectrum, skeletal muscle hypertrophy is the predominant adaptation elicited by resistance training exercise. Skeletal muscle hypertrophy is governed predominantly by the redox sensitive anabolic mammalian target of rapamycin (mTOR) and its downstream initiation factors ribosomal protein S6 kinase (p70S6k) and eukaryotic translation initiation factor 4E (eIF4E) (citations). Antioxidant supplementation with a vitamin C and vitamin E cocktail blocked the anabolic signaling increase from a resistance training program in both men and women (Paulsen et al., 2014). This is in agreement with another study that showed impaired skeletal muscle hypertrophy in a rat overload model following antioxidant supplementation (Makane et al., 2013). Dutra et al., 2018 observed decreased performance measured by peak torque and total work after a…***how long (6 weeks?)...***in individuals supplementing with vitamin C and vitamin E. Like mitochondrial biogenesis, the mechanistic basis for redox control of skeletal muscle hypertrophy is unclear. However, one research group attempted to identify the mechanisms of redox regulated skeletal muscle hypertrophy by using neuronal nitric oxide synthase (nNOS) null mice, nitric oxide synthase inhibitors, and peroxynitrite selective scavengers (Ito et al., 2013-Ito et al., 2018). In a series of studies, the research group demonstrated that NO production from nNOS was required for synergist ablation induced muscle hypertrophy (citation), and that free radical production from NOX was required to open Trpv Ca^{2+} channels thereby allowing Ca^{2+} influx to induce mTORC1-p70S6K1 signaling and consequent muscle hypertrophy (citations). Further studies that confirm this action and the larger role of RONS on muscle hypertrophy are warranted.

Improved Redox Homeostasis

One of the more peculiar adaptations to exercise training is an upregulated antioxidant defense that results in an increased resilience to subsequent oxidative insults (citation). Individuals who perform consistent exercise such as athletes present lower resting levels of oxidative stress, higher antioxidant capacity, and improved repair mechanisms for oxidative damage. Given the high frequency of oxidative stress related pathologies (citations) and the role of free radicals in aging and frailty (citation), upregulated antioxidant defenses play a critical role in slowing the onset of disease and aging and provide a mechanism for one of

the many health benefits of exercise. These redox specific adaptations to exercise are governed by transcription factors, namely nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor kappa B (NF-κB) which function as the master regulators of antioxidant and inflammatory responses, respectively (citation). Nrf2 and Nf-kb are both sequestered in the cytosol by their respective inhibitory proteins, kelch-like ECH-associated protein 1 (Keap1) and inhibitor of Nf-kb (IkBa) which tag the transcription factors for ubiquitination and degradation by the proteasome (citrations). The transient increase in reactive species from exercise stimulates Nrf2 and Nf-kb by interacting with amino acid residues on Keap1 and IkBa causing the dissociation of these transcription factors from their inhibitory proteins. Once free in the cytosol, the transcription factors can translocate to the nucleus to act on their respective promoter regions.

Exercise stimulates reactive species production and subsequent activation of the antioxidant response element (ARE) via Nrf2. Increased nuclear localization of Nrf2 and binding to DNA increases the expression of various endogenously expressed antioxidant enzymes. These antioxidant enzymes include glutathione-s-transferase (GST), γ-glutamylcysteine synthetase (γ-Gcl) heme oxygenase-1 (HO1), glutathione reductase (Gr), glutathione peroxidase (Gpx), thioredoxin (Txn), and thioredoxin reductase (TxnR); all of which are important for protecting against cell damage and preventing the onset of disease (Buddapalli et al., 2012). Exercise and production of reactive species also induces transient activation of Nf-kb. Nf-kb activation is a critical element of innate immunity though chronic activation is associated with low grade chronic inflammation and various metabolic disorders. Increased antioxidant expression from repeated exercise bouts assists in the downstream regulation of Nf-Kb activity, pro-inflammatory cytokine release, and systemic inflammatory state (Suzuki et al., 2020). Increased activation of Nrf2 from a single bout of exhaustive exercise decreases pro-inflammatory cytokine release by increasing antioxidant expression (Ruhee et al., 2020). Nrf2 knockout mice also show significantly higher levels of NF-kb activity and inflammatory gene expression than their wild-type counterparts (115,116-systemic OS/inflammation paper). The molecular cross-talk between Nrf2 and Nf-kb contributes significantly to the preconditioning effects of exercise that result in adaptive changes including the increased antioxidant defenses and increased repair mechanisms seen in athletes and trained individuals. These adaptations help explain the important health benefits of exercise including better resistance to environmental stressors and improved cytoprotective responses (Vargas-Mendoza et al., 2019). In alignment with literature that investigated the role of antioxidant supplementation on muscle responses and exercise adaptations, it appears that exogenous antioxidant consumption may similarly disrupt reactive species signaling necessary for improvements in redox homeostasis (Done & Traustador, 2016). In a double blind, randomized study Morrison et al., (2015) showed that a Vitamin C and E cocktail attenuated exercise induced increases in antioxidant expression (SOD1 and SOD2) following a 4 week exercise program. Some of the other hallmark health improvements that result from exercise such as improved glucose tolerance and decreases in blood pressure may also be negatively impacted by antioxidant supplementation indicating redox mechanisms that govern these responses and the critical nature of redox signaling (Ristow et al., 2009; Wray et al., 2009).

Limitations in Exercise Redox Biology

The study of exercise redox biology has considerable value in the realms of sport performance, health, and disease prevention but is still relatively young. However, similar to shortcomings in menstrual cycle verification, various technological limitations and methodological oversights have confounded the literature and limit the translational value of many findings. Reactive species have an incredibly short half

life (<2 seconds) making them difficult to measure (citation). In addition no single reactive species or oxidized macromolecule can be used to provide a comprehensive systemic analysis. As such, any investigations that consider redox signaling and macromolecular damage must use multiple biomarkers to accurately assess the changes in redox state. Use of improper assays, specifically the TBARS assay to assess lipid peroxidation and TAC to assess antioxidant potential have been critiqued in previous reviews (87, 88, 89) and their discontinuation is recommended (Cobley et al., 2017), however their use in recent exercise redox investigations persists (91, 92, 93). Redox antioxidant enzyme activity is also frequently assayed in the plasma in exercise studies due to its ease and affordability, however, antioxidant enzyme measurements must be corrected for changes in plasma volume which is often neglected (citations). Redox biology is further confounded by the fact that exercise induces several biochemical changes that may influence the biological chemistry of certain reactive species and redox enzymes. For a comprehensive review and list of methodological recommendations regarding exercise redox biology the reader is directed elsewhere (Colby et al., 2017).

Despite these difficulties within exercise redox biology, the field presents many exciting avenues for future research to investigate the biochemistry of redox signaling in response to exercise that may help develop therapeutic targets for health and performance. To facilitate this more studies must attempt to connect the various cellular findings to performance outcomes in order to elucidate their translational potential (Margaretelis et al., 2020; Margaritelis et al. 2016a, Miller et al. 2016). Unfortunately, the study of exercise redox biology in females has been largely overlooked (citation) and redox investigations that consider the endogenous hormonal milieu either throughout the MC or when suppressed due to OCs are almost non-existent (citations). It is critical then to further our understanding of ovarian hormone fluctuations on redox homeostasis in response to various exercise conditions. It is clear from cellular and human studies that redox homeostasis and redox status can significantly impact various elements of exercise physiology. Therefore, redox investigations may provide a mechanistic overview into the influence of hormonal changes on exercise performance and adaptations.

Estrogen Regulates Redox Homeostasis

The precipitous drop of estrogen around menopause suggest estrogen has potent redox effects in multiple cell types (citation). The antioxidant effects of estrogen are at least partly responsible for its cardioprotective effect and protection against negative health outcomes like neurodegeneration and osteoporosis in premenopausal women. Use of hormone replacement therapy (HRT) in postmenopasual women often reverses these outcomes but may conversely increase the likelihood of other negative health outcomes such as stroke or breast cancer through estrogen induced oxidative stress (Kumar et al., 2010). The findings of women's health initiatives imply that estrogen induces a specific physiological redox state that regulates diverse health and performance outcomes.

Estrogen as an Antioxidant

It is generally accepted that estrogen has antioxidant properties (citation). Oxidative stress is consistently higher in postmenopausal women (citations). This suggests that the absence of estrogen is related to antioxidant status. Indeed, estrogen replacement therapy in postmenopausal women appears to restore antioxidant levels to that of premenopausal women (Bellanti et al., 2013). Estrogen deficiency as a result of menopause, ovariectomy, or various pathologies is related with heart disease, bone fractures, neurodegenration, and manifestation of depression and anxiety. How exactly estrogen helps prevent these negative health outcomes is unclear but one leading hypothesis is estrogen's role as an antioxidant.

Previous research supports the concept that oxidative stress contributes to the physiopathological effects of menopause as treatement with exogenous antioxidants has been shown effective in preventing or treating symptoms associated with menopause (Miquel et al., 2006). As such, oxidative stress continues to be researched as a likely cofactor in the pathogenesis of menopausal diseases. It is important to note that exogenous HRT, OCs, and endogenous estrogen can all function quite differently in various aspects. HRT comes in similar formulations as hormonal contraceptives: both are available as a combined estrogen and progestin formula, however hormonal contraceptives are also available as *progestin* only while HRT is available as *estrogen* only. Though these synthetic compounds are very similar in many respects, the prescribed doses of progestin within OCs are much higher than that of HRT in order to stop ovulation which may induce higher levels of oxidative stress (Cartwright et al., 2012; Hickey et al., 2006). The endogenous hormonal actions of eumenorrheic women may differ from that of exogenous hormone users (Jetta et al., 2022) and as such the translational value of HRT to MC performance research is not entirely clear. Despite this, estrogen fluctuations throughout the MC in eumenorrheic women still appear to significantly impact circulating antioxidant levels (citations). Significant cycle dependent changes were found for glutathione (GSH), the most abundant antioxidant in the body, and Gpx, the antioxidant responsible for catalyzing the conversion of GSH to its oxidized form (GSSG), in the endometrium of eumenorrheic women (Servidio et sl., 2002). Specifically, statistically significant correlations (R=.85; P=<.0001) were found between estrogen and GSH and Gpx. This study did not however find increases in oxidative stress throughout the MC. Massafra et al., (2000) found significant increases in Gpx from the late follicular to the mid luteal phase in erythrocytes of naturally menstruating women indicating that the late follicular estrogen peak may influence Gpx activity. In agreement, another study demonstrated that salivary antioxidant status is influenced by circulating estrogen and progesterone levels, however the direction of the change could not be elucidated (Alagendran et al., 2011). The observed phase specific changes in Gpx and CAT suggest an increased susceptibility to oxidative stress at different MC timepoints. Significantly higher markers of lipid peroxidation (TBARS) were observed in the urine of naturally menstruating women during the luteal phase compared to the follicular phase of the MC (Karawociz-Bilisinka et al., 2008), the period during the MC when *both* estrogen and progesterone are high. These findings suggest that progesterone's anti-oestrogenic effects extend to oxidative stress and redox homeostasis. Indeed, progesterone is reported to antagonize the vasoprotective effect of estrogen on antioxidant enzyme expression in vascular smooth muscle. In a time and concentration dependent manner, progesterone downregulated extracellular SOD (ecSOD), mitochondrial localized manganese-dependent (MnSOD), and reversed 17b-estradiol induced overexpression of these antioxidant enzymes. In addition, estrogen replacement in ovariectomized mice prevented ROS increase and oxidative stress, but addition of progesterone negated this effect (Wassman et al., 2005). Contrasting findings do exist: Browne et al., (2008) did not find any difference in antioxidant status or oxidative stress throughout the MC in naturally menstruating women, however this study acknowledged its small sample size (n=7) and large variation in subject BMI (20.9-34.7) as limitations that may mask the true effect of the MC on redox outcomes. When OS was analyzed every 3 days in eumenorrheic women, it was observed that plasma hydroperoxides began to increase around day 6 of the MC before returning to baseline around day 27 of the MC, concluding that women go into a state of OS for ⅔ of the MC (Cornelli et al., 2013). Though oxidative stress was observed in this study, the authors argued that the isolating action of estrogen is not an antioxidant but rather an oxidant. Collectively, these results indicate that in comparison to HRT and menopause, even the relatively small fluctuations in estrogen seen throughout the MC still impact systemic redox status.

Phytoestrogens are a large group of organic compounds found in a variety of plants that resemble the chemical structure of estrogen and provide additional avenues to examine the redox effects of estrogen. In support of the antioxidant actions of estrogen, the majority of phytoestrogens display potent antioxidant and anti-inflammatory properties (99,100,101). Phytoestrogens are effective in treating symptoms of menopause by their binding to estrogen receptors and direct free radical scavenging ability. Phytoestrogens also decrease the risk of osteoporosis, CVD, metabolic syndrome, and various cancers (102,103,104). In one study, the phytoestrogens genistein, daidzein and equol were all effective at scavenging superoxide, hydroxyl radical and H_2O_2 *in vitro* (Kladna et al., 2016). In another study, physiological concentrations of isoflavonoids (a class of phytoestrogens) were effective at attenuating H_2O_2 induced DNA damage in human lymphocytes. Interestingly, in this study isoflavonoids had greater antioxidant properties and were superior in protecting against DNA damage than their endogenous analog 17b-estradiol (Sierens et al., 2001). Phytoestrogen supplementation *in vivo* aso impacts redox homeostasis with concomitant exercise training (citations); in fact, phytoestrogens have been extensively researched as a potential ergogenic aid, though their role in improving exercise performance is equivocal (citation). Phytoestrogens were shown to enhance antioxidant enzyme activities, specifically glutathione dependent activities, in erythrocytes and lymphocytes following an acute swimming session in female swimmers (Mestre-alfaro et al., 2011). In a group of volunteers who underwent 4 weeks of phytoestrogen supplementation in the form of isoflavones, pre-exercise antioxidant levels were significantly increased and supplementation prevented exercise induced decreases in certain antioxidants (Chen et al., 2004). Whether or not this shift in basal redox state towards increased antioxidant capacity following phytoestrogen supplementation is ultimately beneficial is difficult to discern as it is now clear that basal redox state influences exercise induced adaptations (Ostrom & Traustadottir, 2022). This basal redox state helps determine whether or not the exercise induced oxidative stimulus is ultimately a eustress (positive) or a distress (negative). The net influence of phytoestrogen supplementation as an oxidative eustress or distress is still unclear in the context of exercise adaptations and further research is warranted in this arena. Ultimately, phytoestrogens continue to receive extensive attention in health and performance and support the potentially robust antioxidant actions of estrogen.

Estrogen as a pro-oxidant

Despite the large volume of literature that supports the antioxidant functions of estrogen, various studies have outlined the the dual nature of estrogen as both an antioxidant and prooxidant (Kumar et al., 2010; Santanam et al., 1998; Natahn & Chaudhuri, 1998; Markides et al., 1998). Recent evidence points to a central role of oxidative stress following HRT in the development of breast cancer and increased incidence of stroke (113,114). It appears that in post-menopausal women estrogen may have dual effects that protect neuronal tissue against oxidative stress and neurodgenerative disease (Behl et al., 1999) but simultaneously may promote oxidative stress in certain tissues which contribute to the pathogenesis of cancer (114). Some of the original disagreement in the literature regarding estrogen's actions may have come as a result of the type of estrogen that was analyzed. For example, catechol-estrogens are a major estrogen metabolite that have powerful effects in the brain and pituitary gland (Mackluscy et al., 1981). In one study, addition of catechol-estrogens to cell cultures expedited lipid peroxidation measured by conjugated diene formation indicating a pro-oxidant effect. However in the same study other estrogen forms including estradiol, estrone, and metho-estrogen delayed lipid peroxidation and displayed antioxidant effects (Markides et al., 1998). Similarly, another study found that estradiol at physiological concentrations did not show antioxidant properties but rather pro-oxidant properties and only found

antioxidant properties at supraphysiological levels. (Santanam et al., 1998). These findings and the findings of large retrospective studies into the influences of HRT indicate that the specific form of estrogen, estrogen concentrations, and the target tissue jointly mediate the antioxidant/pro-oxidant effects of estrogen (Kumar et al., 2010). Worth noting is the potential for estrogen to promote oxidative stress yet simultaneously be beneficial for tissue health. In the context of cardiovascular disease, estrogen can function as a pro-oxidant increasing the breakdown and oxidation of low density lipoproteins (LDL) which is beneficial to cardiac health (Chiang et al., 2004). Findings like these underscore the previously mentioned issues in redox biology terminology which may cloud interpretation of research data. Though estrogen is increasing the oxidation of LDL this action promotes cardiac health and as such it is wholly inappropriate to associate oxidation or "oxidized" with strictly negative outcomes.

Paradoxical findings underscore the complex nature of estrogen regulated redox biology and highlights several issues: (1) it is clear that estrogen levels influence systemic redox homeostasis, but whether or not estrogen increases are ultimately beneficial or detrimental is contingent on multiple variables; (2) the redox outcome measures of macromolecular damage (LDL, hydroperoxides, MDA, TBARS) and antioxidant activity (SOD, Catalase, GSH, Gpx, etc.) as well as the tissue or cell type (endometrium, plasma etc.) significantly impact interpretations of estrogen's effect; (3) a consensus has yet to be reached regarding the influence of estrogen on redox outcomes.

Potential Mechanisms for Estrogen's Redox Actions

The molecular structure of estrogen has received extensive attention for its potential role in redox homeostasis. Estrogen contains a phenolic hydroxyl group at the C3 position of the A ring (citation) that is critical for neuroprotection and scavenging of excess free radicals *in vitro* (citation). When this phenolic hydroxyl group is eliminated, estrogen provides no neuroprotection against oxidative stress. However, the majority of these *in vitro* studies utilized supraphysiological doses of estrogen far beyond the range that is seen in eumenorrheic women. This suggests that though estrogens in high concentrations can directly quench excess free radicals and confer neuroprotection, this is likely not the mechanism for estrogen protection *in vivo.* Ironically, when metabolized, the same structural phenolic hydroxyl group that scavenges free radicals actually results in the creation of further free radicals and reactive species. The phenol group undergoes various metabolic steps that result in the production of estrogen-quinones via oxidative enzymes or metal ions (citations) during which superoxide and hydrogen peroxide are produced (Yager, 2006 & Bolton, 2008). The increased production of free radicals and oxidative stress via estrogen metabolism has been extensively analyzed in the context of HRT and breast cancer (Bolton, 2008). Interestingly, recent work focused on oxidative stress in oral contraceptive (OC) users shows that women who use OC show significantly higher levels of oxidative stress than their eumenorrheic counterparts (citations). This may partly be due to the increased metabolism of exogenous estrogens and subsequent production of free radicals.

Estrogen exerts genomic effects that play a critical role in the regulation of redox homeostasis by binding to estrogen receptors (ER). ERα and ERβ reflect the two estrogen receptors typically discussed, though various others also exist including ER gamma (ERγ), novel developmentally regulated membrane associated ER (ER-X), and G protein coupled estrogen receptor (GPER). The density of ER subtypes in specific tissues, the expression of one ER type to another (ERa:ERb), and the concentration of different estrogen molecules collectively regulate the signaling actions of estrogen and the modulation of reactive species generation (citation). For example, 17β-estradiol, the most potent and prominent form of estrogen in eumenorrheic women binds equally to ERα and ERβ (citation) but ethinyl estradiol, a synthetic

estrogen used in OC pills preferentially binds to ERα (citation) and significantly increases OS levels (Chen et al., 2012). The genomic actions of estrogen are regulated by the ER-estrogen binding complex in the cytosol and nucleus (29). Once bound, the ER-ligand complex translocates to the nucleus where it can act on the estrogen response element (ERE) and upregulate or downregulate the expression of these genes. The ER-ligand complex also regulates redox homeostasis by interacting with the transcription factors Nrf2 and Nf-kb. There is some disagreement to whether estrogen activates or inhibits Nrf2 and the antioxidant response element (ARE). 17β-estradiol was shown to suppress Nrf2 by causing a ligand dependent interaction with ERα (Ansell et al., 2005) and downregulate phase II detoxification enzymes such as glutathione-s-transferase (GST) and quinone reductase (Ansell et al., 2004). Estrogen-related receptor beta (ERRβ) appears to inhibit Nrf2 by altering the subcellular localization of Nrf2 (Zhou et al., 2007). In contrast, estrogen binding to membrane-associated estrogen receptors and GPERs induced a rapid activation of Nrf2 and phase II detoxification enzymes (Ishi & Warabi, 2019). In addition, it was shown that estrogen increased ARE activity 14 fold and increased the efficacy of Nrf2 activators through estrogen receptor mediated action and that progesterone inhibited this effect (Wu et al., 2014). Many of these investigations were performed in breast cancer cells due to the established link between estrogen, oxidative stress, and breast cancer. To date, no investigations have examined the estrogen-Nrf2 relationship in skeletal muscle following an exercise stimulus. However, one review that examined the molecular mechanisms of estrogen and phytoestrogens on antioxidant genes in smooth muscle concluded that estrogen exerts a protective effect in the vasculature both *in vitro* and *in vivo* via upregulation of the transcription factors NFκB and Nrf2 (Soiw et al., 2007). The equivocal findings of various studies indicate that the ER mediated regulation of Nrf2 and the ARE is ligand, receptor subtype, and cell type specific. Further research is required to confirm an Nrf2 mediated protective effect of estrogen in skeletal muscle and elucidate how estrogens actions may mediate redox regulated exercise adaptations.

Nrf2 assists in the regulation of Nf-kb which is responsible for the expression of inflammatory genes, inflammatory cytokine release, and redox homeostatic regulatory genes (citations). Various studies indicate a molecular cross-talk between ERs and Nf-kb in which ERs repress Nf-Kb activity. (Stein & Yang, 1995, Ray et al., 1997, An et al., 1997). Treatment with 17α-ethinylestradiol blocked induction of Nf-kb and inflammatory genes *in vivo* in response to an inflammatory dietary intervention in hepatic cells and this action was mediated by the ER (Evans et al., 2001). The ER-ligand complex also inhibits inflammatory gene expression in osteoblasts by reducing transcriptional activity of Nf-kb (Stein & Yang, 1995). It was originally believed that ERs repressed Nf-kb by directly blocking Nf-kb binding to promoter regions (Stein & Yang, 1995; Ray et al., 1997), however future studies showed a more complex mechanism in which unliganded ERs displaced binding proteins and coregulators from Nf-kb thereby repressing proinflammatory genes (Cvoro et al., 2011). ER-ligand regulation of Nf-kb may directly regulate skeletal muscle mass and function via decreased release of interleukin-6 (IL-6). IL-6 is a pleiotropic cytokine with inflammatory and anti-inflammatory functions that has established effects on skeletal muscle (VanderVeen et al., 2019…***others)*** and is induced by the transcription factor Nf-kb (citation). The ER-ligand complex has the ability to repress IL-6 expression in osteoblasts (Stein & Yang, 1995…***others in other cell types).*** Since IL6 overexpression increases fatigability and decreases mitochondrial mass independent of muscle fiber type in skeletal muscle (VanderVeen et al., 2019), it stands to reason that estrogen dependent regulation of skeletal muscle mass and function may be partly due to repression of Nf-Kb and subsequent inhibition of IL-6. A full overview of the genomic actions of estrogen are beyond the scope of this review, however, suppression and/or activation of Nrf2 and Nf-kb by estrogen and ERs partly explain estrogen's role in redox homeostasis. Future studies that investigate

the role of estrogen on both transcription factors in skeletal muscle and under exercise conditions are warranted.

The non-genomic effects of estrogen also influence redox homeostasis via plasma membrane bound ERs that activate various signaling cascades (citations)...These distinctive non-genomic actions influence well established signaling pathways that may reduce oxidative stress and inflammation, and prevent apoptosis. Estrogen activates MAPK signaling cascades via binding to membrane bound ERs that phosphorylate ERK 1 and 2, subsequently increasing the expression of SOD/Gpx and decreasing oxidative stress *in vitro* (Borras et al., 2005). Though this study was performed in cultured breast cancer cells, the concentrations of estrogen used were similar to those expressed during the menstrual cycle indicating this mechanism may be seen *in vivo*. Interestingly, ROS can also activate MAPK and induce autophagy and apoptosis via this pathway (He et al., 2017). Estrogen can therefore modulate MAPK activity and redox homeostasis directly by binding to membrane bound ERs and indirectly via upregulating endogenous antioxidant production to scavenge excess ROS that lie upstream of MAPK activation. In contrast, unbound estrogen that gets metabolized is converted to ROS which can activate PI3k signaling and phosphorylate Akt/mTOR (Okoh et al., 2013). In mammary epithelial cells this process is implicated in tumorigenesis (citations), however, in skeletal muscle phosphorylation of Akt/mTOR is a desired outcome necessary for muscle hypertrophy. Further examination of this process in skeletal muscle to understand the role estrogen metabolism and ROS production have on mTOR signaling is warranted.

Estrogens can also increase the production of nitric oxide (NO) by activating endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) (Nuedling et al., 2001; Kumar et al., 2010). NO is then able to scavenge excess ROS by forming peroxynitrite ($ONOO^-$). In one study, the estrogen metabolite 17-epiestriol, upregulated NO in human endothelial cells *in vitro* and attenuated expression of pro-inflammatory and pro-oxidative molecules. This effect was observed in an ERβ dependent manner (Mukherjee et al., 2003). However, peroxynitrite is in itself a powerful free radical that may damage cellular components, increase lipid and protein oxidation markers, inhibit mitochondrial respiration, and contribute to the development of various pathologies (Wang et al., 2003; Pacher et al., 2007)**.** As such, the role of this mechanism on pro/anti oxidative actions of estrogen is equivocal. Estrogen and ERs also influence the thioredoxin (Txn) system. The Txn system plays a critical role in the cellular defense against oxidative stress, apoptosis and regulation of transcription factors including Nf-kb and activator protein-1 (AP-1) (Schenk et al., 1994; Aydin & Uyar, 2021). 17b estradiol was shown to induce neuroprotective effects against oxidative stress by activating the Txn system (Lee et al., 2003). In addition, 17b estradiol binding to nuclear ERa interacts with endogenously expressed Txn and Txn-reductase in the cytosol, mitochondria, and endoplasmic reticulum to assist in the detoxification of H_2O_2 (Rao et al., 2009). Interestingly, in this study the expression of the ERE was influenced by Txn system interactions with nuclear bound ERs. In this way, it appears that estrogen not only regulates redox homeostasis and the ARE through interactions with Nrf2 but the antioxidant status of the cell also modulates the ERE through interactions with ERs.

The influence of estrogen on mitochondrial free radical production has received extensive attention in recent years due to the association between mitochondrial oxidant signaling and various pathologies including muscle atrophy and sarcopenia (citations). Estrogen decreases mitochondrial free radical and H_2O_2 production (Borras et al., 2007; Razmara et al., 2007) in various cell types and this observation is often cited to explain the difference in longevity between males and females. Within skeletal muscle, the subcellular source of ROS production has a significant effect on redox adaptations

and phenotype (Henriquez Olguin et al., 2020). The mitochondria was thought to be the primary source of intracellular ROS production during exercise up until recently. It is now clear that mitochondrial contribution to increased ROS production during exercise is small (citation). Instead, mitochondrial induced oxidant production is greater at rest and mitochondrial sourced free radicals can activate proteolytic systems that result in sarcopenia and frailty (Gomez-Cabrera et al., 2020). The ability of estrogen to attenuate mitochondrial produced free radicals in skeletal muscle may explain the decrease in muscle mass and increases in sarcopenia seen with estrogen deficiency (citation). in post-menopausal women (citation) and warrants further investiagtion.

Alternatively, it is now believed that NADPH oxidase (NOX) is the primary source of free radicals in skeletal muscle (citation) during exercise contributing to redox mediated adaptive responses. Various studies support the idea that high levels of estrogen can increase NOX activity thereby increasing free radical production (Faria et al., 2019; Stephniak et al., 2018). Exogenous 17β-estradiol stimulated NOX expression and increased H_2O_2 production via interactions with ERa in cultured cells (Stephniak et al., 2018). The thyroids of adult female rats also contain higher levels of H_2O_2 and NOX activity than their male counterparts (Fortunato et al., 2013). In contrast, 17b estradiol but not 17a estradiol caused a decrease in NOX activity in endothelial cells (Wagner et al., 2001). Various isoforms of NOX exist and have been identified in various compartments of skeletal muscle (59), however the exact impact estrogen has on NOX sourced free radical production during exercise has yet to be investigated. Another source of free radicals during exercise is the enzyme xanthine oxidase which may be more active during resistance training (citation) and whose activity is modulated by estrogen via receptor independent mechanisms (Budhiraja et al., 2003).

In summary, the redox actions of estrogen are complex and multidimensional. Estrogen can produce ROS that function as signaling molecules to activate specific cell pathways or bind to nuclear or membrane bound ERs which can increase or decrease the expression of genes that control redox homeostasis. The net influence of estrogen is contingent on a variety of factors including the form of estrogen, the concentration of estrogen, and the target tissue. Estrogen's influence on skeletal muscle redox homeostasis is understudied and presents a potential mechanism to explain altered exercise responses and adaptations at different timepoints throughout the MC and between eumenorrheic women and those using exogenous estrogen.

Estrogen, Exercise, and Redox Homeostasis

The redox effects of estrogen indicate that under different hormonal conditions women vary in their ability to withstand oxidative insults and repair damaged cellular compartments which may lead to oxidative stress and disease. Hormonal induced changes in redox homeostasis means redox sensitive signaling pathways that govern exercise performance and adaptations may also be impacted by changes in estrogen. Very few studies have analyzed the influence of estrogen changes on redox homeostasis with concomitant exercise training. Given exercise induced reactive species help determine exercise performance (citations), the magnitude of exercise adaptations (citations) and induce cytoprotective genes that prevent disease (citations); it is critical to understand how estrogen altered redox homeostasis impacts exercise generated reactive species and redox signaling to optimize exercise prescriptions for health and performance.

Endogenous Estrogen

To date, only a handful of studies have investigated the role of MC phase on exercise induced free radical production and antioxidant status (Joo et al., 2004; Akova et al., 2001; Chung et al., 1999; other citations). Joo et al., (2004) found that 30 min at 60% $\dot{V}$O2max on a cycle ergometer induced significantly lower oxidative stress (TBARS) during the follicular phase than the luteal phase of the MC in sedentary women, concluding that when estrogen is high exercise induced free radicals may be easily scavenged. Interestingly, in this study total SOD concentrations were significantly lower in the luteal phase, suggesting a mechanism by which the antagonistic effects of progesterone may modulate endogenous antioxidant concentrations. In contrast, no change in plasma MDA or TBARS were found following 30 min of exercise at 75-80% VO2max during the follicular and luteal phases (Chung et al., 1999). The reason for this discrepancy is likely in the timing of samples taken during the follicular phase. Chung et al., (1999) did not differentiate between the EF phase, when estrogen and progesterone are low, and the LF phase when estrogen is high but progesterone is low; since resting estradiol was significantly higher during the luteal phase than follicular phase this indicates that samples were taken during the early follicular or menstruation phase. Joo et al., (2004) differentiated between the menses, follicular, and luteal phases indicating that follicular samples were taken during the late follicular phase and likely included the estrogen peak. Together, these studies suggest that during continuous moderate intensity exercise, exercise induced free radicals may be more easily eliminated during the late follicular phase than either the early follicular or luteal phases. As such, exercise tolerance and time to fatigue during moderate intensity aerobic exercise may be greater during the late follicular phase, a conclusion reported in previous investigations (De Jonge et al., 2003). This effect may be modulated by additional variables, namely BBT. Prolonged exercise capacity is reduced during the mid-luteal phase in hot conditions but not in temperate conditions, indicating that when BBT is higher during the ML phase, cardiovascular strain may be increased and exercise performance subsequently decreased (Thompson et al., 2012). Interestingly, BBT modulates the acute redox responses to exercise in men. Specifically, increased core body temperature significantly increased leukocyte and antioxidant activity following exercise to counteract increased oxidative stress from a hot environment (Mestre-Alfaro et al., 2012). Though this effect must be investigated in women, increased BBT during the ML phase of the MC may influence systemic redox state and induce a "low grade" oxidative stress. This may increase exercise induced reactive species production and contribute to the decreased exercise capacity observed during the luteal phase in prior experimental models. In line with this hypothesis leukocyte concentration after a high intensity time trial performance was significantly greater during the luteal phase than the follicular phase of the MC despite no decreases in time trial performance (Hashimoto et al., 2014). Redox responses were not included in this analysis, as such it is unclear if oxidative stress or antioxidant activities also increased in the ML phase. The same research group investigated the role of the MC on systemic IL6 following 60 minutes of cycling at 75% of anaerobic threshold (Hayashida et al., 2016) and 60 minutes of cycling at ventilatory threshold (Hayashida et al., 2015). IL6 levels increased during all MC phases following exercise but were significantly upregulated only during the menstruation (early follicular) phase of the MC (Hayashida et al., 2016). These findings support an anti-inflammatory and potentially an antioxidant action of estrogen during aerobic exercise. Estrogen is at its lowest during the early follicular phase (menstruation), at which time the proinflammatory cytokine IL6 shows the largest increase following continuous aerobic exercise. IL-6 is upregulated in response to Nf-kb which is simultaneously inhibited by estrogen and Nrf2 (citation). More comprehensive analyses are needed to confirm the molecular cross-talk between estrogen, Nrf2, and Nf-kb following aerobic exercise.

Unfortunately, very few studies have looked at redox outcomes throughout the MC following resistance or strength training in females making any comparison with aerobic exercise difficult. No change in oxidative stress (plasma MDA) was found in sedentary women throughout the MC who underwent exercise to exhaustion on an isokinetic dynamometer (Akova et al., 2001). This study did report decreases in erythrocyte SOD and Gpx concentrations after exercise in both the menstrual and preovulatory phases but found less significant decreases in endogenous antioxidants following exercise when estrogen was higher in the preovulatory phase. This indicates a potential role of estrogen in modulating antioxidant responses following exercise. In addition, Gpx showed significant positive correlations with estrogen both pre (r=0.58, $P < 0.05$) and post-exercise (r=0.73, $P < 0.001$). The actions of Gpx are of particular interest since Gpx functions as an antioxidant in the detoxification of H_2O_2 but also in a redox cycle converting GSH to GSSG. GSH has a variety of systemic functions including defense against OS, nutrient metabolism, regulation of gene expression, DNA and protein synthesis, protein modification through glutathionylation, apoptosis, signal transduction, and modulation of inflammatory and immune responses. (Wu et al., 2004). The functions of Gpx and previous investigations that show Gpx is strongly associated with estrogen fluctuations throughout the MC should prompt further research into the role of differing estrogen concentrations on GSH homeostasis and GSH regulated signaling pathways (18-21-redox bio: H202 edition). Similar to estrogen, it was previously argued that the actions of Gpx should be considered as prooxidant due to its role in catalyzing GSH to GSSG (Cornelli et al., 2013). However, due to its concomitant function in detoxifying H_2O_2 it is difficult to ascertain if increased Gpx activity should be considered as prooxidant or antioxidant activity. Gpx may ultimately serve as an important downstream marker of estrogen redox actions that help clarify a prooxidant or antioxidant effect under different conditions. Future analyses that include H_2O_2 measurements, the activity of glutathione reductase (Gr), the enzyme that catalyzes GSSG back to GSH and the Gpx:Gr ratio will help clarify this issue.

Recently, the effects of resistance exercise type (power, hypertrophy, strength) on serum oxidative stress were investigated in resistance trained females (Motamedi et al., 2020). The results from this study seemed to indicate there was no impact of resistance training type on redox outcomes H_2O_2 or MDA. Since training status influences the magnitude of redox responses it is possible no effect was seen because the training intervention did not sufficiently overload the subjects. In addition, there was no consideration of the MC or OC usage in the subject criteria which may have clouded the impact of the training interventions of redox outcomes. Another recent study investigated the relationship between cortisol, redox homeostasis, and metabolic profile in young female basketball players during in season training (Millitello et al., 2021). When compared to healthy controls, biological antioxidant potential (BAP) was significantly higher in female basketball players during in season training. Conversely, reactive species measured by reactive oxygen metabolites (d-ROM) and cortisol were significantly lowered in female athletes. Taken together, it appears that on average the subjects were receiving an adequate training dose to upregulate endogenous antioxidant defenses and not in a state of overtraining/overreaching as indicated by increased reactive species and cortisol (citations). Unfortunately, all women were enrolled randomly in respect to their MC and no consideration was given to OC usage. In a rare comparison of redox outcomes between sports in elite female athletes, the redox balance in elite female football players was compared to elite female water polo players (Arsic et al., 2015). The findings in females corroborated what is already known in male athletes, the redox status of elite athletes is significantly different than in sedentary controls and the type of sport activity impacts redox homeostasis (***citations for male athletes).*** Markers of lipid peroxidation (MDA) and antioxidant capacity (TAC) were both significantly higher in sport

participants than sedentary controls. Interestingly, reactive species production significantly differed between sports, with football players demonstrating significantly higher superoxide anion radical ($P<.001$) and H_2O_2 ($P<.05$) levels than their water polo counterparts. These findings can be explained by specific adaptive responses induced by the respective exercise types (citation). Unfortunately a big limitation of this study is the lack of hormonal data to confirm eumenorrheic status. In addition, blood samples were taken at a single arbitrary time point instead of at multiple times throughout the training year, this means that values were likely influenced by training load and no actionable training recommendations can be developed.

Rather than collecting redox information at a single time point which only provides a transient snapshot of internal metrics, longitudinal redox monitoring collects redox data at multiple time points in order to more effectively evaluate if individuals are approaching OS and subsequently increasing risk of disease or injury. This practice is gaining popularity in sport and more studies are being published that show the impact of dynamic training variables on redox homeostasis and ultimately preparedness to train, injury risk, and illness prevention (citations). However, almost all of these studies were performed in men (citations), and the few that investigated females did not consider the MC (Varramenti et al., 2013; Martinovic et al., 2011; citations). Even without considerations for the MC, elite female water polo players display altered GSH and TAC, increased OS, and increased inflammation at various time points throughout a competitive season (citation). These findings are in agreement with studies in men that show training load changes throughout a season impact redox homeostasis in various sports (***men training load redox homeostasis***). When oxidative stress was monitored for 6 weeks in elite female volleyball players without consideration for the MC, subjects showed decreased oxidative stress markers when consuming an antioxidant cocktail (vitamin E, vitamin C, zinc gluconate, and selenium) during the pre competition period. Though the antioxidant treatment effectively scavenged exercise induced free radicals, it was not investigated if this treatment did anything functionally relevant to enhance performance, improve readiness to train, or impact cellular machinery governing exercise adaptations. Furthermore, it is unclear if an antioxidant cocktail would demonstrate the same level of efficacy at different time points throughout the MC like when estrogen and endogenous antioxidant potential is greater. One research group analyzed the role of estrogen levels on antioxidant status, inflammation, and heat shock protein 70 (HSP70) throughout a year of training in competitive handball players (Weber et al., 2012). The results suggested that the redox, inflammatory, and cytoprotective adaptations that result following chronic exercise training were influenced by plasma estrogen levels. Ultimately, it is difficult to determine if the altered cellular responses between the low and high estradiol group in this study resulted in a positive or negative effect in the athletes without concomitant performance outcome measures. As such, future research must not only consider the MC in longitudinal redox monitoring but must make a concerted effort to relate the redox biomarkers and hormone levels with expected performance and adaptive responses to develop recommendations for tailoring training to the hormonal and cellular milieu.

Exogenous Estrogen: Oral Contraceptives

The use of exogenous estrogen via OC can result in significant changes in redox homeostasis that elevate several indices of oxidative stress (Kowalska & Milnerowicz, 2016). Oxidative stress was found in 93% of female athletes using OC when assessed by blood hydroperoxides and twofold higher than non-OC athlete counterparts. OC usage was also inversely related to antioxidant status (Cauci et al., 2016). In a study by the same research group, oxidative stress was again found in 77% of subjects, a significantly higher rate than their non-OC counterparts (1.6%). Significantly higher markers of inflammation were

also found in subjects using OC in the same study (Cauci et al., 2020). In line with these findings, elite female athletes using OC that competed in the Rio games had significantly higher levels of inflammation in the lead up to the Rio games than did naturally menstruating women (Larsen et al., 2020). Significantly higher resting oxidative stress and lipid peroxidation levels and significantly lower levels of glutathione peroxidase were observed in female judoists using OC than naturally menstruating female judoists (Massart et al., 2012). In agreement, longitudinal analysis of female soccer players using combined OC throughout a competitive season showed significantly higher markers of inflammation throughout a season than non-OC users despite similar training programs (Bozzini et al., 2021). It appears that oxidative stress and inflammation in OC users may also be influenced by the inactive or active pill phase. A recent study investigated the temporal changes in redox status across the MC against redox changes over a month of OC usage (Quinn et al., 2021). Oxidative stress and inflammation were both higher during the active pill phase than during the inactive pill phase and higher than eumenorrheic women during the mid-luteal phase. Altogether, the current body of evidence strongly supports that females who use OC may be at risk for increased systemic oxidative stress and inflammation which may have implications for exercise performance, adaptations, and recovery. In addition, elevated oxidative stress and low grade chronic inflammation are linked to several chronic diseases (citations), yet more research must be performed to understand the relationship between increased OS following habitual OC usage and redox related pathologies. Redox monitoring may prove helpful not only in athletic populations to monitor training load and recovery, but also in the early detection of disease.

Conclusion

Estrogen concentrations influence the redox and inflammatory cellular responses that contribute to the performance, recovery from, and adaptations to exercise. Since changes in redox and inflammatory homeostasis relate to training stress and recovery, these internal metrics may prove important outcomes for understanding the influence of ovarian hormones on exercise workloads and developing optimized prescriptions for health and performance outcomes.

In lieu of current evidence that estrogen regulates redox homeostasis and that quintessential exercise responses and adaptations are redox regulated, a strong rationale exists for investigating the redox consequences of acute and chronic exercise in women. Unfortunately, the vast majority of studies in exercise redox biology have been performed in men (citations) and of the few performed in women, many used inappropriate assays to measure redox status (TBARS/TAC) or did not consider the varying hormonal milieu. As such, it is not clear how the ovarian hormones may influence production of reactive species that impact exercise performance and govern magnitude of cytoprotective responses; or how the endogenous antioxidant potential influences the signaling responsible for archetypal adaptations to aerobic or resistance training.

Since it is known that estrogen's effects on redox homeostasis are receptor subtype, estrogen type, estrogen concentration, and cell type specific, all of these variables must be considered. These variables also support specific considerations for women who are eumenorrheic, amenorrheic, premenopausal, postmenopausal, and taking OCs. Future studies must utilize valid methodological strategies that confirm endogenous ovarian hormone concentrations and consider the potential effects of various exogenous hormonal contraceptives. In addition, redox responses to exercise are multifactorial and influenced by training variables (intensity, volume, duration, frequency etc.), individual training status, diet, and genetics. As such, exercise redox investigations in women must appropriately account for all these variables. Equivocal findings surrounding the MC and increasing evidence that OC use increases systemic

oxidative stress and inflammation necessitate more mechanistic investigations into the relationship between, exercise, ovarian hormones, and redox homeostasis. These insights could help tailor exercise and training programs to the ovarian hormonal changes to maximize the positive health and performance consequences of exercise.

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