The Effects of Inorganic Nitrate Supplementation on Exercise Performance and Vascular Health in Females

A Dissertation

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by

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Abstract

Nitric oxide (NO) is a lipid-soluble molecule found in various organ systems that assists in regulating blood pressure, vascular function, and exercise performance. Inorganic nitrate (NO_3^-) supplementation has been touted as both an exercise ergogenic as well as a clinical therapeutic, as NO_3^- reduces to NO via the NO_3^--NO_2^--NO pathway. While NO_3^- has shown to improve exercise economy, time to exhaustion, and muscular power, these studies have predominately only studied healthy young males. Females are largely understudied, perhaps because of the intricacies of the hormonal fluctuations that occur throughout the menstrual cycle. Of these hormones, estrogen fluctuates cyclically in young healthy females ranging from lower levels during the early follicular (EF) phase of the menstrual cycle and peaking typically during the late follicular (LF) phase. This is important as estrogen is known to directly improve NO bioavailability, yet despite this, the interactions of NO_3^- supplementation with estrogen fluctuations in young healthy females has not been understudied. Further, as females undergo menopause, estrogen is largely reduced, as is the NO bioavailability afforded by estrogen. This results in a loss of vascular protection in post-menopausal (PM) females and an exacerbated cardiovascular disease (CVD) risk. While exercise training is known to improve vascular function, this does not occur in PM females, potentially due to a loss of bioavailable NO that comes with the loss of estrogen. Improving bioavailable NO either by increasing exercise intensity or by supplementing with NO_3^- supplementation may provide interventional options for PM females to prevent the elevated CVD risk.

Thus, the purpose of manuscript 1 of this dissertation was to determine the effects of NO_3^- supplementation on aerobic exercise economy and endurance capacity across follicular phases of the menstrual cycle. The results of this placebo-controlled randomized control trial (RCT) revealed that NO_3^- supplementation (BRJ) elevated plasma nitrite and nitrate, but plasma nitrate was higher in the LF phase of the menstrual cycle (MC). Exercise economy was unaltered by BRJ or the MC, however exercise endurance was significantly worsened by 40 seconds (~9%) after BRJ supplementation ($p = 0.04$) but was not different across the MC with no interaction effects.

The purpose of manuscript 2 of this dissertation was to determine the effects of NO_3^- supplementation on isokinetic peak muscular power, maximal voluntary isometric contraction (MVIC) force, muscular endurance, and recovery from fatigue across the follicular phases of the menstrual cycle. Isokinetic peak power was worsened by NO_3^- supplementation ($p = 0.02$), with maximal knee extensor power (Pmax) to being significantly worsened in the LF+BRJ condition compared to the EF+PL condition ($p = 0.04$). Maximal knee extensor velocity (Vmax) was also worsened by NO_3^- supplementation ($p = 0.03$). Muscular endurance, MVIC, and fatigue recovery was unaltered by BRJ or the MC (all $p > 0.05$).

Finally, manuscript 3 of this dissertation was to determine the effects of NO_3^- supplementation and different intensities of exercise on acute changes to vascular health in PM females. BRJ + high intensity exercise (HIE) improved Peak Δ Flow-Mediated Dilation (FMD) compared to all control (CON) conditions ($p < 0.05$), while BRJ + moderate intensity exercise (MIE) improved Peak Δ FMD compared only to BRJ+CON. Neither PL+HIE nor PL+MIE improved Peak Δ FMD compared to PL+CON ($p > 0.05$). Plasma NO_2^- was positively correlated with Peak Δ FMD, while body fat percentage with inversely correlated. Exercise prevented the increase in MAP and PWV over time independent of treatment.

These dissertation data taken together suggest a potential hormetic relationship with estrogen and NO in which NO_3^- supplementation may harm exercise performance in young healthy females who already have sufficient NO, whereby NO_3^- supplementation may be further elevating NO to an extent where it is no longer beneficial. Contrarily, in PM females where endogenous NO is lower due to the loss of estrogen, NO_3^- supplementation and higher intensity of exercise may bring NO bioavailability back into a healthy range, whereby NO_3^- supplementation is beneficial. These data have revealed a fascinating relationship between estrogen and its relationship on both endogenous and exogenous NO that had never been explored previously. Understanding these relationships allows for a precision approach such that NO_3^- supplementation can be implemented in the context of underlying low endogenous NO bioavailability. Hopefully, these data will provoke further study in this area and will invoke curiosity towards studying these relationships in a nuanced manner.

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Background

Introduction

In 1998, Furchgott, Ignarro, and Murad were awarded the Nobel Prize in Physiology or Medicine for their discoveries in identifying and uncovering the role of nitric oxide (NO) on the cardiovascular system[1–3]. Subsequently, research related to NO has grown exponentially and its role in mammalian physiology has become increasingly understood. NO, once known as endothelium-derived hyperpolarizing factor (EDHF), is a systemic gaseous molecule that can be created via endogenous, oxygen-dependent, enzymatic means such as through the three NO synthases (NOSs). NO has several effects on the cardiovascular system including acute vasodilation and reducing vascular smooth muscle cell (VSMC) proliferation and platelet aggregation[4,5]. Thus, NO decreases the risk of atherosclerosis and offers a protection to many cardiovascular diseases (CVD), which are the leading causes of mortality[6]. NO is generated from the precursor L-arginine, in the presence of essential cofactors such as tetrahydrobiopterin (BH_4) and oxygen, via enzymes such as endothelial NOS (eNOS)[7]. If oxygen and BH_4 are not available, eNOS can become "uncoupled", preventing the creation of NO, and creating harmful free radicals and reactive oxygen species (ROS) such as superoxide (O_2^-). NO bioavailability is in constant flux, and numerous diseases elevate ROS and are associated with decreased NO bioavailability, thus worsening CVD-risk[8]. Importantly, there are modifiable lifestyle factors known to improve NO bioavailability and combat the vascular dysfunction associated with these comorbidities. Factors such as aerobic exercise training and eating a diet high in fruits and vegetables increases NO bioavailability and benefits the cardiovascular system[9]. This occurs in part by increasing antioxidant bioavailability which can "quench" oxidative stress, thus decreasing eNOS uncoupling associated with ROS[10–12]. Aerobic exercise offers an additive benefit to NO beyond improvements in antioxidant capacity, as exercise directly increases eNOS expression and transcription[13–17] and induces increases in arterial shear stress which directly mediate endothelial adaptation[18]. Interestingly, improving NO bioavailability benefits exercise performance (as summarized later). As such, exercise training benefits NO bioavailability, and improved NO bioavailability benefits exercise performance. As exercise training offers not only improvements in exercise performance but also amelioration of CVD-risk[19], the relationship between NO and exercise is of key interest. Notably, improving NO bioavailability has shown to improve both aerobic and resistance training aspects of exercise, and recently exogenous non-enzymatic methods of increasing NO bioavailability have been explored.

Inorganic Nitrate

Inorganic nitrate (NO_3^-) supplementation offers an exogenous approach to increase NO bioavailability in a manner that is complimentary to the oxygen dependent eNOS pathway. NO_3^- is

naturally found in high concentrations in green leafy vegetables and roots such as spinach, kale, and beetroot[20]. When NO_3^- is consumed orally, it is swallowed and absorbed into the circulation. Although a majority is excreted by the kidneys, some is sequestered into the salivary glands and secreted for an extended period into the oral cavity where it is reduced via entero-salivary commensal bacteria in the mouth and gut to nitrite (NO_2^-)[21,22]. NO_2^- is then absorbed into the blood, where under conditions of low oxygen, increased H^+, deoxygenated hemoglobin, or in the presence of enzymes such as xanthine oxidoreductase (XOR), NO_2^- is further reduced to NO (Figure 1, left side)[1]. Exercise is known to induce a more hypoxic and acidic environment in the exercising muscle, especially in fast-twitch fibers during high-intensity exercise[23]. Thus, NO_3^- seems primed to impact NO bioavailability during high intensity exercise. This has been confirmed by numerous (though not all) studies showing a beneficial impact of oral NO_3^- on exercise performance[24].

The first evidence that NO_3^- would benefit exercise was in 2007 when Larsen and colleagues showed that for a fixed sub-maximal workload, there was a reduction in submaximal oxygen (VO_2) consumption after NO_3^- supplementation, a result that has since been replicated[25–27]. NO_3^- supplementation has since been shown to improve other aspects of exercise such as aerobic endurance capacity, as well muscular power output[24,28,29]. Additionally, as NO_3^- improves NO bioavailability, supplementation has revealed potent effects on measures of vascular health such as blood pressure, flow-mediated dilation (FMD), and measures of arterial stiffness such as pulse wave velocity (PWV)[30,31]. This has led to a large commercial market for supplements that may increase NO bioavailability including beetroot juice (BRJ).

Despite the increasing popularity of NO_3^- supplementation as a potential ergogenic aid, a severe disparity in the literature exists regarding the effects of NO_3^- in the females. A recent meta-analysis reported that only 5% of all studies were exclusively studying females, with a further 15% having included females and males. Very few studies are adequately powered to compare the effects of inorganic nitrate supplementation between-sex comparisons[24]. This is important as males and females exhibit known sex differences to both acute and chronic exercise due to differences in their hormonal milieu, skeletal muscle fiber phenotypes, and circulatory factors such as differences in cardiac output and blood volume[32]. A key physiological difference is that females demonstrate fluctuations in the hormone estrogen throughout both the menstrual cycle and the lifespan. Estrogen fluctuations occur cyclically throughout each month until menopause occurs, a period represented by estrogen deficiency. Estrogen is a key component relating to sex as a biological factor, and plays a role in both exercise performance[33] and vascular health[34], and thus merits further exploration alongside NO_3^- supplementation.

Estrogen and NO

Estrogens in females are released primarily by the ovaries and, in health and in the absence of pregnancy, fluctuate cyclically across the menstrual cycle until women reach the menopausal transition in middle-age (around age 50), at which point the ovaries no longer produce estrogen at significant concentrations. Of the estrogens that exist in humans, estradiol is the most abundant and potent estrogen in humans, and its vascular effects are exerted by the binding to estrogen receptors (ERs) present on vascular endothelial[35] and smooth muscle cells (VSCMs)[36], as well as in the heart[37]. Estrogen impacts the vasculature in numerous ways including alterations to inflammation, vasoconstrictors, ion channel alterations, kidney function, sympathetic activity, and genetic and molecular alterations[38]. Importantly, estrogen increases endogenous NO bioavailability[39] by preserving eNOS via complex interactions that both upregulate, as well as spare, bioavailable NO[38,40–42]. As such, the effect of estrogen on NO bioavailability is of key interest as it relates to NO_3^- supplementation. Estrogen improves NO bioavailability via a myriad of mechanisms. Estradiol (E2) circulates in the blood after release from the ovaries and binds to ERs on both the endothelium and VSMCs, after which NO bioavailability can be increased by two separate major pathways: i.) Genomic pathway; ii.) Non-genomic pathways[38]. The genomic pathway involves estrogen binding to the ER, and the ER to a specific DNA sequence known as estrogen response elements (ERE), eliciting expression of various estrogen-responsive genes and protein expression. Namely, ER activates eNOS mRNA and protein expression[43], and thus NO bioavailability can be directly increased by estrogen and estrogen binding to ERs. The non-genomic pathways involve estrogen binding to ERs, resulting in increased enzymatic activity and activation of alternative pathways, all of which upregulate eNOS[44–48]. A summary of this is presented in Figure 1 (right). Thus, estrogen increases NO bioavailability by several pathways, all of which require mediation via the presence of ERs. Importantly, ERs are dramatically diminished post-menopause[49,50], and thus a major pathway for increasing NO bioavailability is entirely ceased, warranting need for other pathways for enhancing NO.

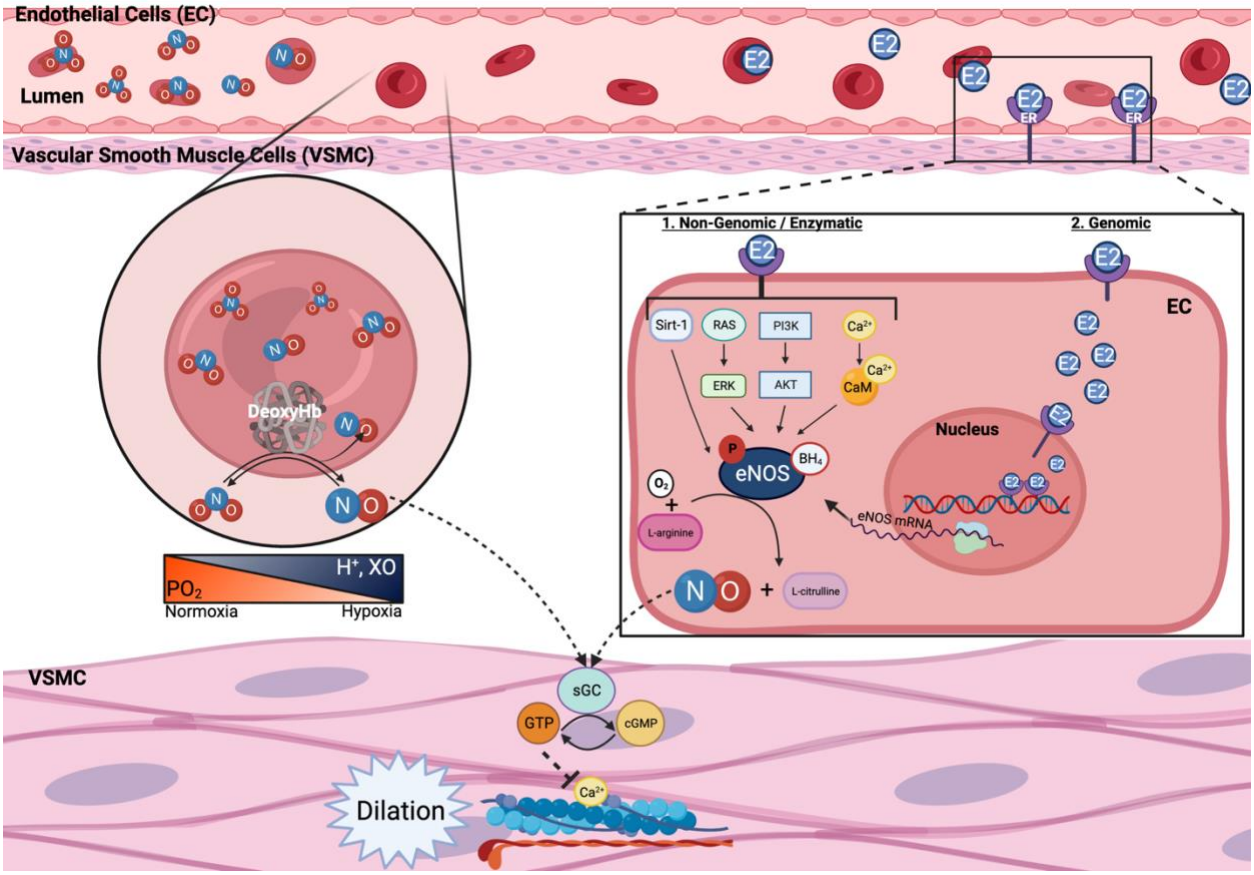

Figure 1: The effects of NO_3^- supplementation (left) and estrogen (right) on NO bioavailability.

Left: NO_3^-, NO_2^-, and NO circulate in the blood, whereby in situations of hypoxia or in the presence of elevated H^+ or enzymes such as xanthine oxidoreductase (XO), NO_2^- can convert to NO via deoxygenated-hemoglobin (DeoxyHb). NO can then diffuse into the VSMC to cause vasodilation. Right: Estrogen (E2) circulates in the blood and can bind to E2 receptors (ER) on the endothelial cells (EC). E2 can then increase NO bioavailability by i.) a non-genomic pathway and ii.) a genomic pathway. The non-genomic pathway involves upregulation of enzymes involved in Sirt-1, RAS-ERK, PI3K-AKT, and calcium calmodulin (CaM) pathways, all of which can increase eNOS activity to synthesize NO. The genomic pathway involves E2 binding to ERs on the nucleus before entering the nucleus and binding to estrogen response elements (EREs), directly increasing eNOS mRNA and protein expression. NO from estrogen can then diffuse out of the ECs and into the VSMC to cause vasodilation.

Estrogen and the Menstrual Cycle

While post-menopausal females are at a high-risk for CVD development at least in part due to the exponential drop in endogenous estrogen production and loss of ERs (detailed later), pre-menopausal women also experience fluctuations in estrogen that occur monthly. Menstruation in a typical eumenorrheic cycle can be divided into phases that represent a different hormonal milieu with fluctuations in estrogen, progesterone, luteinizing hormone, and follicle stimulating hormone (FSH). While hormones other than estrogen play an important role in female reproduction, less emphasis on these

hormones has been studied as estrogen appears to be the primary hormonal driver of potential alterations to vascular function[51–53] and exercise[33,54–56]. Briefly, at the start of menses (typically deemed "day 1" and indicated often as the start of the "early follicular phase" - EF), there is relatively low circulating estrogen, which slowly rises until a peak around days ~10-14 (in the "late follicular phase" - LF) after which the follicular phase ends, ovulation occurs, and the luteal phase begins (see Figure 2). At this point, estrogen tends to fluctuate more unpredictably than in the follicular phase, but generally remains elevated but with simultaneous elevation of progesterone, a hormone known to counteract some of the beneficial impacts of estrogen[57]. Because of this, most research in females has focused on recruiting participants for observation during the EF phase only, or during both the EF and LF phases to explore the impact of estrogen fluctuations alone. This allows for exploration of any impact of cyclic estrogen fluctuations on factors such as exercise and vascular health.

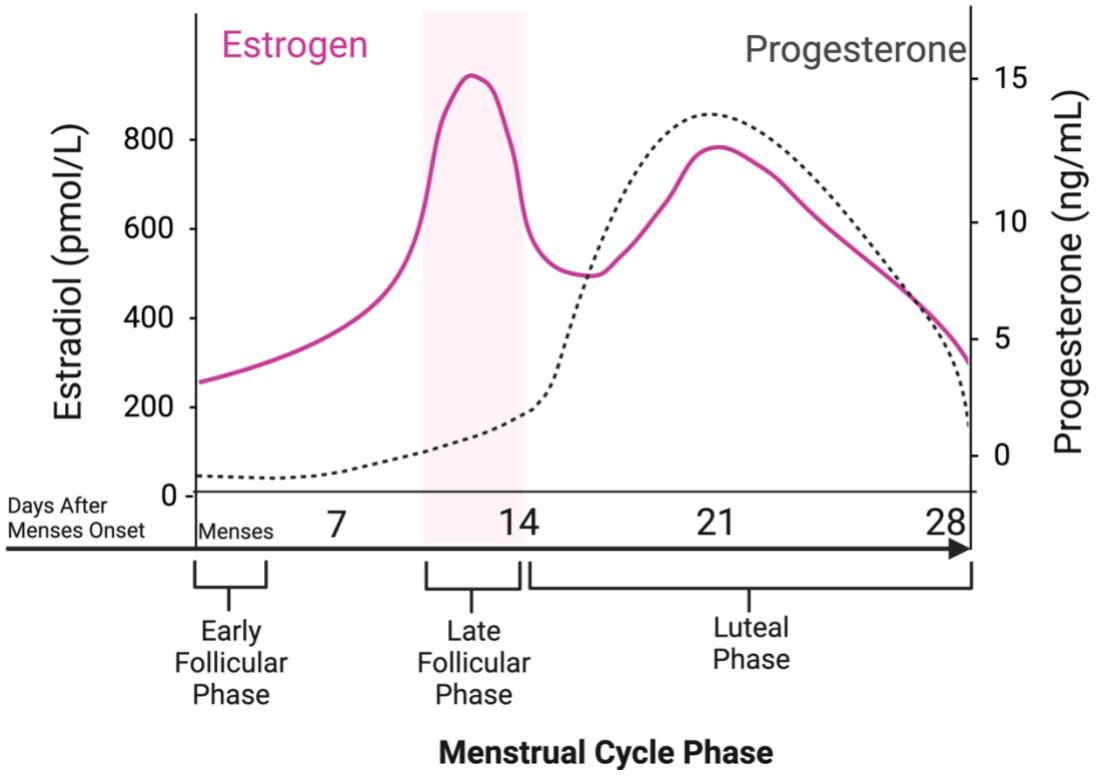

Figure 2: The ovarian cycle in a normally menstruating, eumenorrheic, pre-menopausal female

Estrogen and Menopause

As estrogen fluctuations occur cyclically in pre-menopausal years across the menstrual cycle, this can only occur a finite number of times in a females' lifespan as ovarian follicles are lost with each ovulation occurance. Typically, around age 45 the ovarian follicles are depleted to the extent that estrogen

production from the ovaries is severely diminished. This results in a feedback loop in which the hypothalamus, via GnRH signaling, and pituitary gland releases excessive FSH in an attempt to raise the decreasing estrogen release. Eventually, due to lack of follicles, estrogen will no longer be released. At this point, females will have either an inconsistent (peri-menopause) or a complete lack of a menstrual cycle (menopause).

As estrogen is diminished, so are estrogen's cardio-protective properties and thus CVD risk rises rapidly[6,58]. As such, post-menopausal (PM) females have impaired FMD[59–61] and worsened arterial stiffness[62] compared to age-matched pre-menopausal females. FMD also worsens at nearly double the rate in PM females compared to age-matched males[63]. Importantly, this worsened FMD in PM can be attenuated by chronic replacement of estradiol[60,64] further emphasizing the benefits of estrogens on vascular health. Endothelium-dependent vasodilation[65,66] and coronary artery atherosclerosis[67] are also improved by exogenous estrogen in PM females. Estrogen replacement likely improves vascular health in PM females in a manner coinciding with endogenous NO pathways, as amelioration of oxidative stress via acute antioxidant vitamin C[60] and acute increases in BH_4[68] both improve FMD similar to estrogen in PM females. Importantly, the combination of antioxidants with estrogen replacement did not result in additive improvements, suggesting a similar pathway between the two. Because the presence of estrogen receptors (which are lost in menopause due to a lack of binding activity) have shown to correlate highly with eNOS expression and phosphorylation[50], it is likely that the loss of estrogen leads to the loss of eNOS. This is further supported by studies in which estrogen therapy improves FMD less in those with a longer time-since-menopause (and thus less estrogen receptors and lower expression of eNOS)[69]. As such, it appears that the loss of vascular health in PM females may be mediated by endogenous NO pathway downregulation.

Interestingly, despite aerobic exercise training typically improving FMD in older adults, exercise training following recommendations *at moderate intensity* does not seem to improve FMD in PM females unless administered with concomitant estrogen[64,70] or unless baseline FMD is severely blunted[71]. This suggests estrogen-dependent (or perhaps NO-dependent) adaptations to moderate-intensity exercise training exist for improving vascular health and CVD-risk in PM. Because estrogen therapy brings potential secondary health risks[72], alternatives to increase NO bioavailability and perhaps rescue the exercise-induced benefits to vascular health in PM females are paramount. There are limited pharmacological or lifestyle interventions that improve the vascular health responses in PM females without simultaneously potentially causing other health risks. Thus, implementation of other strategies to increase NO bioavailability and cardiovascular health should be explored, one of which is NO_3^- supplementation.

NO_3^- and Exercise in Pre-Menopausal Females

While NO_3^- research has begun to include young adult females, only a few studies have controlled for menstrual cycle (MC) phase[73–76], whereas the majority have not[77–82]. A meta-analysis concluded that MC phase may alter exercise performance[33], although to a small degree. As such, some authors have used this evidence as reason to *not* control for MC phase when studying females. This approach may result in confounding our understanding of female physiology. It is possible that some females experience differences in exercise via alterations in estrogen and NO, while others may not. This topic has fueled a Point:Counterpoint debate in the literature recently[83] further emphasizes the importance of addressing these potential disparities in knowledge. Ultimately, based on existing data indicating differences in exercise outcomes across the MC, controlling for the MC when studying females is important in understanding potential hormonal interactions with exogenous supplements, such as NO_3^-.

To our knowledge, there are no studies to date which explore the impact of NO_3^- supplementation across the MC on any exercise outcomes, and studies observing the effects of NO_3^- in females in general is very limited. The majority of data thus far suggests that NO_3^- may be less effective at improving exercise capacity in healthy females compared to males[84], with largely equivocal results.

i) Submaximal VO_2 in males is lowered after inorganic nitrate supplementation, indicating improved exercise economy[25,27]. Similar results have been reported in females in some[85,86], but not all[87] studies. However, studies in this area have either not controlled for the MC[86], tested females only during the luteal phase[73], or tested females on hormonal contraceptives with no mention of testing on placebo or active pill phase[87]. As such, whether NO_3^- improves exercise economy differently across the MC has yet to be explored.

ii) Exercise performance as measured by endurance capacity and time trial performance is improved after NO_3^- supplementation[24]. However, studies testing the impact of NO_3^- on time trial performance in females are equivocal[79,87–89]. One study reported BRJ improved time trial performance in females performing a 500 meter kayaking event (n = 5)[89]. In contrast, cycling time trial performance was not improved regardless of BRJ supplementation, or BRJ combinations with other ergogenics such as carbohydrates or caffeine[79,87,88]. Overall, due to this widespread lack of consistent control of the MC, it is unknown whether this may impact the ability of NO_3^- to improve endurance in females.

iii) Measures of maximal power have important implications in both exercise performance and clinical factors such as the ability to perform activities of daily living. NO plays a role in muscular contraction[90,91], and thus the impact of NO_3^- on these outcomes warrants exploration. A recent meta-analysis concluded that both acute and chronic NO_3^- supplementation increases maximal power by ~5%, with no differences in sex on these

outcomes[29]. NO_3^- also improves isokinetic peak power, especially in knee extensor muscles when performed at higher angular velocities (i.e., 360◦/s and at an estimated max power)[77,92–94]. This is perhaps due to the fast-twitch fiber nature (and thus relative hypoxic environment of the muscle) of such contractions. As previously mentioned, this would facilitate conversion of NO_2^- to NO. Despite these benefits, there still lies a paucity of data in females, as only one study in this area has explored sex differences[77]. Interestingly, in this study, Coggan et al. studied a heterogeneous group of older adults (47 $\pm$ 20 yrs) and showed that acute BRJ improved maximal power 4.4% in pooled data, with females trending towards greater improvements (p = 0.079). However, the authors note that this studied was not powered to determine sex differences. Thus, while it appears that NO_3^- may improve factors contributing to maximal power and torque, the data in females is insufficient as it stands, and the menstrual cycle has not been considered in these studies.

Ultimately, the data examining the effects of NO_3^- supplementation on exercise are mostly in males, with the few studies in females showing inconsistencies in study design, equivocal findings, and an overall lack of observation into any impacts that estrogen fluctuations throughout the MC may play on these outcomes. This represents a large gap in the literature and has important implications on whether or not females would benefit from NO_3^- supplementation.

NO_3^- and Exercise in Post-Menopausal Females

While the disparity in sex research is ubiquitous, the known interaction that estrogen has with NO bioavailability paired with the importance of NO for both exercise and vascular health makes this an important and novel path for exploration. Whether NO_3^- can benefit the effects of exercise on vascular health in a manner that is similar to (but potentially safer than) estrogen therapy, offers a potential therapeutic avenue for exercise combined with NO_3^-.

Exercise training improves central and peripheral vascular health in healthy, diseased, and aging individuals via a combination of factors including arterial remodeling, increases in capillary density, mitochondrial biogenesis, and enhancement of NO bioavailability[95–99]. This ultimately contributes to exercise-mediated improvements in mortality[100] and CVD risk[101]. Despite this, because the exercise-induced benefits in FMD are lost PM (unless estrogen is administered), alternative therapies are needed for this at-risk population. Importantly, a meta-analysis of 7 randomized controlled trials concluded that NO_3^- improves FMD by ~0.62%[31]. While NO_3^- improves NO bioavailability, FMD is also potentially enhanced by improving both oxidative stress and inflammation[102–107], and thus NO_3^- represents an affordable, feasible, long-term method to possibly improve FMD and subsequently CVD-risk in PM

females. Additionally, the data that has revealed a lack of beneficial impact of exercise training on FMD in PM females have only explored exercise at moderate intensities[70]. As exercise recommendations suggest 150 min/week of moderate intensity exercise, or 75 min/week of high-intensity exercise[108], it appears that at least the moderate intensity interventions may be not sufficient for improving vascular health in PM females. However, it is possible that high-intensity exercise may offer a greater stimulus for shear-induced vasodilation[18,109] in PM females. This may result in greater NO bioavailability and vascular health adaptations. Further, as NO_2^- reduces to NO to a greater extent in situations of hypoxia[110], it's possible that high-intensity exercise (which uses more fast-twitch muscle fibers shown to benefit more from NO_3^-[111]) paired with NO_3^- supplementation may result in additive benefits to vascular health.

Conclusion

While NO_3^- supplementation appears to offer potentially ergogenic and therapeutic benefits to both exercise and vascular health, this is largely unstudied in females. Any potential interaction that NO_3^- supplementation may have with estrogen either across the menstrual cycle, or with estrogen-depletion in post-menopause is unknown. As NO_3^- supplementation has increased dramatically in recreational and competitive athletes, any differential impact that NO_3^- may have in females must be elucidated to give evidence-based guidance towards supplementation and to maximize the risk to benefit ratio. Finally, the current recommendations to perform moderate intensity exercise for improvements in cardiovascular health appear inadequate in PM females. Thus, exploration of whether high-intensity exercise, addition of NO_3^- supplementation, or perhaps a combination of the two can improve the exercise-mediated responses to vascular health in PM females are novel. These findings may have important implications and have the potential to change the current recommendations for exercise in this at-risk population.

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Manuscript 1:

The Effects of Inorganic Nitrate Supplementation on Exercise Economy and Endurance Capacity Across the Menstrual Cycle

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Abstract:

Oral inorganic nitrate (NO_3^-) supplementation has been shown to increase bioavailable NO and provide potential ergogenic benefits in males, however data in females is scarce. Estrogen is known increase endogenous NO bioavailability and to fluctuate throughout the menstrual cycle (MC) being lowest in the early follicular phase (EF) and highest during the late follicular phase (LF). This study examined the effects of oral NO_3^- supplementation on exercise economy, endurance capacity, and vascular health in young females across the MC. Twelve females with eumenorrheic MC were tested in a double-blinded, randomized design. Participants consumed ~13mmol NO_3^-, in the form of 140ml beetroot juice (BRJ) or an identical NO_3^--depleted placebo (PL) for ~3 days prior to lab visits and 2 hours prior to testing on lab visits. Plasma nitrate, nitrite, and estradiol were assessed, as was blood pressure and pulse wave velocity. Moderate intensity exercise economy and severe intensity time to exhaustion (TTE) were tested on a cycle ergometer. As expected, plasma estradiol was elevated in the LF phase, and plasma nitrite and nitrate were elevated in the BRJ condition. Exercise economy was unaltered by BRJ or the MC, however TTE was significantly worsened by 40 seconds (~9%) after BRJ supplementation ($p = 0.04$) but was not different across the MC with no interaction effects. In conclusion, NO_3^- supplementation did not affect exercise economy or vascular health and worsened aerobic endurance capacity (TTE), suggesting healthy females should proceed with caution when considering supplementation with BRJ.

New and Noteworthy:

While inorganic nitrate (NO_3^-) supplementation has increased in popularity as a means of improving exercise performance, data in females at different phases of the menstrual cycle is lacking despite known interactions of estrogen with NO. This study revealed neither NO_3^- supplementation nor the menstrual cycle influenced exercise economy or vascular health in healthy young eumenorrheic females, while NO_3^- supplementation significantly worsened endurance capacity (9%) independent of the menstrual cycle.

1. Introduction:

Nitric oxide (NO) is a pluripotent lipid-soluble molecule with a myriad of physiological effects (1). NO is produced endogenously in the presence of oxygen by the conversion of L-arginine via nitric oxide synthases (NOS) (2, 3). NO bioavailability can also be exogenously increased via entero-salivary reduction of oral inorganic nitrate (NO_3^-) to nitrite (NO_2-), with subsequent reduction to NO (4). This reaction is favored in conditions of low pH and reduced tissue pO_2, such as exercise (5, 6). Increased NO bioavailability is purported to improve exercise performance via increased tissue blood flow, mitochondrial function, skeletal muscle calcium handling, and a reduction in the ATP cost of work (7–10). This has led to the widespread use of oral NO_3^- supplementation as an ergogenic aid. However, the impact on oral NO_3^- supplementation on exercise outcomes is equivocal, with results differing between samples of clinical patients compared to healthy participants (11) with the vast majority of data reported in males.

Despite initiatives by the National Institutes of Health to improve the inclusion of females in research, large sex disparities persist in studies examining the ergogenic effect of nitrate supplementation. A recent a meta-analysis indicated that only ~5% of trials studied female participants, while a further ~15% contained mixed sex groups and generally did not examine potential sex differences (11). The lack of female representation in these studies may be related to the difficulty in controlling for estradiol which is altered during the menstrual cycle. However, estrogen is known to increase bioavailable NO and could impact exercise outcomes (13).

Most studies recruiting female participants have either not controlled for the menstrual cycle or have opted to recruit exclusively during the early follicular (EF) phase when estrogen levels are at a nadir and are most similar to males (14, 15). This approach ignores a large component of female physiology and therefore, may be too restrictive for practical recommendations (16–18). During the late follicular phase of the menstrual cycle, when estradiol is elevated, there is increased endothelial NOS expression via multiple physiological pathways which can directly increase NO bioavailability (19–21). As such in a background of increased endogenous NO bioavailability during LF, supplementation via oral NO_3^- may be less beneficial as an ergogenic aid than during EF.

Thus, the purpose of the present study was to examine the effect of oral NO_3^- supplementation on exercise economy and endurance capacity in healthy young females across the menstrual cycle. The primary hypothesis was that exercise economy and exercise capacity would be a) greater during the low estrogen EF phase when consuming the high NO_3^- beverage (EF+BRJ) when compared to EF+PL and LF+BRJ, and b) not impacted during the LF phase where consuming the high NO_3- beverage may have limited impact due to the presence of elevated estrogen on endogenous NO.

2. Methods:

2.1. *Experimental Design and Protocol*

This study was a randomized, double-blind cross-over design (Figure 1, NCT04588740). Participants reported to the laboratory for initial screening and baseline exercise testing prior to randomization to either inorganic NO_3^- supplementation in the form of beet root juice (BRJ) or placebo (PL) (See description under *Supplementation procedures*). Participants were tested during both the EF (1 – 3 days since menses onset) and LF phase (11 – 14 days since menses onset) of the menstrual cycle, as determined by self-report and with later confirmation by plasma estradiol measurements. All study visits were performed by the same researcher, at the same time of day (± 1 hour), and under the same sensory conditions. Females were tested on two consecutive months allowing for a washout period between supplemental time-frames. Participants were recruited from the University of Virginia and surrounding Charlottesville, Virginia area. All procedures were approved by the Institutional Review Board at the University of Virginia and all participants provided written informed consent.

2.2. *Subjects*

Subjects were included if they exercised ≤ 3 days/week for <u>less than</u> 30 minutes, were apparently healthy and not aerobically trained (i.e., VO_{2peak} ≤ 45 mL/min/kg), normotensive (<120/80 mmHg), and had no orthopedic limitations to exercise testing. Subjects must have had regular menstrual periods (minimum of 10-12 menses per year) and could not be on any type of contraception with the exception of a monophasic oral contraceptive pill consistently for at least 6 months prior to the study.

Subjects were excluded if they had any known pulmonary, cardiovascular, or metabolic conditions or diseases, any food allergies, if they were taking any medications or supplements that could impact study outcomes in the last 6 months (i.e., pre-workout supplements, L-citrulline, L-arginine, etc.), if they used tobacco, had abnormal blood pressure, were currently or recently pregnant or lactating (< 1 year), or if they contracted Covid-19 during the experimental period.

2.3. *Screening visit*

During the screening visit prior to exercise testing, anthropometric measurements and resting blood pressures were obtained for all subjects. These measurements were followed by an incremental exercise test on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). The exercise test consisted of a 2-minute warm up at an initial power output of 20 watts followed by 25-watt increases every 2 minutes until volitional exhaustion. Subjects were instructed to maintain a pedaling rate around 80±5 rpm. During all exercise bouts metabolic measures were obtained via standard open circuit spirometry (Vmax Encore metabolic cart, Carefusion, Yorba Linda, CA), heart rate (HR) was constantly

monitored with a HR chest strap linked to a Polar A300 watch, and rating of perceived exertion (RPE) (22) was collected. VO_{2peak} was calculated as the highest 30 second average attained. Gas exchange threshold was determined by the V-Slope method (23). The power output associated with 75% of the GET (moderate intensity) and the power output corresponding the 70% of the difference between GET and peak power output (70% Δ - severe intensity) were calculated for subsequent exercise testing.

2.4. *Supplementation Procedures*

Subjects were assigned to consume 70mL of beet root juice (BRJ - ~6.5mmol NO_3^-) twice/day (~13mmol total NO_3^-) for ~3 days or an identical NO_3^--depleted placebo (PL). On testing days, subjects were instructed to ingest the last two 70mL shots 2h prior to their laboratory arrival time while remaining otherwise fasted. Both supplements were provided by the same company to guarantee similar taste and appearance (Beet It Pro, James White Drinks, LTD). Subjects were given additional bottles and were instructed to start consuming the juice the day before the estimated day of menses to consider any changes in the start of the menstrual cycle and guarantee consumption of at least 3 days of supplementation before experimental visits. Between menstrual cycle phases (i.e., EF and LF phase), participants stopped supplementation until ~3 days prior to the next testing visit. Each participant was given a sufficient washout period between testing sessions, based on the time-course and half-life of NO_3^- and NO_2^- (24–26).

2.5. *Experimental Procedure*

During experimental visits, subjects underwent ~10min of supine rest prior to vascular assessments. First, as per manufacturer's instructions, Pulse Wave Analysis (PWA) was performed using a SphygmoCor Xcel system where an arm cuff was placed on the subjects' upper arm, aligning the designated markings with the brachial artery. The automated system then measured pulsations recorded at the brachial artery to produce central aortic pressure waveforms. Pulse wave velocity (PWV) was measured via a simultaneous comparison of the carotid pulse (via applanation tonometry) and the femoral pulse via a specialized thigh cuff.

After completion of the vascular tests, subjects completed two bouts of 2-minutes of cycle ergometer exercise at 20watts followed by 4-minutes at 75% GET (moderate-intensity). Each bout was separated by a 5 min passive recovery.

Upon completion of the two moderate-intensity exercise bouts and after an additional 5-minutes passive recovery, subjects performed an exercise bout at the power output set at 70% Δ GET and VO_{2peak} (severe intensity domain) until exhaustion. They were encouraged to pedal for as long as they could and were blinded to the time expired. When pedaling rate fell by >10rpm despite verbal encouragement by the

researchers the test was terminated and time to exhaustion (TTE) task recorded. Data from the two moderate-intensity bouts were averaged to improve signal-to-noise ratio.

2.6. *Blood sampling– Plasma N-oxides and estradiol*

Prior the exercise test, blood was drawn from an antecubital vein into a NO_3^- free syringe (BD Luer-Lok™) (~5mL) for subsequent NO_3^- and NO_2^- analysis. Plasma NO_3^- and NO_2^- were assessed via ozone-based chemiluminescence using a Sievers NOA model 280i (GE Analytical Instruments, Boulder, CO, USA) as previously described (27). Briefly, plasma samples for NO_3^- analysis were deproteinized using cold ethanol precipitation in a 1:3 dilution (plasma:ethanol) followed by a 30-minute incubation before being centrifuged at 14,000g for 10-min. The supernatant was removed for the subsequent NO_3^- analysis in the presence of vanadium chloride in hydrochloric acid at 95°C. The NO_2^- of the undiluted (non-deproteinized) plasma samples was determined by its reduction to NO in the presence of glacial acetic acid and potassium iodide as previously explained(28). Estradiol was measured by RIA in the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core using a commercial kit (MP Biomedicals; Orangeburg, NY; Cat #07-238102). Assay characteristics were as follows: sensitivity = 10 pg/ml; intra-assay CV = 6.5%; inter-assay CV = 9.2%.

2.7. *Statistical Analysis*

A mixed-model analysis of variance (ANOVA) with Tukey's adjustments for multiple comparisons was used to determine differences between treatment (PL vs BRJ) and between menstrual cycle phases (EF vs LF) for VO_2, HR, RPE, TTE (sec), and vascular measures. Paired T-Test was used to test differences in estradiol across menstrual cycle phases. For HR and RPE at several timepoints within the same TTE test (3-min, 5-min, and at exhaustion) a three-way ANOVA (Supplement*Sex*Timepoint) was used. All statistical analysis was conducted using GraphPad Prism Version 9.3 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). GraphPad Prism was also utilized for the creation of all graphs and figures. Biorender (www.biorender.com) was used to create the study design figure. Data are reported as mean ± SD unless otherwise stated, with $p<0.05$ required for statistical significance.

3. Results:

3.1. *Participant Characteristics*

Twelve young healthy females participated in the study (Table 1). Of those, the majority (n = 10) were not on any type of contraception, while the remainder (n = 2) were on a monophasic oral contraceptive.

3.2. *Plasma Estradiol, Nitrate, and Nitrite*

Plasma estradiol was significantly elevated in the LF phase compared to the EF phase ($p < 0.05$; Table 1). Plasma NO_3^- was significantly different across treatments ($p < 0.01$; Figure 2A), menstrual cycle phases ($p < 0.01$), and an interaction effect was found revealing NO_3^- was significantly higher in the LF+BRJ condition ($p < 0.05$; Figure 2A). Plasma NO_2^- was significantly different across treatments ($p < 0.01$) but did not differ across menstrual cycle phases ($p = 0.90$) and no interaction effect was found ($p = 0.45$; Figure 2B.

3.3. *Vascular Measurements*

Regardless of supplementation or menstrual cycle phase, there were no significant differences in resting HR, SBP, DBP, MAP, aortic SBP, aortic DBP, or PWV (Table 2).

3.4. *The Effect of Inorganic Nitrate on Oxygen Uptake During Moderate Intensity Exercise*

Gross exercise economy (Figure 3) was not significantly different across the menstrual cycle phases ($p = 0.42$) or between treatments ($p = 0.67$), and no interaction effect was observed ($p = 0.93$). There were no significant differences in HR or RPE at during either the warm-up or the moderate-intensity exercise bout (Table 3).

3.5. *The Effect of Inorganic Nitrate on Time to Exhaustion Task in the Severe Domain*

Time to exhaustion (Figure 4) was significantly decreased within for the BRJ treatment compared to PL (404 ± 147 v 444 ± 166 sec, respectively $p < 0.05$), with no significant differences between menstrual cycle phases (EF: 415 ± 148 sec; LF: 433 ± 165 sec; $p = 0.34$), and no interaction effect ($p = 0.34$). There were no statistical differences between conditions for VO_{2peak}, HR, or RPE during the severe intensity exercise (Table 4).

4. Discussion:

To our knowledge this is the first study to examine the effects of oral NO_3^- supplementation on exercise economy and exercise capacity in healthy young females across the eumenorrheic menstrual cycle. Our major findings were that there were no effects of either treatment (BRJ or PL) or MC phase (EF or LF) on exercise economy (Table 3; Figure 3), but a worsened exercise endurance capacity (TTE) during cycling at a "severe" intensity when consuming oral NO_3^- (Table 4, Figure 4). As expected, estradiol levels were significantly greater in the LF phase of the MC and participants significantly increased plasma nitrate and nitrite concentrations following supplementation.

We hypothesized that NO_3^- supplementation may be less beneficial as an ergogenic aid during a background of higher estradiol, and hence higher bioavailable NO (29), during the LF phase of the MC. The present data suggest no exercise economy benefit of NO_3^- supplementation in healthy young females and a potential detrimental exercise capacity effect regardless of estradiol concentrations throughout the EF and LC phase of the MC. These data differ from previous reports in males that suggest that NO_3^- supplementation may improve exercise economy (7, 30, 31).

Previous research of exercise economy in females is limited with mixed results showing benefits in some studies (14, 15), but not others (32). A potential factor for these conflicting results is a lack of standardization or accommodation of the timing of exercise testing during the menstrual cycle. One study did not standardize testing by phase of the cycle (15), another tested during the luteal phase when progesterone is elevated and can blunt estrogen's binding to estrogen receptors (14), and the third study included females taking hormonal contraceptives who experience reduced estrogen fluctuations (32).

A further complication is the lack of standardization of nitrate dosage and workloads between studies. Wickham et al. administered double the dose of NO_3^- of the present study (~26 vs ~13 mmol/day, respectively) but showed no improvements in exercise economy at either 50% or 70% of VO_{2peak}. In contrast, Rienks and colleagues administered the same absolute daily NO_3^- dose as the present study in females of similar fitness. However, their subjects weighed 10kg more than the current group and as such had a lower relative dose, when adjusted for bodyweight. They saw improvements in exercise economy after supplementation at a 75W absolute power output. Our individualized workload based from GET averaged 46W (Table 2). Whether there is a dose response relationship with regard to supplementation and exercise economy cannot be addressed from the present study or previous data in the literature. We also explored whether NO_3^- supplementation impacts exercise economy differentially across the menstrual cycle. Our findings suggest that neither NO_3^- supplementation, phase of the menstrual cycle, impacts moderate intensity exercise economy in young healthy females.

An unexpected finding was that NO_3^- supplementation impaired endurance capacity in the severe domain in young healthy females (Figure 4). NO_3^- supplementation has resulted in either improvements or a lack of change to TTE in studies testing predominantly male or mixed sex participants (33). In the present study TTE was reduced by 40 seconds (~9%) after BRJ supplementation, with no differences in VO_{2peak}, HR, or RPE (Table 4). As mentioned previously, we had originally hypothesized that NO_3-supplementation may be less beneficial as an ergogenic aid during the LF phase as there is greater background of higher estradiol and likely greater endogenous NO at this timepoint. Although not statistically significant there was a 58 second reduction in TTE during the LF phase compared with 22 seconds in the EF phase. This could potentially represent a substantial effect on "real world" exercise performance at different times in the MC and is likely worthy of further exploration.

It is unclear why NO_3^- supplementation worsened exercise performance in the females in the present study. It is possible that there is a dose response relationship between bioavailable NO and exercise performance. It is also possible that in young healthy females there is already adequate bioavailable NO and the addition of endogenous supplements creates a deleterious environment. To date, most research studies assigned an absolute dosage of NO_3^- often based on supplement packaging sizes. Perhaps adjusting for body mass and endogenous hormone concentrations may provide a more nuanced and beneficial approach. We have previously suggested that NO_3^- supplementation is likely more beneficial in participants with reduced or dysfunctional endogenous NO production such as patients with cardiovascular diseases (11).

Another interesting observation from the present study is that plasma NO_3^- was significantly greater when consumed as BRJ during the LF phase versus the EF phase, however, plasma NO_2^- increased similarly in both phases (Figure 2A and 2B). This could be related to a higher endogenous NO bioavailability during the LF phase when estradiol is highest (29) and perhaps a lower conversion rate from NO_3^- to NO_2^- by commensal bacteria in the mouth and gut at that menstrual cycle phase, although this was not explored here.

While NO_3^- has shown to improve measures of vascular health (34, 35), the participants included in this study did not experience improvements in blood pressure or PWV (Table 2). While this may indicate that neither the menstrual cycle nor NO_3^- supplementation would impact vascular health in females, the participants in this study were young and healthy, having good vascular responses at baseline. It is possible that benefits may be observed in females with impaired vascular health (e.g. post-menopausal females or those with resting hypertension).

Limitations:

There are a few limitations to this study. Testing during the menstrual cycle phases was completed based on self-reported timing, although this was confirmed with plasma estrogen samples run at the end of the study. Additionally, two of the twelve participants were on oral contraceptive pills which may have diminished the effects in the LF phase due to a lack of natural estrogen peak. There was no method to measure ovulation such as with transvaginal ultrasound, urinary ovulation testing, or measurement of progesterone to predict the luteinizing hormone surge for more accurate testing of the LF phase. It is possible that testing characterized here as the LF phase was not completed during the highest peak of estrogen.

Participants were not tested during the luteal phase of the MC when estrogen is generally higher than the EF phase, but lower than the LF phase, and when progesterone is elevated. As progesterone is known to block the actions of estrogen (36), and estrogen plays a role on NO bioavailability, the EF and

LF phases were specifically chosen for study here. It is possible based on the results of this study that the ability of progesterone to decrease the action of estrogen may result in different effects on exercise performance after NO_3^- supplementation. Finally, urinary, skeletal muscle and oral NO_3^- and NO_2^- concentrations were not measured in this study, so we are unable to fully account for potential changes in N-oxides in these compartments and if they are different across the menstrual cycle.

5. **Conclusions**:

The present study indicates that NO_3^- supplementation did not affect exercise economy or vascular health and worsened aerobic endurance capacity (TTE) in young healthy females, regardless of MC phase. The possibility of an optimal dose response relationship to improve the aforementioned variables merits further investigation. In the interim, young healthy females should proceed with caution when considering supplementation with BRJ, particularly in light of equivocal findings at present and the cost of supplementation.

Acknowledgements:
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Figure 1: Study Schematic

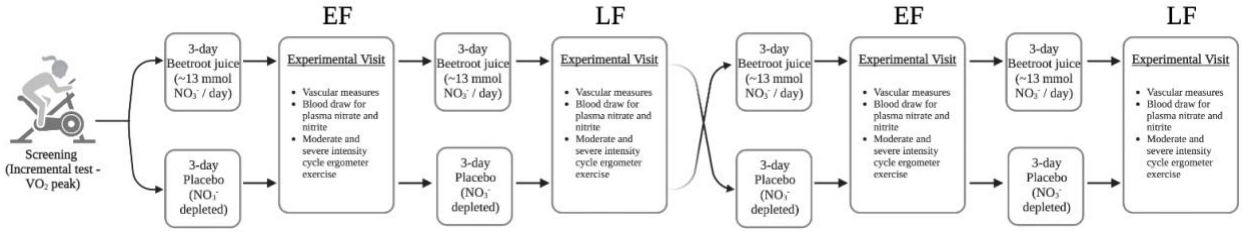


Table 1: Participant Characteristics

Variable	Mean ± SD
N	12
Age	23.4 ± 3.5
Height (m)	1.60 ± 8.1
Weight (kg)	61.4 ± 7.4
BMI (kg/m^2)	24.2 ± 3.5
VO_{2peak} (ml/kg/min)	34.4 ± 5.9
Peak Power Output (watts)	157.1 ± 42.7
EF Estradiol (pmol/L)	185 ± 71
LF Estradiol (pmol/L)	242 ± 107*

BMI – Body Mass Index. EF – Early follicular phase. LF – Late follicular phase. Data presented as mean ± SD. * $p \leq 0.05$ compared to EF Estradiol.

Figure 2: N-oxides across the menstrual cycle phases and following supplementation.
Figure Legend: Absolute values for (A) plasma nitrate and (B) plasma nitrite. Values are mean ± SD. * significant difference compared to EF ($p < 0.05$). **** significant difference compared to PL ($p < 0.01$).

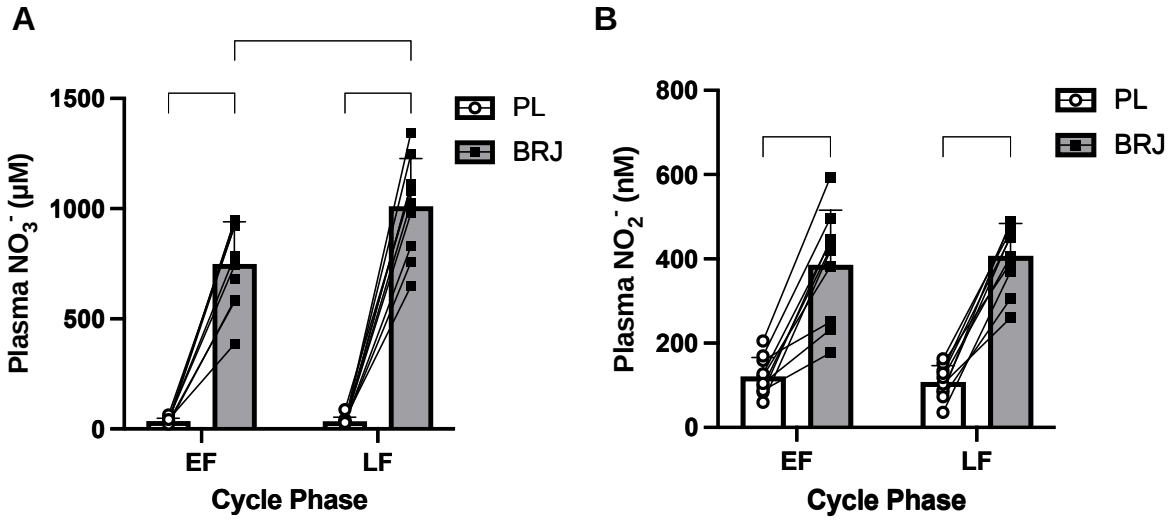


Table 2: The Effects of Inorganic Nitrate or Placebo and Menstrual Cycle Phase on Cardiovascular Measures

	Placebo		Nitrate		*p*		
	EF	LF	EF	LF	Phase	Treatment	P x T
SBP (mmHg)	110 ± 8	107 ± 7	109 ± 4	108 ± 8	.26	.71	.43
DBP (mmHg)	66 ± 9	66 ± 5	66 ± 6	66 ± 9	.99	.69	.94
MAP (mmHg)	78 ± 9	78 ± 8	78 ± 6	79 ± 9	.73	.96	.59
Aortic SBP (mmHg)	96 ± 9	95 ± 7	96 ± 5	96 ± 10	.79	.99	.35
Aortic DBP (mmHg)	68 ± 9	66 ± 5	67 ± 6	67 ± 9	.90	.35	.81
PWV (m/s)	4.4 ± 0.6	4.5 ± 0.8	4.6 ± 0.7	4.7 ± 0.8	.69	.21	.95

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, Mean arterial pressure; EF, Early follicular; LF, Late follicular; PWV, pulse wave velocity; P x T, Interaction effect. Data presented as mean ± SD. $n = 12$.

Figure 3: Effects of NO_3^- and the menstrual cycle on exercise economy during moderate intensity exercise

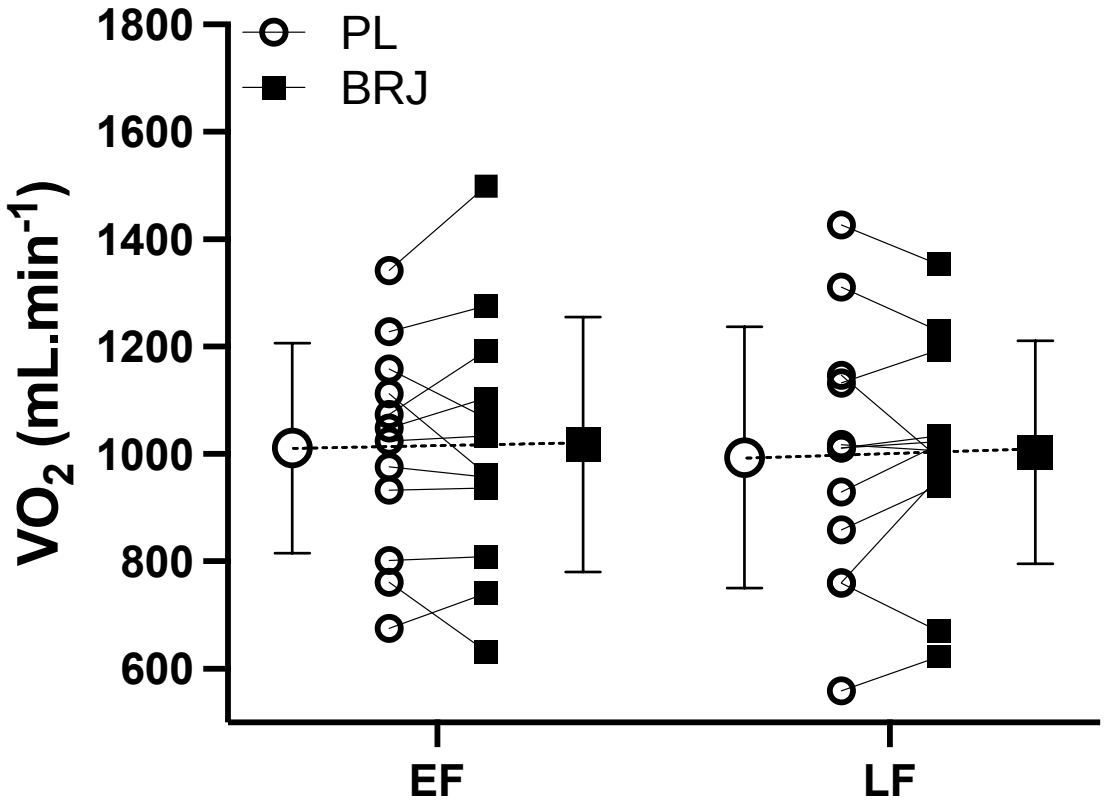


Table 3: The Effects of Inorganic Nitrate or Placebo and Menstrual Cycle Phase During Submaximal Exercise

	Placebo		Nitrate		*p*		
	EF	LF	EF	LF	Phase	Treatment	P x T
Warm-Up (20 ± 0 Watts)							
VO_2 ($mL.min^{-1}$)	723 ± 95	742 ± 133	757 ± 118	705 ± 113	.34	.93	.06
Heart Rate (bpm)	103 ± 15	103 ± 16	105 ± 19	104 ± 19	.52	.69	.80
RPE	8 ± 1	8 ± 1	8 ± 1	8 ± 1	.48	.30	.30
***Moderate-Intensity* (46 ± 25 Watts)**							
VO_2 ($mL.min^{-1}$)	1011 ± 195	994 ± 244	1018 ± 238	1004 ± 208	.42	.67	.93
Heart Rate (bpm)	126 ± 21	126 ± 22	128 ± 23	124 ± 23	.33	.88	.41
RPE	10 ± 1	10 ± 2	10 ± 2	10 ± 1	.73	.76	.64

VO_2, oxygen uptake; EF, Early follicular; LF, Late follicular; P x T, Interaction effect. Data presented as mean ± SD. *n* = 11 or 12.

Figure 4: Effects of NO_3^- and the menstrual cycle on change in time to exhaustion (TTE) during the severe-intensity exercise.
Figure Legend: * = ($p < 0.05$) for treatment effect (BRJ vs PL)

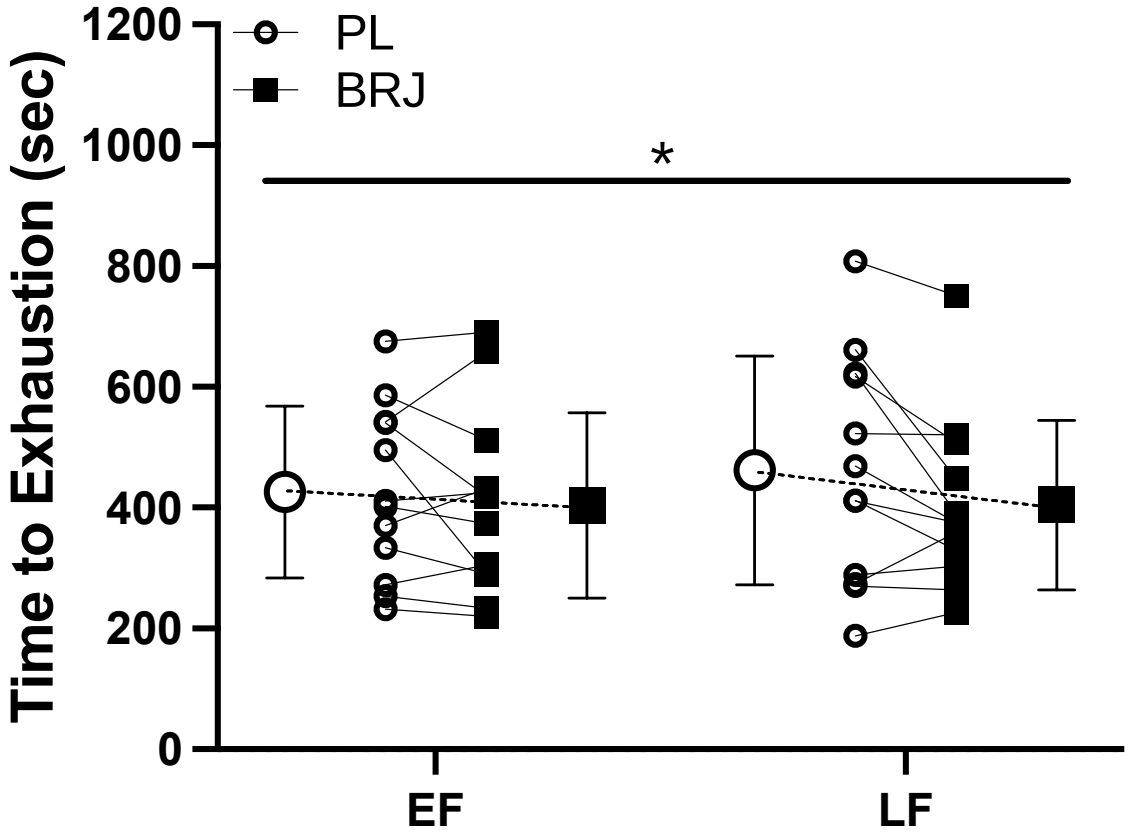
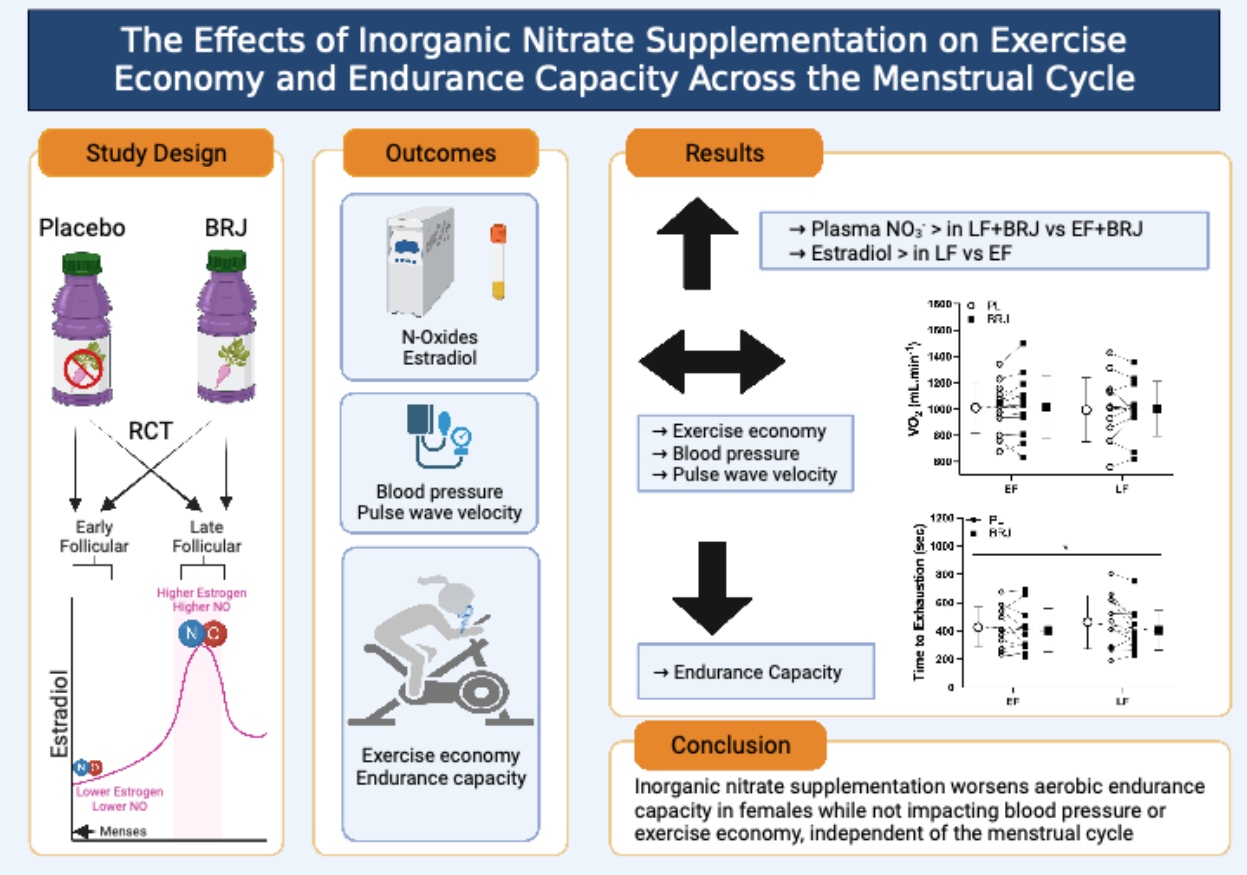


Table 4: The Effects of Inorganic Nitrate or Placebo and Menstrual Cycle Phase During Severe Intensity Exercise

	Placebo		Nitrate		*p*		
	EF	LF	EF	LF	Phase	Treatment	P x T
Heart Rate							
3 min (bpm)	170 ± 13	169 ± 12	170 ± 14	170 ± 12	.72	.86	.89
5 min (bpm)	177 ± 12	174 ± 10	180 ± 14	179 ± 11	.33	.27	.29
Peak (bpm)	181 ± 11	180 ± 11	181 ± 12	183 ± 10	.63	.36	.09
RPE							
3 min	15 ± 2	15 ± 2	15 ± 2	15 ± 2	.21	.99	.99
5 min	17 ± 1	17 ± 2	17 ± 2	16 ± 2	.15	.32	.88
Failure	19 ± 1	19 ± 1	18 ± 1	19 ± 1	.95	.06	.93
Exhaustion							
VO_2 ($mL.min^{-1}$)	2085 ± 521	2089 ± 485	2064 ± 495	2089 ± 450	.72	.76	.75
TTE (s)	426 ± 142	462 ± 189	404 ± 153	404 ± 140	.34	**.04**	.34

EF, Early follicular; LF, Late follicular; TTE, Time to exhaustion; RPE, Rating of perceived exertion; P x T, Interaction effect. Data presented as mean ± SD. *n* = 11-12. Power output: 132 ± 35 Watts

Graphical Abstract



Manuscript 2:

The Effects of Inorganic Nitrate Supplementation on Muscular Power and Endurance Across the Menstrual Cycle

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Abstract

Supplementation with oral inorganic nitrate (NO_3^-) increases nitric oxide (NO) bioavailability. Studies have shown that NO_3^- improves muscular power in males and females, although data in females are limited. Estrogen improves NO and fluctuates throughout the menstrual cycle (MC), ranging from low levels in the early follicular (EF) phase and peaking during the late follicular (LF) phase. This study examined the effects of NO_3^- supplementation on isokinetic peak power, maximal voluntary isometric contraction (MVIC) force, muscular endurance, and recovery from fatigue in healthy young females across the MC. Twelve females with eumenorrheic MC were tested in a double-blinded, randomized design. Participants consumed ~13mmol NO_3^-, in the form of 140ml beetroot juice (BRJ) or an identical NO_3^--depleted placebo (PL) for ~3 days prior to lab visits and 2 hours prior to testing on lab visits. Plasma nitrate, nitrite, and estradiol were assessed. As expected, plasma estradiol was elevated in the LF phase, and plasma nitrite and nitrate were elevated in the BRJ condition. Isokinetic peak power was worsened by NO_3^- supplementation ($p = 0.02$), and there was velocity x treatment interaction ($p < 0.01$). Multiple comparisons revealed power at maximal knee extensor power (Pmax) to be significantly worse in the LF+BRJ condition compared to the EF+PL condition ($p = 0.04$). Maximal knee extensor velocity (Vmax) was also worsened by NO_3^- supplementation ($p = 0.03$). Muscular endurance, MVIC, and fatigue recovery was unaltered by BRJ or the MC (all $p > 0.05$). In conclusion, NO_3^- supplementation worsened maximal knee extensor power and velocity without impacting muscular endurance, MVIC, or fatigue recovery. Further, NO_3^- supplementation during the LF phase of the MC worsened peak power compared to EF+PL, suggesting that NO_3^- may interact with estrogen fluctuations in the MC. For young healthy eumenorrheic females, it may be unwise to supplement with oral NO_3^- if the goal is to augment muscular performance.

1. Introduction

Nitric oxide (NO) is a gaseous molecule known to play a role in various aspects of human physiology including exercise (1, 2). While NO is endogenously synthesized from NO synthase (NOS) enzymes (3, 4), an exogenous avenue to increase NO bioavailability known as the entero-salivary pathway or the nitrate-nitrite-NO pathway has been increasingly utilized for enhancing exercise performance (5). In this pathway, oral consumption of inorganic nitrate (NO_3^-) can be reduced to form nitrite (NO_2^-) by oral bacteria, and subsequently reduced to NO (6). Conversion of NO_2^- to NO is facilitated in acidic and hypoxic situations such as during exercise (7–9). NO_3^- supplementation has been purported to improve exercise outcomes including exercise economy (10–13), time-to-exhaustion (10), and muscular contractions (11, 14–17), findings which are likely dependent on improvements in tissue perfusion (9), ATP turnover (11) and calcium handling (7, 18). Despite this, evidence of the potential impact of NO_3^- supplementation on muscular force production in humans is scarce and equivocal (19–21), with some results suggesting an enhanced effect in females over males (22).

Reproductive age eumenorrheic females experience cyclic fluctuations in hormones throughout the menstrual cycle, characterized by fluctuations in estrogen (23). In normally menstruating women, estrogen is lowest at the time of menses during the early follicular (EF) phase and tends to peak ~10-14 days later during the late follicular (LF) phase. Estrogen has shown to improve nitric oxide (NO) bioavailability (6, 24–30), possibly explaining differences in exercise performance across the menstrual cycle (31). Despite known effects of the menstrual cycle on NO bioavailability and exercise performance, few studies measure females and those that do oftentimes only examine responses during the EF phase in an attempt to control for estrogen fluctuations (32). This gap in our understanding of the totality of the female physiology minimizes the practical translation of research findings. Understanding the relationship between the potential impact of NO_3^- supplementation across the menstrual cycle phases in females may reveal important precision strategies for improving exercise performance.

Thus, to the purpose of this study was to explore differences in the effects of NO_3^- supplementation on maximal force, muscular endurance, and recovery from fatigue in females across the follicular phase of menstrual cycle. The primary hypothesis was that NO_3^- supplementation would improve muscular strength, endurance, and recovery only during the EF

phase when estrogen and NO are lowest, and that NO_3^- supplementation will have a limited affect during the LF phase of the menstrual cycle when estrogen and NO are already elevated.

2. Methods

2.1. Experimental Design and Protocol

This study was a randomized, double-blind cross-over design (NCT04588740). Participants reported to the laboratory for initial screening and baseline exercise testing prior to randomization to either inorganic NO_3^- supplementation in the form of beet root juice (BRJ) or placebo (PL) (See description under *Supplementation procedures*). Participants were tested during both the EF (1 – 3 days since menses onset) and LF phase (11 – 14 days since menses onset) of the menstrual cycle, as determined by self-report with later confirmation by plasma estradiol measurements. All study visits were performed by the same researcher, at the same time of day (± 1 hour), and under the same sensory conditions. Females were tested on two consecutive months allowing for a washout period between supplemental time-frames. Participants were recruited from the University of Virginia and surrounding Charlottesville, Virginia area. All procedures were approved by the Institutional Review Board at the University of Virginia. All subjects provided written informed consent.

2.2. Participants

Subjects were included if they exercised ≤ 3 days/week for less than 30 minutes, were apparently healthy and not aerobically trained (i.e., VO_{2peak} ≤ 45 mL/min/kg), normotensive (<120/80 mmHg), and had no orthopedic limitations to exercise testing. Subjects must have had regular menstrual periods (minimum of 10-12 menses per year) and were not on any type of contraception with the exception of a monophasic oral contraceptive pill consistently for at least 6 months prior to the study.

Subjects were excluded if they had any known pulmonary, cardiovascular, or metabolic conditions or diseases, any food allergies, if they were taking any medications or supplements that could impact study outcomes in the last 6 months (i.e., pre-workout supplements, L-citrulline, L-arginine, etc.), if they used tobacco, had abnormal blood pressure, were currently or recently pregnant or lactating (< 1 year), or if they contracted Covid-19 during the experimental period.

2.3. Supplementation Procedures

Subjects were assigned to consume 70mL of beet root juice (BRJ - ~6.5mmol NO_3^-) in the morning and at night (~13mmol total NO_3^-) for ~3 days or an identical NO_3^--depleted placebo (PL). On testing days, subjects were instructed to ingest two 70mL beverages 2h prior to their laboratory arrival time while remaining otherwise fasted. Both supplements were provided by the same company to guarantee similar taste and appearance (Beet It Pro, James White Drinks, LTD). Subjects were given additional bottles and were instructed to start consuming the juice the day before the estimated day of menses to consider any changes in the start of the menstrual cycle and guarantee consumption of at least 3 days of supplementation before experimental visits. Between menstrual cycle phases (i.e., EF and LF phase), participants stopped supplementation until ~3 days prior to the next testing visit. Each participant was given a sufficient washout period between testing sessions, based on the time-course and half-life of NO_3^- and NO_2^- (33–35).

2.4. Experimental Procedure

Prior to all experimental visits and supplementation, a full familiarization visit was completed. All testing was performed on the dominant leg (right leg for all participants) of each individual using a multimodal dynamometer (Model System 3; Biodex Medical Systems Inc, Shirley, NY) as described previously (36). Briefly, subjects sat upright with the right knee at 90° flexion for calibration and a correction for limb weight was used. All positioning settings were noted and repeated for each study visit. The dynamometer was adjusted to place the axis of rotation of the lever arm alongside the lateral femoral epicondyle. To limit undesired muscular contraction, a strap was secured across the hips throughout testing, and participants crossed their arms across their chest. The experimental protocol consisted of four separate testing protocols [i. Isokinetic peak power, ii. Pre-fatigue-protocol maximal voluntary isometric contraction (Pre-MVIC), iii. Fatigue-protocol, iv. Post-fatigue-protocol MVIC (Post-MVIC)] in succession and explained below (Figure 1).

i. Isokinetic peak power

During the isokinetic peak power protocol, participants were instructed to “kick out and pull back as hard and fast as possible” for 10 repetitions, across three different isokinetic angular velocities (180, 270, and 360°/s), each separated by 30 second rest (36) (Figure 1A). Participants were given real-time visual feedback and verbal encouragement was given to ensure maximal

effort. Isokinetic peak power (W/kg) was calculated via native Biodex Advantage software. The highest power generated at each velocity was plotted to obtain a power-velocity relationship which was then fitted to a 2nd-order polynomial as previously described (14, 15, 37). The polynomial function allowed for estimation of maximal knee extensor velocity (Vmax), as well as maximal knee extensor power (Pmax).

ii. Pre-fatigue-protocol maximal voluntary isometric contraction (Pre-MVIC)

Following a 5-minute rest subjects were instructed to perform maximal isometric knee extension contractions with range of motion locked at 90° (Figure 1B). Participants were instructed to perform at least three MVIC's (until a clear peak was obtained), with 1-minute rest between each, and the highest value was recorded and compared across study visits.

iii. Fatigue-protocol

Participants were given another 5-minute rest prior to starting the fatigue protocol. The fatigue-protocol consisted of performing 3-second isometric contractions set to 60% of the pre-MVIC force from the first familiarization visit (prior to any supplementation) (Figure 1B). This was repeated consecutively with 2-second rest in between each repetition (i.e., 12 repetitions/minute with 3 seconds of contraction and 2 seconds of rest). Participants were instructed to perform this protocol until failure and both visual feedback and verbal encouragement were provided to ensure the 60% MVIC goal was met each repetition. When participants could no longer maintain the 60% MVIC (as determined by three failed repetitions, with a failed repetition determined when <50% of the assigned workload was met), the exercise was stopped, and time-to-exhaustion (TTE) was recorded as the measure of muscular endurance.

iv. Post-fatigue-protocol MVIC (Post-MVIC)

After exhaustion, a 1-minute rest was allotted before participants finally performed three separate Post-MVIC's each separated by 1 minute rest to determine recovery from fatigue (Figure 1B).

2.5. Blood sampling– Plasma N-oxides and estradiol

Prior the exercise test, blood was drawn from an antecubital vein into a NO_3^- free syringe (BD Luer-Lok™) for subsequent NO_3^- and NO_2^- analysis. Plasma NO_3^- and NO_2^- were assessed via ozone-based chemiluminescence using a Sievers NOA model 280i (GE Analytical Instruments, Boulder, CO, USA) as previously described (38). Briefly, plasma samples for NO_3^- analysis were

deproteinized using cold ethanol precipitation in a 1:3 dilution (plasma:ethanol) followed by a 30-minute incubation before being centrifuged at 14,000g for 10-min. The supernatant was removed for the subsequent NO_3^- analysis in the presence of vanadium chloride in hydrochloric acid at 95°C. The NO_2^- of the undiluted (non-deproteinized) plasma samples was determined by its reduction to NO in the presence of glacial acetic acid and potassium iodide as previously explained (39). Estradiol was measured by RIA in the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core using a commercial kit (MP Biomedicals; Orangeburg, NY; Cat #07-238102).Assay characteristics were as follows: sensitivity = 10 pg/ml; intra-assay CV = 6.5%; inter-assay CV = 9.2%.

2.6. *Statistical Analysis*

Peak power and MVIC over time were analyzed using three-way mixed linear models (Menstrual Cycle x Treatment x Velocity or Timepoint, respectively). Paired T-Test was used to test differences in estradiol across menstrual cycle phases. All other outcome measures were analyzed via 2-way (Treatment x Menstrual Cycle) analysis of variance (ANOVA) with Tukey's adjustments for multiple comparisons. All statistical analysis was conducted using GraphPad Prism Version 9.3 (GraphPad Software, La Jolla, CA, USA). Data are reported as mean ± SD, and statistical significance was determined *a priori* at $p < 0.05$.

3. Results

3.1. *Participant Characteristics*

Twelve young healthy females participated in the study (Table 1). Of those, the majority (n = 10) were not on any type of oral contraception, while the remainder (n = 2) were on a monophasic oral contraceptive pill.

3.2. Plasma estradiol and N-oxides

Plasma estradiol was significantly elevated in the LF phase compared to the EF phase ($p < 0.05$; Table 1). Plasma NO_3^- was significantly different across treatments ($p < 0.01$; Table 2), menstrual cycle phases ($p < 0.01$), and an interaction effect was found revealing NO_3^- was significantly higher in the LF+BRJ condition compared to the EF+BRJ condition ($p < 0.05$; Table

2). Plasma NO_2^- was significantly different across treatments ($p < 0.01$) but did not differ across menstrual cycle phases ($p = 0.90$) and no interaction effect was found ($p = 0.45$; Table 2).

3.3. Skeletal Muscle Contractile Function

Isokinetic peak power (Figure 2) was significantly different across angular velocities ($p < 0.01$), and treatments ($p = 0.02$), but not across menstrual cycle phases ($p = 0.39$). There was a velocity x treatment interaction ($p < 0.01$). Subsequent multiple comparisons revealed Pmax to be significantly worse in the LF+BRJ condition compared to the EF+PL condition ($p = 0.04$). Vmax (Figure 3) was significantly higher in the PL treatment compared to the BRJ treatment ($p = 0.03$) with no differences between menstrual cycle phases ($p = 0.86$) or any interaction effects ($p = 0.81$).

3.4. Skeletal Muscle Endurance Capacity

Muscular endurance (Figure 4) as determined by TTE during the fatigue protocol was not different between treatments ($p = 0.69$) or menstrual cycle phases ($p = 0.12$), and there were no interaction effects ($p = 0.46$).

3.5. Maximal Voluntary Isometric Contractions Before and After Fatigue

Figure 5 shows MVIC's before, and 1-, 2-, and 3-min post-fatigue protocol. Pre-MVIC showed no significant differences between treatment ($p = 0.37$), MC phase ($p = 0.84$), or any interactions ($p = 0.84$). MVIC's did not differ between treatments ($p = 0.52$) or menstrual cycle phases ($p = 0.86$) but did differ across time ($p < 0.001$) with each timepoint different from one another (all $p < 0.001$). There were no significant interactions between treatment and menstrual cycle ($p = 0.28$), treatment and time ($p = 0.52$), or time and menstrual cycle ($p = 0.84$), and there was no 3-way interaction (all $p = 0.65$).

4. Discussion

To our knowledge, this is the first study to examine the effects of NO_3^- supplementation on muscular power or endurance in females across the menstrual cycle. The major findings of this study are that isokinetic peak power (Figure 2) and Vmax (Figure 3) were significantly worsened by NO_3^- supplementation in females, and that this negative effect is more pronounced

in the LF phase of the menstrual cycle (Figure 3) when relative plasma estradiol concentrations are at their highest. The present study also showed that neither NO_3^- supplementation nor the menstrual cycle had any impact on muscular endurance capacity during an intermittent isometric leg fatigue protocol (Figure 4), or maximal voluntary isometric force production either before, or 1-, 2-, or 3-minutes post-fatigue protocol (Figure 5). These findings were contradictory to our hypothesis that NO_3^- supplementation would improve exercise performance in females in the EF phase, but not during the LF phase when endogenous estrogen is already elevated.

NO_3^- supplementation has been suggested to improve muscular performance (21), and previous studies in isokinetic testing showed an improvement in power at faster angular velocities (i.e., 360°/s) and at Pmax after NO_3^- supplementation (14, 15). Despite this, equivocal effects of NO_3^- supplementation on muscular performance exist (19, 20), and only one study compared sex differences in isokinetic power and suggested that females are more likely to benefit from NO_3^- supplementation than males (22). This study consisted of a heterogenous group of middle-aged adults (13 males 7 females, age 47 ± 20 yrs) and did not control for the menstrual cycle or report menopause status. A second study employing electrically stimulated plantar flexor muscles showed no difference in peak torque or MVC after NO_3^- supplementation in females (40). This study did not control for the menstrual cycle, and all females included were taking hormonal contraceptives.

Our data show that Pmax (Figure 2) and Vmax (Figure 3) (15, 22, 41), was worsened after NO_3^- supplementation. This was driven by differences in EF+PL compared to LF+BRJ (p = 0.04). These data are the first to show that NO_3^- supplementation may worsen muscular performance in females, and that the elevated estrogen in the LF phase of the menstrual cycle may contribute to detrimental effect on muscular performance.

Another finding of this study was that neither NO_3^- supplementation nor the menstrual cycle influenced muscular endurance (TTE) during a fatiguing, intermittent isometric exercise protocol (Figure 4). NO_3^- supplementation has shown to improve isometric muscular endurance in some studies (42, 43), however, to our knowledge this has only been tested in males. As the reduction of NO_2^- to NO is facilitated in hypoxia (44) and NO_3^- supplementation may only improve contractile force in fast-twitch muscle fibers (18), it is possible that a less hypoxic exercising tissue in females (who have higher concentrations of fatigue-resistant type I skeletal muscle fibers (45)) could be associated with a more limited reduction of NO_2^- to NO and thus decreasing

the beneficial effects of NO_3^- supplementation. Indeed, the improved ATP cost of exercise and improved phosphocreatine and oxygen kinetics after NO_3^- supplementation has only been studied in males (11, 46), and thus it is unknown if these same ATP-sparing and metabolic benefits persist in females.

This study also showed that neither NO_3^- supplementation nor the menstrual cycle phase impacted MVIC either prior to, or following, a muscular fatiguing protocol (Figure 5). While data suggest either a lack of benefit or small improvement of MVIC with NO_3^- supplementation (19), the findings here confirm the work of Wickham et al. who showed no difference in MVC with electrical muscle stimulation in females (40). While fatigue-recovery as determined by post-fatigue MVIC has shown to be improved with NO_3^- supplementation in males (47), our data show that this may not be the case in females nor across the menstrual cycle.

It is unknown why NO_3^- supplementation alone, and when supplemented in the LF phase of the menstrual cycle in particular, may result in worsened muscular performance. It is possible that the combination of elevated endogenous NO from higher estrogen paired with elevated exogenous NO from NO_3^- supplementation may depress contractile function. An excess of NO may result in an environment that is harmful to muscular performance, and this effect may be dependent on the fiber type characteristics of the exercising skeletal muscle (48–50).

Limitations:

There are a few limitations to this study. Testing during the menstrual cycle phases was completed based on self-reported timing, although this was confirmed with plasma estrogen samples run at the end of the study. Additionally, two of the twelve participants were on oral contraceptive pills which may have diminished the effects in the LF phase due to a lack of natural estrogen peak. There was no method to measure ovulation such as with transvaginal ultrasound, urinary ovulation testing, or measurement of progesterone to predict the luteinizing hormone surge for more accurate testing of the LF phase. It is possible that testing characterized here as the LF phase was not completed during the highest peak of estrogen.

Participants were not tested during the luteal phase of the MC when estrogen is generally higher than the EF phase, but lower than the LF phase, and when progesterone is elevated. As progesterone is known to block the actions of estrogen (51), and estrogen plays a role on NO bioavailability, the EF and LF phases were specifically chosen for study here. It is possible that

the ability of progesterone to decrease the action of estrogen may result in different effects on exercise performance after NO_3^- supplementation during the luteal phase. Finally, urinary, skeletal muscle and oral NO_3^- and NO_2^- concentrations were not measured in this study, so we are unable to fully account for potential changes in N-oxides in these compartments and if they are different across the menstrual cycle.

5. Conclusion

The present study shows for the first time that NO_3^- supplementation did not impact muscular endurance or fatigue-recovery, but impaired Pmax and Vmax in young females, and that these negative effects are exacerbated in the LF phase of the menstrual cycle. Any improvements of exercise performance that have been seen with NO_3^- supplementation may be related to the lower basal NO found in males and higher proportion of fast-twitch skeletal fibers. Studying females across different phases of the menstrual cycle has revealed a potential sexual dimorphism and interactive effect of sex hormones in this relationship. This data suggests that for young healthy eumenorrheic females, it may be unwise to supplement with oral NO_3^- if the goal is to augment muscular performance.

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Table 1: Participant Characteristics

Variable	Mean ± SD
Age	24 ± 4
Height (cm)	161 ± 9
Weight (kg)	62 ± 7
Body Mass Index (kg/m^2)	24 ± 3
VO_{2peak} (ml/kg/min)	35 ± 6
Peak-MVIC (Nm)	159 ± 40
EF Estradiol (pmol/L)	182 ± 70
LF Estradiol (pmol/L)	242 ± 105*

N = 12; MVIC = Maximal Voluntary Isometric Contraction. EF = Early Follicular. LF = Late Follicular. * - signifies $p < 0.05$ compared to EF Estradiol.

Table 2: The Effects of Inorganic Nitrate and the Menstrual Cycle on N-Oxides

	Placebo		Beetroot Juice	
	EF	LF	EF	LF
Nitrate (μM)	37 ± 12	36 ± 19	749 ± 192	**1,012 ± 216***
Nitrite (nM)	122 ± 45	108 ± 39	366 ± 129	407 ± 77

EF, Early follicular. LF, Late follicular. *denotes significantly different from BRJ EF.

Figure 1: Experimental protocol schematic. The experimental visit was divided into two major testing blocks; (A) Maximal isokinetic knee extension and (B) pre- and post-MVIC with the fatigue protocol in between.

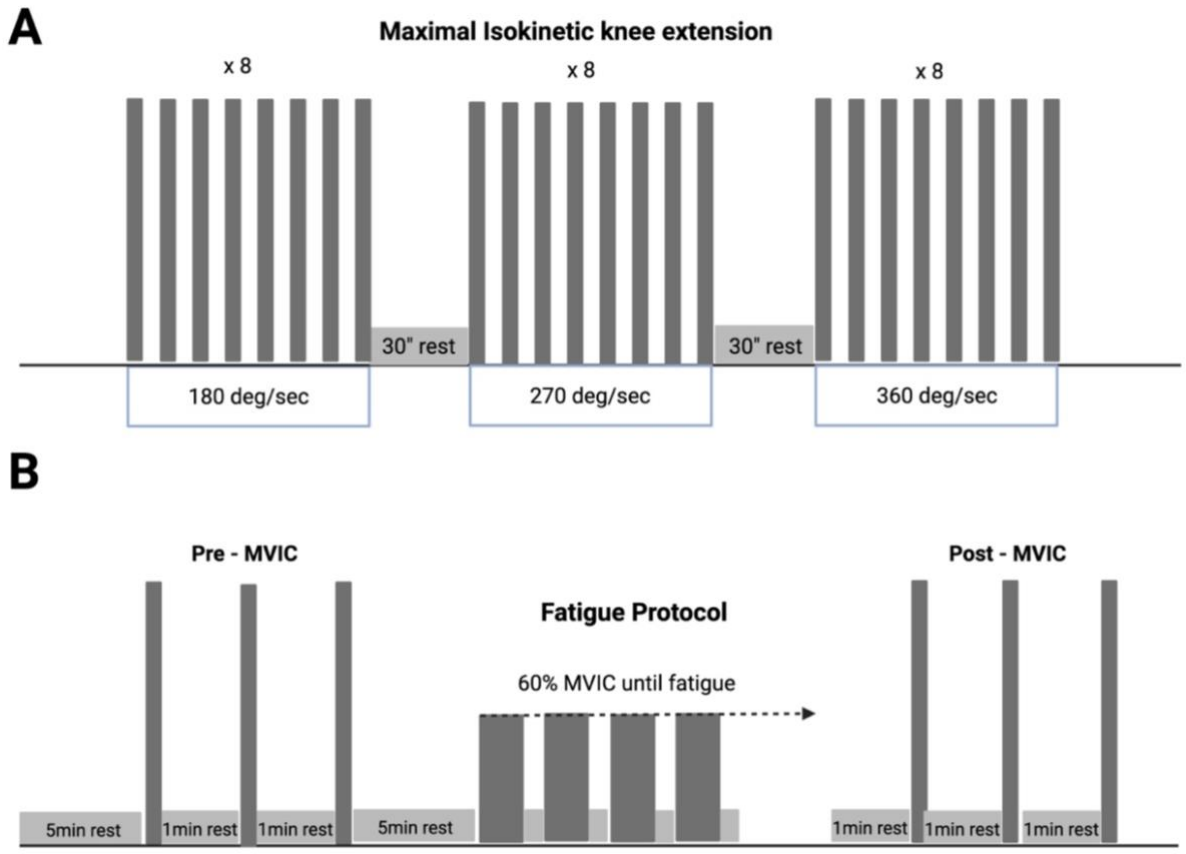


Figure 2: The Effects of Inorganic Nitrate and the Menstrual Cycle on Isokinetic Peak Power

Figure Legend: * denotes significant difference between EF+PL vs LF+BRJ.

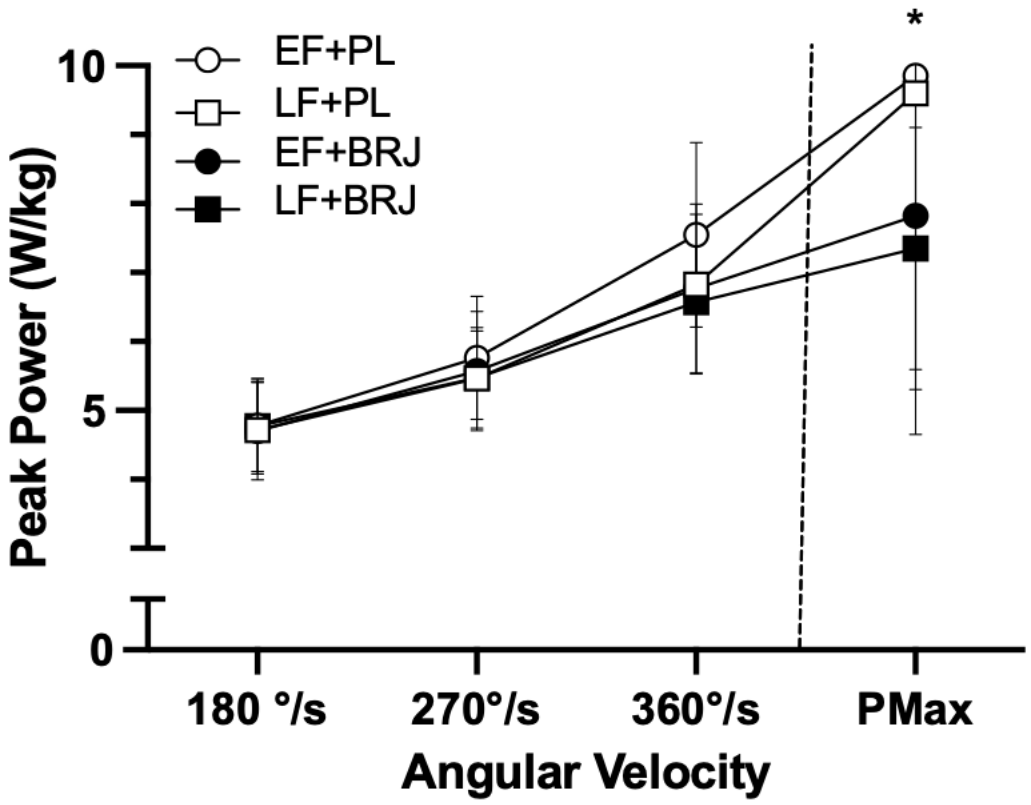


Figure 3: The Effects of Inorganic Nitrate and the Menstrual Cycle on Vmax
Figure Legend: * denotes a significant treatment effect.

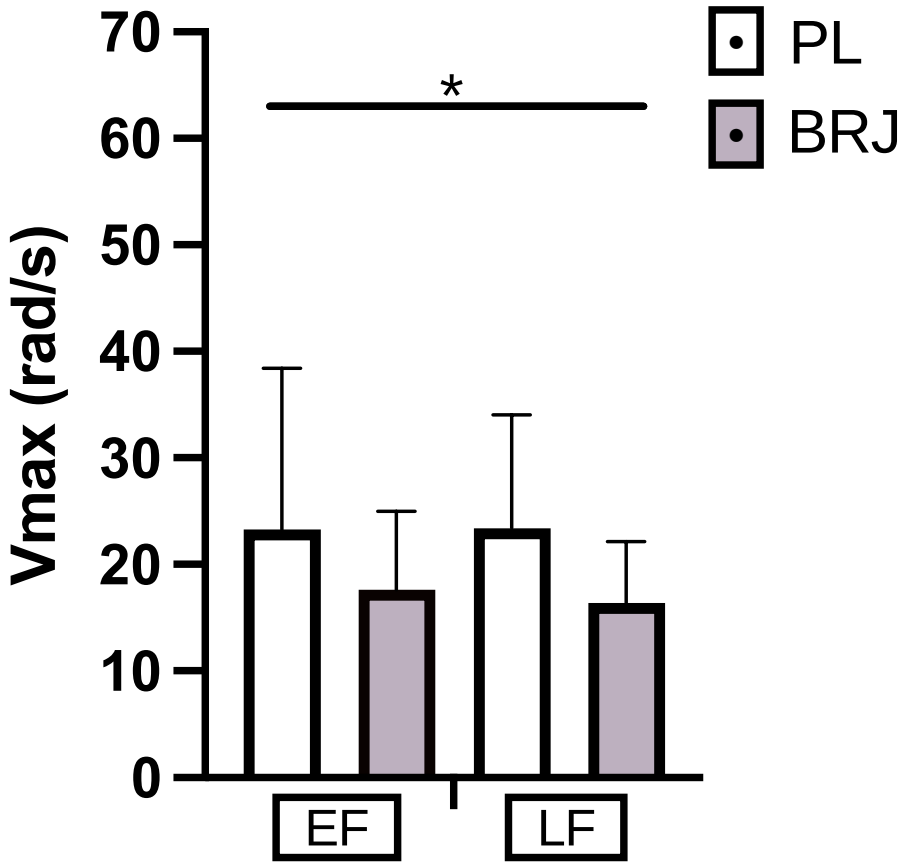


Figure 4: The Effects of Inorganic Nitrate and the Menstrual Cycle on Time to Exhaustion during Intermittent Isometric Muscular Contractions

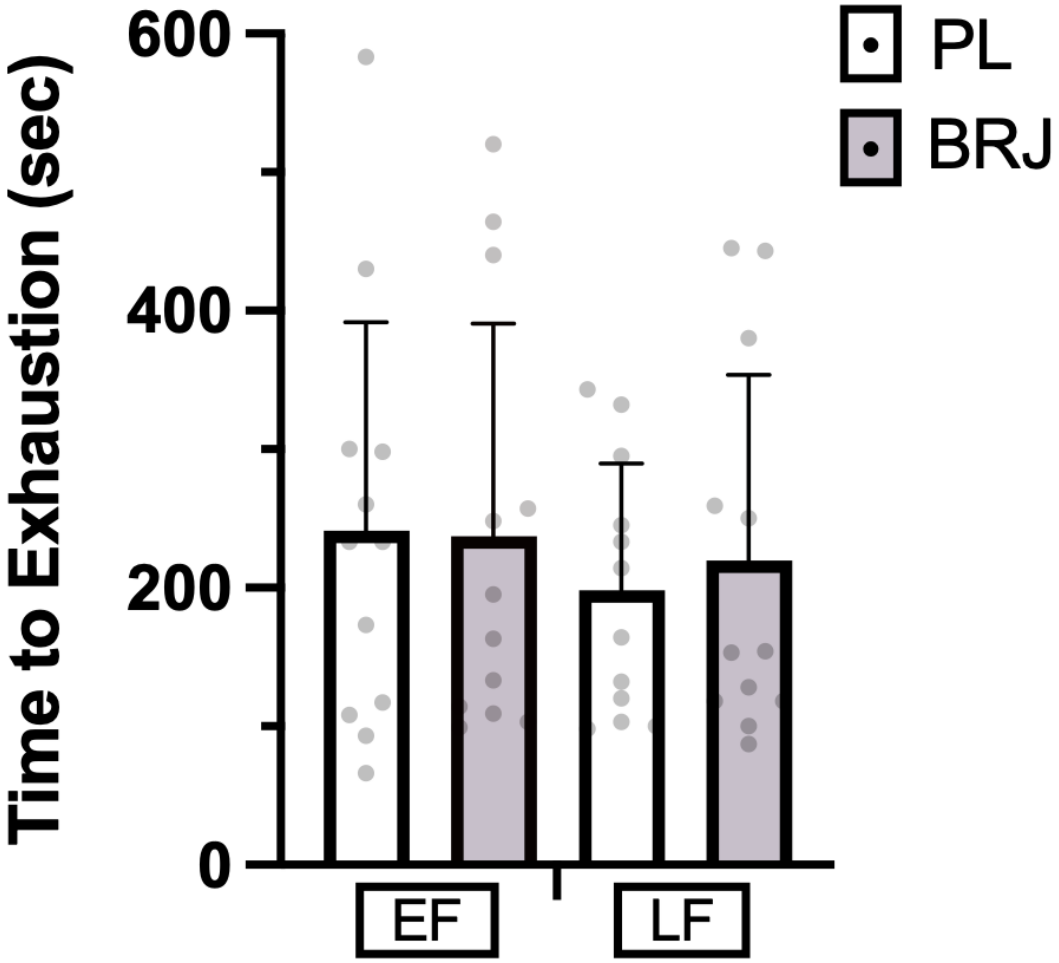
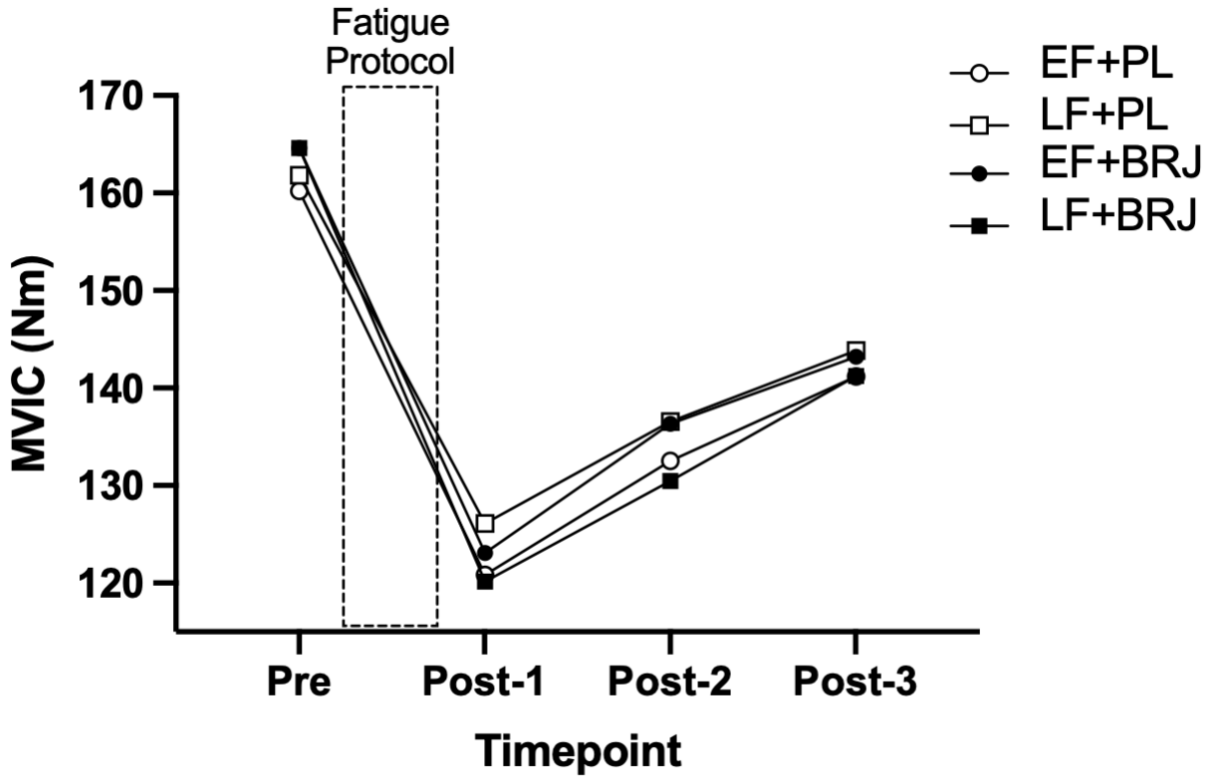


Figure 5: The Effects of Inorganic Nitrate and the Menstrual Cycle on Maximal Voluntary Isometric Contraction (MVIC) Force Before and After Intermittent Isometric Fatigue Protocol



Manuscript 3:

Inorganic Nitrate, Exercise Intensity, and Vascular Function in Post-Menopausal Females

Austin Hogwood, Joaquin Ortiz de Zevallos Muñoz, Nathan Weeldreyer, Vincent Mazzella, James R. Clark, Dylan Myaing, Lauren Cain, Kaitlin Love, Arthur Weltman, Jason D. Allen

Abstract:

Moderate intensity aerobic exercise improves vascular health in older males but not in post-menopausal (PM) females unless they either have severely impaired vascular health at baseline or unless estradiol treatment is co-administered. Estradiol may improve the vascular adaptations to exercise via improvements in nitric oxide (NO) bioavailability, however, estradiol treatment may convey separate health risks. Oral inorganic nitrate (NO_3^-) supplementation provides an exogenous source of NO and may offer an alternative avenue to increase vascular responsiveness in PM females. Further, high intensity exercise (HIE) may provide a greater stimulus for vascular adaptation than moderate intensity exercise (MIE), especially when combined with NO_3^- supplementation. This study examined the effects of acute NO_3^- supplementation and different exercise intensities on flow-mediated dilation (FMD), mean arterial pressure (MAP), and pulse wave velocity (PWV) in PM females. Twenty-two participants were tested in a double-blinded, block-randomized design. Participants consumed ~13mmol NO_3^-, in the form of 140ml beetroot juice (BRJ; n = 11) or an identical NO_3^--depleted placebo (PL; n = 11) for ~3 days prior to lab visits and 2 hours prior to lab testing. Study visits consisted of vascular health measures before (Timepoint 0) and every 30 minutes after (Timepoints 60, 90, 120, 150, 180) high intensity exercise (HIE), moderate intensity exercise (MIE), and a non-exercise control (CON). Blood was taken at rest to determine plasma NO_2^- between treatment groups. As expected, plasma nitrite was elevated in the BRJ group ($p < 0.05$). BRJ+HIE improved Peak Δ FMD compared to all CON conditions ($p < 0.05$), while BRJ+MIE improved Peak Δ FMD compared only to BRJ+CON. Neither PL+HIE nor PL+MIE improved Peak Δ FMD compared to PL+CON ($p > 0.05$). Plasma NO_2^- was positively correlated with Peak Δ FMD, while body fat percentage with inversely correlated. Exercise prevented the increase in MAP and PWV over time independent of treatment. In conclusion, NO_3^- supplementation combined with high intensity exercise provided the greatest improvement in Peak Δ FMD in post-menopausal females. NO_3^- supplementation combined with moderate intensity exercise improved FMD and may offer a feasible alternative if participants are not willing to perform high intensity exercise. Future studies should test whether long term exercise training at high intensities with NO_3^- supplementation can enhance vascular health in PM females.

1. Introduction

Approximately 1.2 billion females will be postmenopausal (PM) by 2030 (1). Menopause involves the loss of endogenous estradiol which greatly increases metabolic and cardiovascular disease (CVD) risk (2). Specifically, PM is associated with an increase in visceral fat (3, 4) and reduced bioavailable nitric oxide (NO) (5), both of which impact metabolic and vascular health.

NO bioavailability may play an important role in reducing CVD risk through its impact on lipid accumulation, glucose uptake, vasodilation, flow regulation, and platelet function (6, 7). Estrogen increases bioavailable NO by promoting NO synthesis, and may reduce NO consumption via increasing antioxidant status and reducing inflammation (7). Estradiol treatment has been shown to improve NO bioavailability and vascular health in PM females, however not to the levels observed in the premenopausal state (8–10). Importantly, following data from the Women's Health Initiative showing potentially increased risk of blood clots, stroke, and breast cancer without a reduction in the risk of CVD (11–13), many PM females are advised against estradiol treatment. Consequently, novel interventional approaches that increase NO bioavailability to promote vascular health in PM females are paramount.

Exercise is a low-cost approach to improving vascular health (14). Although moderate intensity exercise (MIE) training combined with estradiol treatment lowers CVD risk (8), the benefits of exercise training alone on vascular health in PM females are equivocal. This is partly due to confounding study differences such as length of PM status prior to training, levels of visceral fat, metabolic/vascular impairment at baseline, and training intensity/duration (9, 13, 15–26). A viable approach to exercise training in PM may be to utilize exercise intensities above the lactate threshold, as this high intensity exercise (HIE) is known to induce greater vascular adaptations (27–29).

Another innovative approach to increase bioavailable NO is through the conversion of inorganic nitrate (NO_3^-) and nitrite (NO_2^-) anions. This method is attractive as it is biologically distinct from endothelial-NO synthase, and can be achieved easily via oral beetroot juice (BRJ) administration (30). Briefly, inorganic nitrate is swallowed, absorbed into the circulation, concentrated in the salivary glands and then re-secreted into the oral cavity, where commensal bacteria reduce NO_3^- to NO_2^- (31). This NO_2^- is then swallowed and absorbed into the circulation. BRJ supplementation improves vascular health (32, 33) and exercise tolerance (34) in many clinical populations. Combining higher intensity exercise training with exogenous NO

supplementation may optimize the beneficial effects of each treatment individually but remains to be tested in PM females**.**

Thus, the purpose of the present study was to determine whether acute calorically-matched HIE improves vascular health more than acute MIE in PM females, and whether NO_3^- supplementation impacts these responses. Further, factors such as plasma NO_2^-, body fat, time since menopause (TSM), baseline vascular health, and visceral adipose tissue (VAT) were studied to determine whether they could affect these responses. The primary hypothesis was that that HIE would improve brachial artery flow-mediated dilation (FMD) more than MIE, and that NO_3^- supplementation will further enhance these improvements. The secondary hypothesis was that a greater amounts of plasma NO_2^-, longer time since menopause (TSM), lower baseline FMD, lower fitness, and worse body composition will affect changes in FMD.

2. Methods

Experimental Design and Protocol

This study was a randomized, double-blind, placebo-controlled trial (NCT05221905). Following a screening visit, participants were randomized to one of two treatment arms; i) ~13 mmol of NO_3^- in the form of 140ml beetroot juice (BRJ); ii) or identical placebo (PL) with the nitrate extracted (<0.1 mmol nitrate), for 2 days prior to each testing visit, as well as 2-hours prior to each study visits (see *Supplementation Protocol* below). Each arm of the trial involved three randomized experimental visits that consisted of HIE, MIE, and a non-exercise control (CON) visit (see Figure 1). All study visits were performed by the same investigator, at the same time of day, and under the same sensory conditions. Participants were encouraged to maintain physical activity and diet habits throughout the study period. Participants were recruited from the University of Virginia and surrounding Charlottesville, Virginia area. All procedures were approved by the Institutional Review Board at the University of Virginia, and the study was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent.

Participants

A total of 22 (11 per group) estrogen-deficient PM females were included in this trial, defined as not having had a menstrual cycle for at least 1 year. Participants were nonsmokers,

sedentary or recreationally active (<3 days/week of exercise), with no use of hormone replacement therapy in the last year, and who had not had a hysterectomy or oophorectomy. Participants were normotensive (resting blood pressure <140/90 mmHg) and were not taking any medications that might interfere with NO_3^- supplementation (e.g., antihypertensives, nitrates, proton pump inhibitors, H_2 blockers).

Screening Procedures

Participants were phone screened for eligibility. Prior to all testing, participants abstained from all food or drinks other than water for at least 6 hours, caffeine for at least 12 hours, and exercise or alcohol for at least 24 hours. Participants met at the Clinical Research Unit (CRU) of the University of Virginia hospital and were consented, baseline measures taken (blood pressure, height, weight), and they were screened by a study physician. After enrollment, a venous catheter was placed in the antecubital fossa for serial blood lactate sampling during exercise. Participants performed an incremental symptom-limited cycle ergometer exercise test at the Exercise and Physiology Core Laboratory. Open-circuit spirometry was used to measure respiratory gases (Viasys Vmax Encore, Yorba Linda, CA). The exercise test consisted of 3-minute, 20-watt stages until participants reached volitional fatigue. The criteria for achieving VO_{2peak} specific for older females included maximum HR within 10 bpm of age-predicted HR-max, respiratory exchange ratio (RER) > 1.10, and a rating of perceived exertion (RPE) > 17) (35). Blood lactate was sampled in the last 30 seconds of each stage, analyzed using a YSI 2300 STAT Plus (Yellow Springs, OH) and the resultant data plotted to determine that lactate threshold (LT) (36).

On a separate day (at least 48 hours later), participants completed a DEXA scan (Hologic Horizon) and baseline vascular testing visit which consisted of measures of blood pressure (BP), pulse wave velocity (PWV), and brachial artery flow-mediated dilation (FMD) prior to any beetroot juice supplementation. This visit started with participants resting in a supine position in a dark, quiet thermoneutral room for 10 minutes. BP and PWV testing was completed with a SphygmoCor Xcel model (AtCor Medical, Itaska, IL) following manufacturer instructions.

Subsequently, FMD was performed according to established guidelines (37, 38). Briefly, participants laid in a supine position with their arm extended and with their hand supinated, while an automatic inflation blood pressure cuff (AI6 Arterial Inflow System, Hokanson Inc., Bellevue, WA) was placed on the forearm directly below the elbow. Brachial artery diameter and

blood velocity were recorded at rest with a high-resolution 7.5MHz linear array transducer using doppler ultrasound (uSmart 3300 Ultrasound System, Terason, Burlington, MA). A probe holder was used to stabilize the transducer, and both anatomical notes as well as images of the artery were taken to ensure accurate repeat testing placement. An EKG trigger was used to capture images during end-diastole of the cardiac cycle. After resting measures, the cuff was inflated to 200 mmHg for 5-minutes while the participant was encouraged to remain as still and relaxed as possible. Occlusion measures were taken the final 10 seconds of the occlusion period, and measures continued for 2-minutes after cuff deflation. Automated edge detection software (Medical Imaging Applications, Coralville, IA) was used to evaluate diameter measurements. All images were coded to ensure blinding for analysis. Participants were then given either BRJ or PL by a blinded study investigator. Participants were given instructions about when to consume the supplement prior to the remaining experimental testing visits, and provided a form to log supplementation times.

Within each experimental condition (HIE, MIE or CON) Δ FMD for each timepoint was calculated as the difference in FMD at each timepoint (i.e., 60, 90, 120, etc.) from the FMD at timepoint 0 for the same study visit. Peak Δ FMD within each condition was determined as the largest Δ FMD for each individual participant across timepoints.

Supplementation Protocol

Participants were block-randomized (matched for baseline characteristics of FMD, PWV, VO_{2peak}, age, BMI, abdominal visceral fat (VAT), and time-since-menopause) to one of two treatment arms. Participants were assigned to either consume ~13 mmol of NO_3^- daily via two 70 mL bottles (morning and night) of nitrate-rich beetroot juice (BRJ), or a nitrate-depleted beetroot juice (PL) (<0.1 mmol) with identical taste and appearance (Beet It, James White Drinks Ltd., Ipswich, United Kingdom). Participants consumed the beverages for two days prior to study visits and consumed two bottles two-hours prior to the beginning of each study visit to account for the time course of nitrate and nitrite bioavailability (39, 40). Participants were instructed to avoid high nitrate foods (i.e., spinach, arugula, celery, etc.) throughout the study period, as well as to avoid factors that could impact oral and microbial environment, such as mouthwash or antibiotics (41–43). A 24-hour dietary recall was employed during the study visits to ensure low-nitrate diets were adhered to (data not reported).

Experimental Visits

Each treatment arm of the study (BRJ or PL) consisted of three experimental visits; HIE, MIE, and a CON visit, assigned in a randomized order. Upon arrival to the laboratory, participants laid supine for 5-minutes before undergoing a blood draw. Participants then underwent a repeat of the vascular tests administered during the screening visit (Timepoint 0 min). Following vascular testing, participants were unblinded to the experimental visit allocated to that day (HIE, MIE, or CON).

Exercise sessions consisted of calorically-matched exercise at either HIE or MIE, with the goal of reaching 200 kcal of energy expenditure. Gas exchange and heart rate were recorded continuously during the exercise sessions (Cosmed Quark, Cosmed USA Inc., Concord, CA). Time to expend 200 kcal was estimated based on the VO_{2peak} test, with real-time adjustments were made if energy during the exercise sessions was expended at a rate other than expected.

a) HIE occurred at a power output associated with the power output that was 75% of the difference between LT and VO_{2peak} (75% Δ). If participants could not maintain this power output until 200 kcal were expended, power output was decreased to 50% Δ and then to 25% Δ if necessary.

b) MIE occurred at the power output associated with LT.

c) CON visits consisted of passive rest.

After each experimental condition and 5 minutes of supine rest, participants underwent a post-condition blood draw to determine changes in blood markers from pre- to condition-exercise. Vascular tests were repeated every 30-minutes for 2 hours (e.g., timepoints 60, 90, 120, 150, 180 min).

Statistics

Unpaired T-tests were used to determine differences between participant characteristics at baseline. The highest change in FMD from pre- to post-experimental condition (Peak Δ FMD) was determined for each participant. Two-way ANOVA was used to determine any differences between treatments and time within experimental conditions (i.e., exercise intensity visits), as well as to compare exercise parameters between experiment conditions. Simple linear regression models were utilized to examine the relationship between supplemented plasma nitrite, body fat,

VO_{2peak}, VAT, time-since-menopause, and baseline FMD with Peak Δ FMD. All statistical analysis was conducted using GraphPad Prism Version 9.3 (GraphPad Software, La Jolla, CA, USA). The study schematic was created using BioRender.com. Statistical significance was determined *a priori* when $p < 0.05$, and Cohen's *d* was interpreted as having trivial effects (0.0 – 0.19), small effects (0.20-0.49), medium effects (0.50 – 0.79) and large effects (>0.80) (44).

3. Results

Participant Characteristics

A total of 22 post-menopausal females completed the study (11 per treatment arm). Groups were matched for age, time-since-menopause, body composition, fitness, and vascular health (Table 1).

Blood Markers

As expected, plasma nitrite (NO_2^-) was significantly elevated in the BRJ treatment (Figure 2).

Exercise Parameters

There were no differences between treatment groups for exercise parameters during the screening VO_{2peak} testing (i.e., peak power, heart rate, RER, RPE, blood lactate concentration, or % of VO_{2peak} that lactate threshold occurred in; all $p > 0.05$; data not shown). Data from the experimental exercise bouts are displayed in Table 2 showing similar energy expenditure between groups, while higher exercise intensity resulted in higher heart rate, RPE, and a shorter duration ($p < 0.05$).

Endothelial Function

Peak Δ FMD revealed a significant treatment ($p = 0.03$) and exercise intensity effect ($p < 0.01$), with a trending interaction effect ($p = 0.09$; Figure 3). Multiple comparison's revealed significant differences between BRJ+HIE and all other conditions, as well as BRJ+MIE vs CON+BRJ (all $p < 0.05$).

During the CON visit, two-way ANOVA for treatment x time revealed no significant effects of treatment ($p = 0.62$), time ($p = 0.97$), or an interaction ($p = 0.57$). Cohen's *d* effect size

calculations revealed trivial effects on Δ FMD throughout the 180 mins of serial testing during the CON testing visit, regardless of treatment (d < 0.5 for PL vs BRJ; Figure 4A).

During moderate intensity exercise, a significant treatment effect (p = 0.03), but not effect of time (p = 0.61) or an interaction (p = 0.68) was revealed. Cohen's d effect size calculations revealed no effects for Δ FMD in the PL group for the duration of the testing (180mins) but moderate to large effects for Δ FMD between the BRJ treatment versus PL immediately after exercise (~60min timepoint) and throughout the remainder of the study visit (~1% improvement; Figure 4B).

During high intensity exercise, there was a significant time effect (p < 0.01), but not treatment effect (p = 0.48), or an interaction (p = 0.28). Multiple comparisons revealed a significant difference between 120min and both 60min (p = 0.02) and 90min (p = 0.01) timepoints. Cohen's d calculations appeared to display a biphasic response on Δ FMD in the BRJ treatment group compared to the PL group with an initial decrease (d = -0.37), followed by a subsequent increase (~2% improvement; Figure 4C). Differences between PL vs BRJ peaked at the 180min timepoint in which BRJ had a medium effect on elevating FMD (d = 0.73).

Predictors of Peak Δ FMD

Post-supplemented plasma NO_2^- had a positive correlation with Peak Δ FMD (R^2 = 0.49; p = 0.001), while body fat (%) had a negative correlation with Peak Δ FMD (R^2 = -0.29; p < 0.01). No other factors predicted Peak Δ FMD (Table 3). Further exploration revealed a larger baseline brachial artery diameter in those with higher body fat (R^2 = 0.18; p = 0.05), and that those with a larger brachial artery diameter had a lower Peak Δ FMD (R^2 = 0.22; p = 0.03).

Blood Pressure and Pulse Wave Velocity

MAP was significantly different across time (p < 0.01) for all three experimental conditions, with no treatment or interaction effects (p > 0.05). Multiple comparisons revealed significant differences between timepoint 0 and 180mins (p < 0.001) during CON, suggesting a slow increase in MAP over the course of testing (~2mmHg). During MIE, MAP at 90min was significantly reduced from timepoints 0 (p < 0.01), 60min (p < 0.01), and 180min (p < 0.01), while timepoint 120min was lower than 180min (p < 0.01). A similar pattern was seen during HIE, with MAP during timepoint 90min was significantly lower than from timepoints 0 (p <

0.01), 60min ($p < 0.01$), and 180min ($p < 0.01$), while MAP during timepoint 120min was different from timepoints 0 ($p < 0.01$), 60 ($p < 0.01$), and 180min ($p < 0.01$).

PWV significantly increased over time during the CON visit ($p < 0.001$), with no treatment ($p = 0.27$) or interaction effects ($p = 0.48$). Multiple comparisons showed that timepoints 150 ($p < 0.01$) and 180mins ($p < 0.01$) were significantly higher than timepoint 0. PWV was not significantly altered by time ($p = 0.16$), treatment ($p = 0.58$), and there was no interaction ($p = 0.50$) during MIE. Similarly, PWV was not significantly altered by time ($p = 0.07$), treatment ($p = 0.50$), and there was no interaction ($p = 0.87$) during HIE (Figure 5).

4. Discussion

To our knowledge, this is the first study to examine the effects of NO_3^- supplementation and different intensities of exercise on vascular health in PM females. The primary findings of this study are that oral NO_3^- supplementation augments acute post-exercise Peak Δ FMD in estrogen-deficient PM females. This augmentation appeared to be greater after high intensity exercise (Figure 3). When taking into consideration the effects of time, NO_3^- supplementation improved FMD after MIE (Figure 4B), whereas FMD was improved after HIE regardless of NO_3^- supplementation (Figure 4C). In agreement with our hypothesis, plasma NO_2^- levels correlated positively with Peak Δ FMD, while body fat percentage correlated negatively with Peak Δ FMD (Table 3). However, we did not see relations with time-since-menopause, fitness, VAT, nor baseline FMD. Finally, both MAP and PWV increased over time during CON, which was ameliorated by exercise regardless of treatment (Figure 7).

The primary hypothesis of this study was that HIE would improve FMD compared to MOD and CON, and that NO_3^- supplementation would augment these responses. As changes in FMD post-acute exercise predict the exercise training-induced changes in FMD (45). the present findings suggest that long-term exercise training at high intensities may provide a greater stimulus for vascular adaptation than moderate intensity exercise in PM females, and that the combination of BRJ+HIE seems to offer the greatest improvements in FMD. This is notable as a 1% improvement in FMD is considered clinically meaningful, and is associated with a relative risk reduction of CVD events of ~10% (46). Consuming an exogenous source of NO may aid with the decrease of endogenous NO that occurs secondary to the loss of estrogen in menopause.

Effects of Exercise Intensity and Inorganic Nitrate on FMD

Studies examining acute changes to FMD after exercise in PM females are equivocal and results appear to be dependent on factors such as adiposity and time-course of FMD measurement post-exercise (47, 48). We hypothesized that HIE would produce greater changes to acute post-exercise FMD responses because of the greater elevation of shear-stress and overall larger stimulus that this exercise induces when compared to MIE. Supporting this, previous data from our laboratory in 2014 have shown that in younger lean adults, an improvement in FMD is observed 2-hours post-HIE ($\Delta 3.2 \pm 0.5\%$) which is largely sustained 4-hours post-exercise (47). However, other studies have shown that in PM females, acute post-exercise FMD was improved only ~1% after high-intensity interval training (HIIT) and was unchanged with MIE, findings which were not statistically significant (48). Interestingly, the improvements in FMD after HIIT in that study were seen immediately after exercise, with FMD returning to baseline 1 hour later.

In the present study, FMD trended towards a decrease during the initial timepoints after HIE in the BRJ treatment group (i.e., 60-90 min) before rebounding and improving afterward in the later timepoints in both HIE conditions (120-180 min). These rebounding improvements in FMD resulted in an elevation in FMD in the BRJ+HIE condition that was greater than the PL+HIE group when evaluating the data as Peak Δ FMD post-exercise (Figure 3). Interestingly, 2-way ANOVA for HIE showed a time but not a treatment or interaction effect. This was likely driven by the bi-phasic FMD response of both treatment groups over time. There was an effect size of 0.73 for FMD response between treatments at 180mins with BRJ increased ~2% and PL 1% from baseline (Figure 4C). The initial lack of response or worsening of FMD after HIE is commonly reported in the literature (45, 49) and has been attributed to the elevated exercise-induced increased shear rate resulting in a larger baseline diameter of the artery (49). Indeed, the initial decrease in FMD after BRJ+HIE observed here was accompanied by the greatest improvement in BL diameter throughout the post-exercise period, although this was not significantly different (BRJ+HIE: +0.17mm; BRJ+MIE: +0.14mm; PL+HIE: +0.06mm; PL+MIE; +0.07mm; data not shown).

While studies have reported that FMD after acute MIE increased FMD as high as ~4.6% in PM females (which was not further enhanced by estradiol (50, 51)), long-term exercise training at moderate intensities have failed to improve FMD (13) unless baseline FMD is impaired (17, 19) or unless co-administration of estrogen is administered (9). The present study

showed no change over time from baseline in the PL+MIE condition on average compared to pre-exercise FMD, results which have been shown previously in PM females (52). In the BRJ+MIE condition however, sustained improvements in FMD were noted (Δ 0.5-1%) and were significantly different from PL+MIE (p = 0.03; Figure 4B). This may suggest that although chronic moderate intensity exercise training has failed to result in improvements in FMD in PM females in some studies, exogenous consumption of direct NO donors such as inorganic nitrate may aid in recovering the exercise-induced improvements in FMD to levels similar to what is seen with concomitant estradiol use. This provides a potential low-cost alternative for PM females who are reluctant to undertake HIE training as the addition of BRJ to MIE appears to provide an additive effect that results in higher FMD than HIE alone. In addition, the improvement in peak FMD observed in the HIE condition may be driven in part by the addition of BRJ, suggesting that PM females willing to undergo HIE training might benefit from BRJ supplementation (Figures 3 and 4).

Predictors of FMD Response

Predicting the acute improvements of FMD after exercise should allow for the pursuit of more precision approaches to exercise prescription in PM females. In the present study, the greatest predictor of Peak Δ FMD was the supplemented plasma NO_2^- level, showing that those with greater plasma NO_2^- had greater changes in FMD (Table 3). This is not surprising as FMD is thought to be ~67% NO-mediated (56) and NO_2^- is reduced to NO in situations of lower partial oxygen pressure and lower pH (57). Interestingly, baseline supplemented FMD did not differ between treatments (Timepoints 0), suggesting that elevated plasma NO_2^- from BRJ alone does not improve resting FMD. As the BRJ+HIE resulted in the greatest Peak Δ FMD, it appears plausible that NO_2^- may convert to NO to a greater extent specifically after HIE. Indeed, HIE is well known to require the recruitment of a greater proportion of fast-twitch muscle fibers. This will result in decreased PO_2 and an increased acidic environment to the muscle, both of which are which are known to facilitate reduction of NO_2^- to NO (58). Whether increasing plasma NO_2^- levels (potentially by a larger dose of inorganic nitrate) would further improve Peak Δ FMD or whether there is a ceiling effect is unknown.

Contrary to our hypothesis, neither baseline FMD, fitness, time-since-menopause, nor VAT were predictive of Peak Δ FMD (Table 3). Body fat percentage was negatively correlated

with Peak Δ FMD (Table 3). Previous work from our lab has suggested that improvements in FMD after HIE were only seen in lean individuals, but not in obese individuals (47). Those findings were likely due to the larger baseline diameter in the obese group, findings which are similar to those seen in the current study ($R^2 = 0.18$, $p = 0.05$; data not shown). Thus, it appears that obesity blunts the exercise-mediated improvements in FMD and that this does not change after BRJ supplementation. Because the same absolute NO_3^- dose was provided to each person (~13 mmol per day), it is unclear if participants who have a higher body fat percentage may experience greater improvements in FMD post-exercise if given a larger dose of NO_3^-.

Effects of Exercise and Inorganic Nitrate on Blood Pressure and Pulse Wave Velocity

Acute exercise often induces elevations in blood pressure during exercise activity, followed by mild rebound hypotension after exercise caused by peripheral vasodilation (59). Post-exercise hypotension has recently been shown not to be altered by NO_3^- supplementation, findings that are similar to ours (Figure 5). Similarly, acute post-exercise PWV has shown to be either decreased or normalized compared to control conditions (60), results that are similar to our findings (Figure 5). While FMD responses to exercise training are blunted in PM females, exercise-mediated improvements in blood pressure and PWV are still possible in PM females (61). Thus, as NO_3^- does not seem to enhance these effects acutely (Figure 5), it appears the beneficial impacts of NO_3^- and exercise on FMD shown here occur via mechanisms not related to changes in blood pressure and arterial stiffness.

Limitations

There are several limitations in this study. First, due to the preliminary nature of this study, there is a small sample of participants (n = 11 per treatment arm) which did not provide adequate power to perform a three-way ANOVA for treatment x intensity x time. Because of this, two-way ANOVAs within each exercise intensity as well as effect sizes were utilized to examine differences between treatments within each condition. There were, however, adequate statistical power to examine the primary outcome of Peak Δ FMD.

Another limitation of this study is that the sample of PM females were in relatively good vascular health. Only 7 of the 22 participants had a baseline FMD that is considered "impaired" for PM females (< 4.5% FMD). This may have caused an under report of the potential FMD

improvements when extended to a less healthy population as previous data suggest that individuals with impaired compared to normal baseline FMD improve to a greater extent (62). Similarly, the participants in the present study were not on any anti-hypertensive medications, and thus it is unknown if these benefits may be blunted in those individuals. The majority of the participants in this study were Caucasian (n=21) with only one participant from a Hispanic background. While resting and postprandial FMD has previously been shown by our group to be unaffected by race in PM females (18), we can't address whether post-exercise FMD may be impacted differently across races with and without BRJ. Finally, the follow-up period for this study was ~2 hours post-exercise, and thus it is unknown for how long these effects on FMD may persist post-exercise.

5. *Conclusion*

These preliminary data suggest HIE combined with BRJ improves Peak Δ FMD responses to exercise in PM females and supports our hypothesis that higher intensities of exercise and/or exogenous sources of NO_3^- may be necessary to increase NO bioavailability and endothelial function in post-menopausal females. When taking into account time course of FMD changes post-exercise, high-intensity exercise improves FMD independent of treatment. Additionally, BRJ+MIE may improve Peak Δ FMD more than MIE alone, and observing the time course of FMD changes post-exercise reveals that BRJ+MIE may offer an option for individuals who are not willing to participate in HIE. The time course of improvements in FMD post-exercise appear to differ with different exercise intensities, and this is not impacted by NO_3^- supplementation. Plasma NO_2^- levels were significantly correlated with Peak Δ FMD, while body fat was negatively correlated with Peak Δ FMD. Finally, blood pressure and pulse wave velocity rises were mitigated by exercise, independent of exercise intensity or supplement treatment. These findings reveal a potential precision approach to improve vascular health in PM females, a population that is growing and is experiencing unmitigated insult to their endothelial function. Further research should examine whether chronic exercise training interventions performed at high vs moderate intensities and paired with NO_3^- supplementation may rescue the impairments in endothelial function that are seen in post-menopausal females.

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Figure 1: Study Schematic.

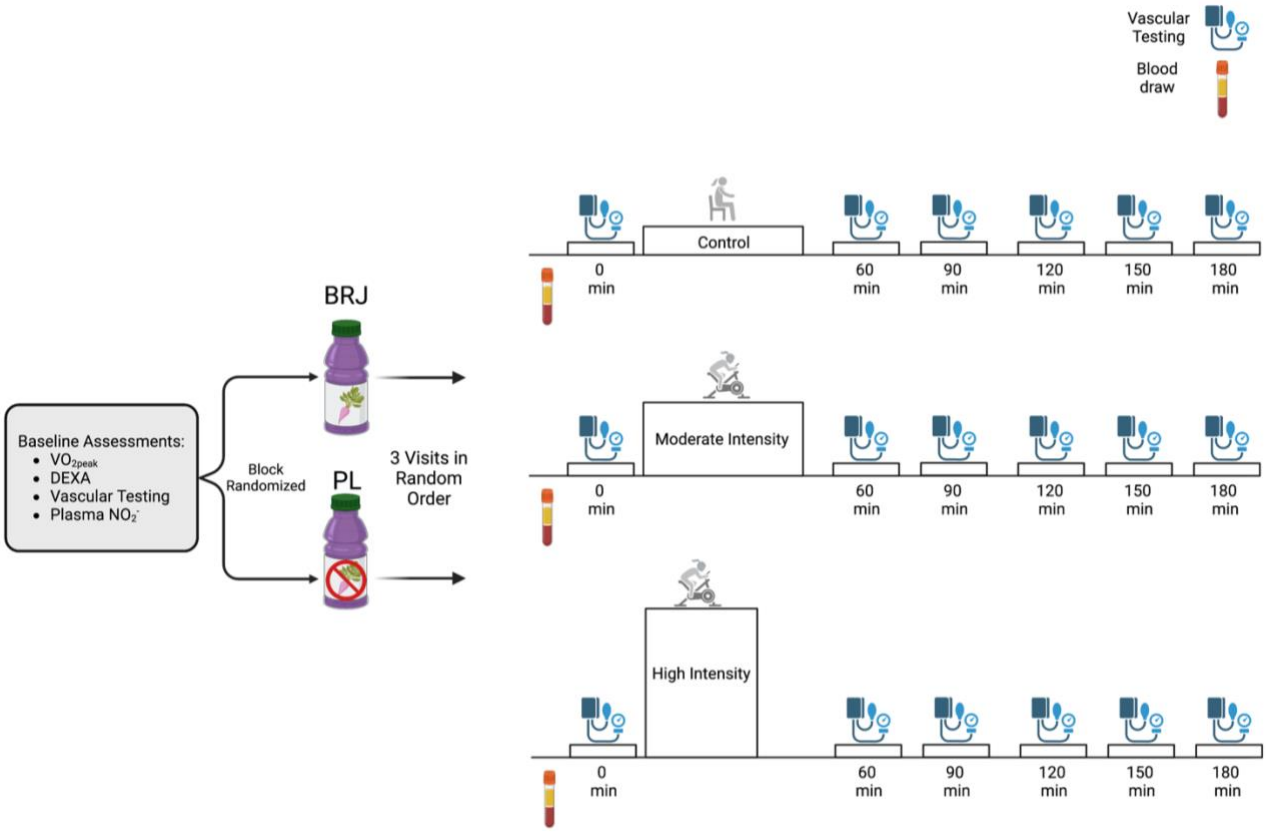

Table 1: Participant Characteristics

Variable	PL	BRJ	*p*-value
Age (yr)	61 ± 2	60 ± 2	0.36
Time Since Menopause (yr)	10 ± 6	9 ± 5	0.20
Body Mass Index (kg/m^2)	28 ± 5	28 ± 7	0.41
Body Fat (%)	37 ± 6	38 ± 6	0.38
Visceral Adipose Tissue (g)	380 ± 180	447 ± 205	0.21
VO_{2peak} (ml/kg/min)	24 ± 4	24 ± 5	0.41
Flow-Mediated Dilation (%)	6.3 ± 2.2	6.0 ± 2.2	0.72
Mean Arterial Pressure (mmHg)	92 ± 7	91 ± 7	0.65
Pulse Wave Velocity (m/s)	7.4 ± 1	7.3 ± 1	0.81

Mean + SD. N = 11 per treatment.

Table 2: Experimental Exercise Parameters

	Moderate-Intensity		High-Intensity	
Variable	PL	BRJ	PL	BRJ
Power, W (% $Power_{peak}$)*	56 ± 15 (42%)	69 ± 22 (48%)	114 ± 18 (85%)	124 ± 31 (87%)
VO_2, ml/kg/min (% VO_{2peak})*	15.8 ± 3.8 (65%)	15.7 ± 2.6 (67%)	19.7 ± 4.1 (82%)	19.7 ± 4.7 (83%)
HR, bpm (% HR_{peak})*	112 ± 25 (69%)	115 ± 15 (72%)	143 ± 7 (87%)	138 ± 9 (86%)
Borg-RPE*	12.0 ± 1	12.2 ± 1	17.2 ± 1	17.3 ± 1
Duration, min*	36 ± 4	33 ± 8	29 ± 2	25 ± 4
Energy Expenditure (kcal)	198 ± 20	200 ± 15	200 ± 22	199 ± 17

Mean + SD. N = 11. *denotes significant difference between exercise intensities.

Figure 2: The effects of inorganic nitrate (BRJ) or placebo (PL) on plasma nitrite.

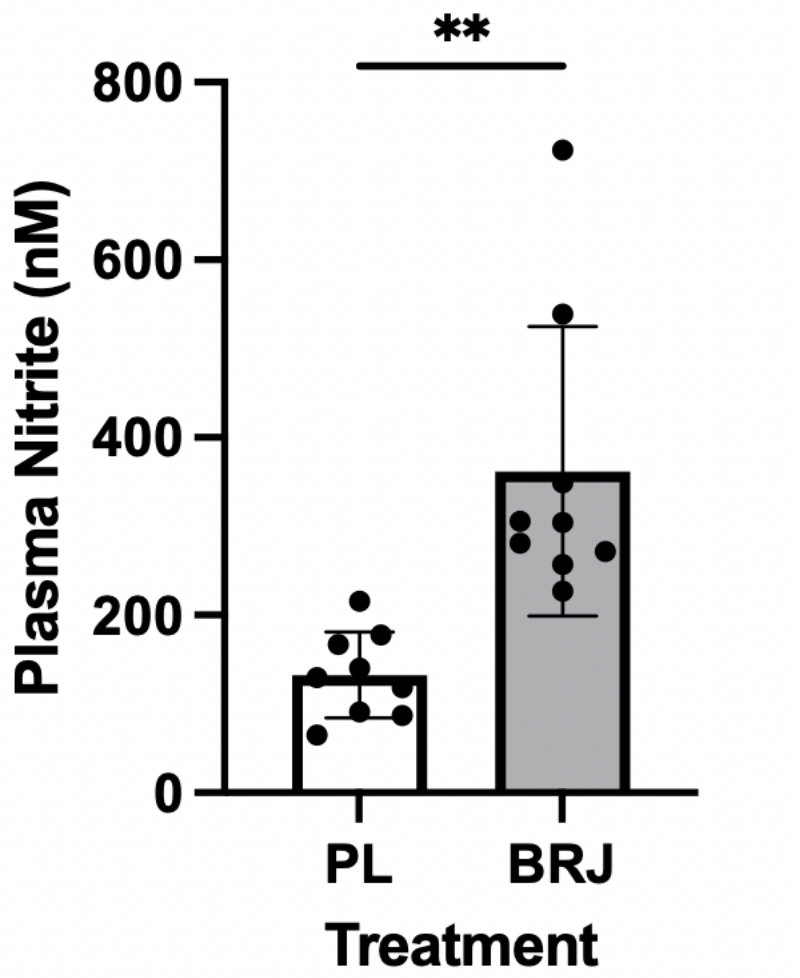

Figure 3: The effects of inorganic nitrate (BRJ) or placebo (PL) and exercise intensity on Peak Δ FMD.
Figure Legend: ***** denotes significantly different from PL+CON, PL+MIE, PL+HIE, and BRJ+CON. † denotes significantly different from BRJ+CON. CON: control; MIE: moderate intensity exercise; HIE: high intensity exercise.

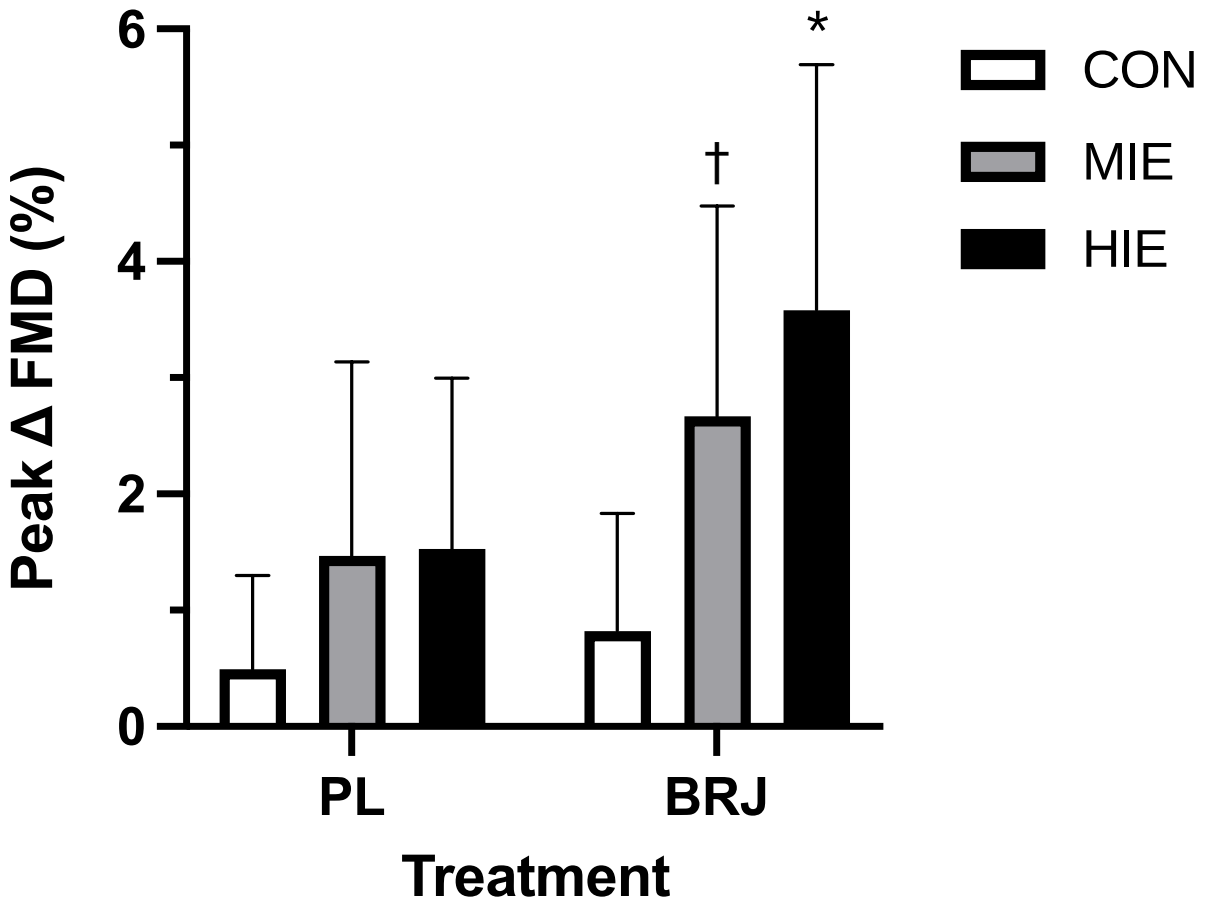

Figure 4: The effects of inorganic nitrate (BRJ) or placebo (PL) on change in flow-mediated dilation (Δ FMD) from timepoint 0 during A. Control, B. Moderate Intensity Exercise, C: High Intensity Exercise.
Figure Legend: * denotes significantly different from PL within exercise intensity. † denotes significantly different from timepoint 120 within exercise intensity.

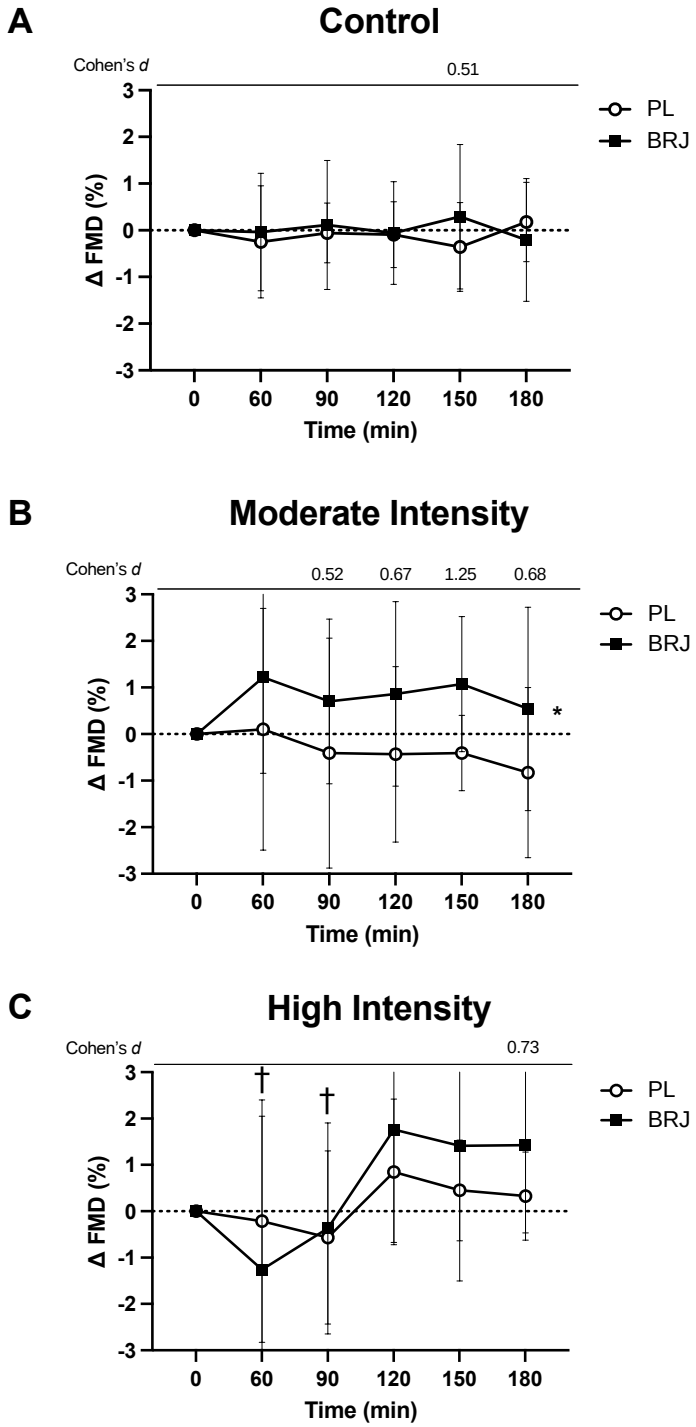

Table 3: Predictors for Peak Δ FMD

Variable	R^2	*p*-value
Supplemented Plasma NO_2^- (nM)	0.49	< 0.01
Body Fat (%)	0.29	< 0.01
Peak Δ MAP (mmHg)	0.11	0.14
VO_{2peak} (ml/kg/min)	0.12	0.12
Baseline FMD (%)	0.04	0.40
Visceral Adipose Tissue (g)	0.01	0.64
Time Since Menopause (yr)	0.01	0.63

Figure 5: The effects of inorganic nitrate (BRJ) or placebo (PL) on mean arterial pressure (MAP; left) and pulse wave velocity (PWV; right) during A. Control, B. Moderate Intensity Exercise, C: High Intensity Exercise.

Figure Legend: Only a significant main effect of time was observed for MAP in each condition, and for PWV during CON. a denotes significantly different within measure from timepoint 0. b denotes significantly different within measure from timepoint 60. c denotes significantly different within measure from timepoint 90. d denotes significantly different within measure from timepoint 120. e denotes significantly different within measure from timepoint 150. f denotes significantly different within measure from timepoint 180.