THE EFFECTS OF OVARIECTOMY AND SOY DIET ON VASCULAR FUNCTION IN FEMALE C57BL6 MICE

by

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As people begin to live longer, studying age-related disease becomes more important. Age is a major risk factor for Alzheimer's disease (AD), a prominent neurodegenerative disease, and cardiovascular diseases. Females develop AD at much higher rates than males and all signs point to sex hormones as a cause. Estrogen drastically decreases post menopause, and it has been suggested that estrogen deficiency is a contributing factor to the sex differences seen in AD and other age-related diseases. The vascular system plays an important role in aging. A characteristic of aging in the vascular system is stiffening of larger arteries. Large artery stiffening is detrimental because it leads to an increase in pulse pressure and stress on the microvasculature. Decreased estrogen activity results in increased production of reactive oxygen species, causing tissue damage and dysfunction. Soy also has been seen to be a protective factor against symptoms of age-related disease due to its role as a phytoestrogen, thus showing the potential importance of soy. This study aimed to explore the effects of estrogen depletion post menopause and the effects of a soy diet in relation with estrogen loss. We utilized a mouse model of ovariectomies to mimic estrogen loss post menopause and studied cognitive function, motor coordination, and vascular function. We found that

soy supplementation positively affected cognition but not motor coordination, while loss of estrogen had no effect on both cognition and motor coordination. Arterial stiffness was not impacted by either ovariectomy or soy, but nitric oxide mediated dilation was impaired with estrogen loss and was recovered with soy diet supplementation. Altogether, these results suggest that even with loss of estrogen, soy could play a protective role in endothelial function while it does not affect cognitive function, motor coordination or in vivo large artery stiffness.

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Introduction

The average age of the United States population is set to triple by 2050 as people are living longer than ever in part due to advancements in medicine and changes to lifestyle habits. Age is a major risk factor for age-related diseases such as Alzheimer's disease (AD) and females comprise two-thirds of patients who have AD (Alzheimer's Association et al., 2013). Previous research has pointed towards the vasculature as a key factor in aging and the development of age-related diseases (Kehmeier & Walker, 2021). Furthermore, estrogen has been seen to be a protective factor against various cardiovascular and AD disease pathologies, with circulating estrogen decreasing drastically after menopause (Kehmeier & Walker, 2021). However, the primary causes for why women are at an increased risk for AD and how estrogen plays a protective role in cardiovascular disease are relatively unknown. Therefore, studying the role of the aging vascular system in such age-related diseases is especially important.

Vascular Function

Age is the greatest risk factor for cardiovascular diseases. Past research has indicated that chronic inflammation is associated with a wide variety of age-related diseases (Chung et al., 2009). Increased inflammation in the vascular system is attributed to a reduction in vascular nitric oxide (NO) availability (Donato et al., 2015) (Thorin-Trescases et al., 2018). NO plays an important role as a vasodilator in the vascular system, and a key reason for its age-related reduction in bioavailability is

oxidative stress. Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) and antioxidants to combat ROS.

An understanding of the vasculature's role in age related disease is also important. A characteristic of aging in the vascular system is stiffening of larger arteries. These large arteries contain important structural proteins such as collagen and elastin. With aging, there is a decrease in overall elastin content and an increase in elastin fragmentation. This is further accompanied by an increase in collagen content and crosslinking (Thorin-Trescases et al., 2018). Collagen crosslinking is a cause for arterial stiffness; thus, collagen plays an important role in aging. Compliance of these large arteries is important for absorbing the pulsatile flow and protecting smaller arteries from turbulent flow and high pressure (Thorin-Trescases et al., 2018). However, compliance is negatively impacted by collagen cross linking and inflammation. When these arteries experience a decrease in compliance, it results in an increase in pulse pressure and stress that occurs with vascular stiffening. This increase in pulse pressure and stress in the larger arteries results in damage to smaller cerebral arteries in the brain. Therefore, studying the vasculature in the brain is necessary to fully understand the mechanisms of aging that evolve could into AD.

The endothelium, or innermost layer of cells that line the vessels, plays an important role in vascular health by adjusting to pulse pressure and regulating myogenic tone. The release of active molecules such as NO from the endothelium helps modulate vasoactive responses (Seals et al., 2011). When healthy, the endothelium is in a delicate

balance of many factors such as oxidants, vasodilators, vasoconstrictors, and inflammatory molecules. The endothelium becomes dysfunctional when this balance is lost (Donato et al., 2015). One way to measure if the endothelium is dysfunctional is by measuring endothelial dependent dilation (EDD). EDD, which is measured through ex-vivo vessel studies, is predictive of vascular diseases through measuring the contributions of the endothelium in vasoactive responses. (Donato et al., 2015). Endothelial dysfunction develops with aging, seen with a decrease in EDD and an increase in inflammation and cognitive impairment (Donato et al., 2015) (Thorin-Trescases et al., 2018). A key reason for reduced EDD and NO bioavailability is oxidative stress, as the superoxide anion can inactivate NO thus leading to impaired EDD. Thus, vascular function is an important facet to understanding such age-related diseases.

Sex Steroid Hormones

Sex differences seen in aging are attributed to the decline in circulation of sex hormones. In females, the primary circulating hormone is estrogen, with progesterone being another prominent hormone. In males the primary circulating hormone is testosterone, a hormone which is also seen in females but at lower circulating amounts (Al-Azzawi & Palacios, 2009). With aging in females, levels of progesterone and testosterone decrease, and these can also have major implications in aging, but levels of estrogen decrease the most drastically post menopause in females. Steroid hormones play pivotal roles in modulating arterial function and while estrogen in particular helps

decrease arterial stiffness, its role in the cerebral vasculature needs to be elucidated (Iorga et al., 2017).

Past research highlights the importance of soy as a diet supplementation in postmenopausal women (Azadbakht et al., 2007). Soy contains phytoestrogens, which have been helpful in treating menopausal symptoms along with having beneficial effects on cardiovascular and metabolic diseases (Sirotkin & Harrath, 2014). Furthermore, phytoestrogens consumed through soy nut were shown to improve certain markers of endothelial function and inflammation (Azadbakht et al., 2007). Additionally, phytoestrogens have been shown to increase the release of NO from the endothelium, further emphasizing the potential protective role of soy and phytoestrogens in aging of postmenopausal women (Azadbakht et al., 2007).

Sex Steroid Hormones and Vascular Function

Despite the various roles sex steroid hormones have on the vasculature, many studies investigating the effects of aging on the cerebral vasculature have neglected to consider the roles of these hormones, whether in high or low concentrations, on cerebral artery dysfunction.

The most dominant form of estrogen is 17β estradiol, which is majorly produced and secreted from the ovaries in premenopausal women. Estrogen production drastically decreases in post-menopausal women (Iorga et al., 2017). The decrease in estradiol is associated with an increase in inflammation in the vasculature due to estrogen's antioxidant effects (Ungar et al., 1993). Estrogen reduces the production of ROS through the production of antioxidants. With a decrease in estrogen, its antioxidant

effects are mitigated, thus leading to an increase in ROS and inflammation. Studies suggest that estrogen is very important for endothelial function along with improved mitochondrial function and reduced oxidative stress (Iorga et al., 2017). Estrogen can activate transcription of endothelial nitric oxide synthase (eNOS), therefore causing an upregulation of NO through both the genomic and non-genomic pathways (Chambliss & Shaul, 2002).

Estrogen can act on two major receptors: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Estrogen can also bind to a newly identified surface receptor, the G-protein coupled receptor, GPR30. ERα aids in neuroprotection and has been shown to decrease post menopause. ERα has been noted as the primary receptor in relation to estrogen and vascular function. ERβ expression increases post menopause (Pavón et al., 2012) leading to the production of ROS, decrease of NO bioavailability, and thus contributes to endothelial dysfunction (Reddy et al 2010 and Uddin et al). GPR30 is involved in the non-genomic pathway. Here, estrogen binds to the membrane bound receptors and rapidly activates transcription factors (Iorga et al., 2017). GPR30 is expressed both in vascular endothelial and smooth muscle cells and it has been hypothesized that GPR30 can enhance endothelium independent and dependent dilation of arteries (Broughton et al., 2010). Furthermore, activation of GPR30 leads to the activation of phosphorylated eNOS which solidifies its helpful role in vasodilation. Finally, studies have also shown the role of GPR30 in helping regulate the inflammatory response of the body (Zilin et al 2015). Female mice have more membrane localized GPR30 than male mice, indicating that GPR30 could play a major role in the vasodilatory response and sex differences seen in certain vascular diseases

(Iorga et al., 2017). Transcriptional levels of each of the three estrogen receptors has varied between different types of tissues as seen in previous studies. Aged female mice had increased mRNA levels of GPR30 in the aorta, kidney, and heart while mRNA levels of ERα decreased in these aortas (Gurrala et al., 2021). Estrogen also acts primarily through ERα and GPR30 to increase antioxidant production and thus reduce ROS and oxidative stress (Iorga et al., 2017).

Female Age-Related Vascular Dysfunction

Women are more predisposed to AD, thus studying the interactions between estrogen and aging in the vascular system is incredibly important. Vascular dysfunction has been shown to trigger a series of events that is associated with the progression of AD. The vascular system has an important role in nutrient supply and clearance of unwanted proteins. Aβ deposition induces endothelial dysfunction through increased ROS production which can lead to disruptions to microcirculation which precedes cognitive impairment (Thomas et al., 1996)(Cortes-Canteli & Iadecola, 2020). Aβ can interact with endothelial cells which in turn produces superoxide leading to prolonged inflammation (Thomas et al., 1996). Furthermore, the vasoactive properties of Aβ are only seen in the endothelium, indicating that Aβ mediated mechanisms function through the endothelium itself (Thomas et al., 1996). Furthermore, long term inflammation can occur, which induces large artery stiffening, endothelium damage, and large artery stiffening, further exacerbating AD symptoms.

As women are more likely to develop AD, current research has turned towards studying development of age-related diseases in women. Estrogen and its various

receptors have been seen to have positive effects on endothelium dependent dilation and inflammation (Azadbakht et al., 2007; Camporez et al., 2011). Ovariectomies, removal of the ovaries, from mice are being used as an animal model to mimic estrogen depletion post menopause in humans and study these interactions. Therefore, this study was focused on understanding the underlying mechanisms of estrogen in the absence of phytoestrogens through the use of ovariectomies and the utilization of a special soy free diet.

Methods

Animals

Female controls and ovariectomized C57BL/6J mice at six months of age were utilized for this study. Ovariectomies were performed at approximately four months of age. Mice were monitored post-operatively to ensure proper healing until discharged by veterinarian on call. Mice were housed together and kept in an animal care facility on a 12/12/-hour light-dark cycle at 24 degrees Celsius and were either on a normal chow diet or a soy-free diet with access to food and water ad libitum. Aortas and mesenteries were obtained for gene expression post euthanasia, and posterior cerebral arteries were collected for pressure studies. Furthermore, hearts, carotid arteries, kidneys, livers, gastrocnemius, and soleus muscles were dissected and stored for further studies. All protocols are approved by the IACUC of University of Oregon.

Pulse Wave Velocity

Aortic stiffness was measured via pulse wave velocity (PWV). Mice were anesthetized through a nose cone supplied constant stream of 2% isoflurane and oxygen and placed on a heating platform (Walker et al 2015). Probes were placed at the transverse aortic arch and abdominal aorta, velocities were measured using 20 MHz Doppler probes (Indus Industries, Webster, TX, USA), and recorded using the Indus software version 1.627. Distances between probes were measured and used to calculate PWV.

Accelerating Rotarod Test

To assess motor coordination, mice were tested using a Rotarod apparatus (Ugo Basile srl Rotarod for mouse model #47650) over a two-day period adapted from Xhako et al (2020). Before each testing day mice were habituated to the testing room for one hour prior to the beginning of testing. On the first day, mice were placed on an elevated rod rotating at 4 rpm to acclimatize them to testing conditions. Mice needed to stay on the rod for 90 seconds in order to acclimate to the rotarod rest. If the mice fell off before 90 seconds elapsed, they were placed on the rod again. On the second day, the mice were placed on the rod that accelerated 4 rpm to 40 rpm over the course of 5 minutes. Mice ran on the rod until they fell off or spun around two consecutive times and the time of fall was recorded by the apparatus. Mice received three trials spaced 10 minutes apart on the second day. Data was analyzed using GraphPad Prism.

Nest Building

Nest building protocol was utilized to assess cognition. Mice were left with a paper nestlet in an individual cage overnight and assessed on their ability to construct a nest. They were assessed the next morning according to a five-point nestling scale with various parameters (**Table 1**).

	Parameters
1	Nestlet is >90% intact
2	Nestlet is partially torn up
3	Nestlet is <50% intact but <90% is within a quarter of cage floor area
4	Flat nest with >90% of nestlet torn up, nest is flat with walls higher than mouse's body height
5	>90% of nestlet is torn up, nest is a crater with walls higher than mouse's body height

Table 1: Nest Building Score Parameters (replicated from Deacon 2012).

Cerebral Artery Endothelial Function

Endothelial function was assessed *ex vivo* in isolated, pressurized posterior cerebral arteries (PCAs) (Walker et al., 2015). First, animals were euthanized by exsanguination under isoflurane. PCAs were isolated from the brain and were cannulated onto glass capillaries inside a myograph chamber (DMT Inc., Hinnerup, Denmark) and tied with nylon (11-0) sutures. The chamber was filled with a physiological solution which contained 145 mM NaCl, 4.7 mM CaCl2, 1.17 mM MgSO4, 1.2 mM NaH2PO4, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, 3.0 mM MOPS buffer, and 1 g/100 mL BSA, pH 7.4. The chamber was kept at 37°C and the artery was pressurized to 50 mm Hg. All endothelium dependent dilation (EDD) was measured by first pre-constricting the artery with phenylephrine (PE). Following pre-constriction, increasing doses of acetylcholine (ACh) were administered, and the

percent dilation of the intraluminal diameter was calculated. Following increasing doses of ACh and insulin, arteries were incubated with N-nitro-L-arginine methyl ester (L-NAME), an eNOS inhibitor, for 30 minutes, after which the ACh and insulin responses were repeated in the presence of L-NAME. Lastly, endothelium independent dilation (EID) was measured by first pre-constricting the artery with PE and then administering doses of increasing concentration of sodium nitroprusside (SNP), an NO donor. Passive responses were also carried out on PCAs, in which pressure was slowly increased and luminal and outer diameter of the artery was measured.

Gene Expression

Expression of estrogen receptors (GPR30, ERα and ERβ) was studied through real-time quantitative PCR. RNA was isolated from the aorta and cerebral arteries of mice using the Qiagen RNAeasy Mini and Micro kit. Using a NanoDrop RNA samples were quantified, and the concentration of RNA per sample (ng/μL) was obtained. RNA was then converted to complementary DNA (cDNA) with the Qiagen QuantiTect Reverse Transcription Kit. Real-time PCR amplified the cDNA using primers that correspond to the estrogen receptors. For each animal, each gene was normalized to the level of expression of 18s rRNA, which is a housekeeping gene. This controls for different tissue concentration across the samples.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism. Group differences were determined using one-way ANOVA and Tukey's post-hoc analysis. For cerebral artery endothelial function group differences were determined by repeated-measured ANOVA and unpaired t-tests. Significance was determined by a p value of less than 0.05. All data is presented as mean ± SEM.

Results

Animal Characteristics

Characteristics of animals used throughout studies. There were no significant differences in characteristics, other than sham mice having significantly less white adipose tissue mass when compared to both OVX and Soy OVX groups.

	OVX	Sham	OVX + Soy Diet
n	17	17	10
Age (months)	6.35 ± 0.06	6.13 ± 0.07	5.84 ± 0.03
Body mass (g)	26.52 ± 0.53	24.20 ± 0.46	27.0 ± 1.14
Heart mass (mg)	0.18 ± 0.05	0.13 ± 0.004	0.13 ± 0.004
Percent heart: body mass	0.66	0.52	0.47
Spleen mass (mg)	0.11 ± 0.01	0.10 ± 0.003	0.10 ± 0.004
Percent spleen: body mass	0.41	0.41	0.36
WAT mass (mg)	1.53 ± 0.12	0.81 ± 0.08*	1.58 ± 0.16
Percent WAT: body mass	5.76	3.32*	5.86
Gastroc mass (mg)	0.18 ± 0.01	0.18 ± 0.01	0.19 ± 0.01
Percent gastroc: body mass	0.68	0.73	0.71
Soleus mass (mg)	0.01 ± 0.001	0.01 ± 0.001	0.01 ± 0.001
Percent soleus: body mass	0.04	0.05	0.05

Table 2: Characteristics of animals used for studies. Data are presented as mean ± SEM. *$p < 0.05$ denotes significant difference between Sham and Soy OVX and OVX

Instinctual behavior but not motor coordination is dependent on soy diet

Normal cognitive behavior was measured by analyzing nest building scores. Soy OVX mice had significantly higher nest building scores when compared to both Sham ($p = 0.0250$, **Figure 1)** and OVX groups ($p = 0.0401$, **Figure 1).** There were no significant differences between the Sham and OVX groups ($p = 0.9948$, **Figure 1)**.

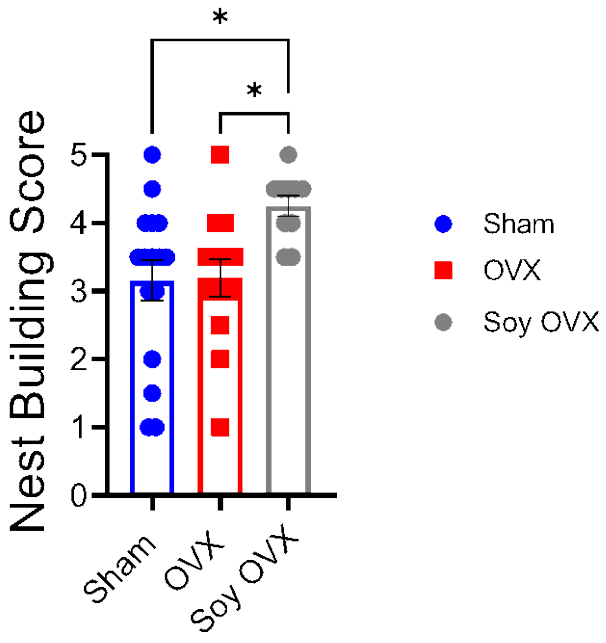

Figure 1: Nest building scores for Sham (n=15), OVX (n=13), and Soy OVX (n = 10) mice. *Indicates $p < 0.05$.

Motor coordination was tested via rotarod trials. Our data show that time spent on the rod did not differ between groups ($p = 0.3017$, $p = 0.3721$, $p = 0.9994$, Figure 2).

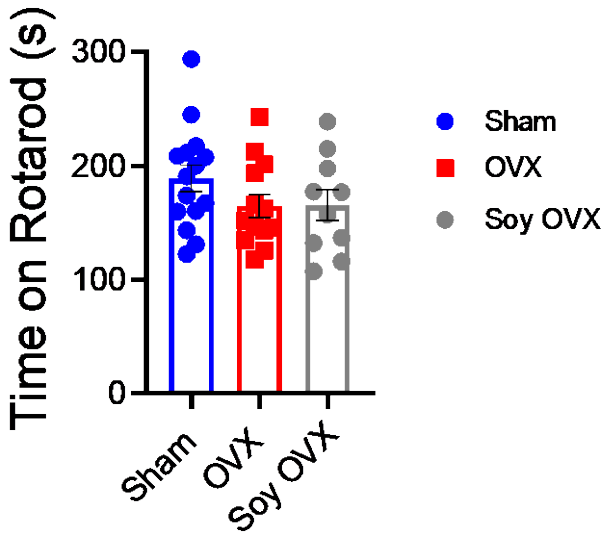

Figure 2: Average time spent on the Rotarod for Sham (n=15), OVX (n=13), and Soy OVX (n = 10) mice.

In vivo large artery stiffness was independent of OVX and soy status

PWV is a direct measure of aortic stiffness. PWV did not differ between groups and are independent of OVX and soy status ($p = 0.9256$, $p = 0.1225$, $p = 0.0738$, **Figure 3**).

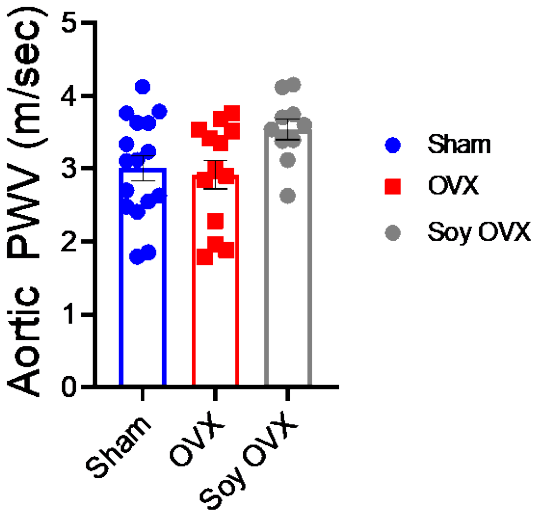

Figure 3: Aortic PWV for Sham (n = 15), OVX (n = 12), and Soy OVX (n = 10) groups.

Ex vivo cerebral artery endothelial function is dependent on OVX and soy

NO endothelium dependent dilation was significantly impaired in the OVX group compared to the sham group ($p = 0.0020$, **Figure 4**). However, there were no differences of the sham compared to the Soy OVX group ($p = 0.3184$, **Figure 4**). In the presence of the eNOS inhibitor, L-NAME, minimal dilation was seen and there were no differences between groups ($p = 0.8162$, $p = 0.8766$, 0.9480, **Figure 4**).

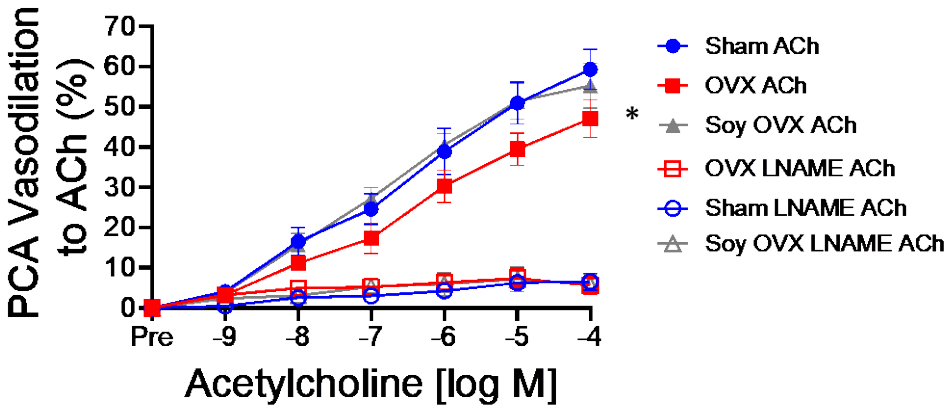

Figure 4: Endothelium-dependent dilation to acetylcholine in the absence or presence of L-NAME in posterior cerebral arteries in Sham (n = 15), OVX (n = 13), and Soy OVX (n = 10) groups. *Indicates $p < 0.05$.

We further investigated NO mediated dilation in response to increasing doses of insulin. NO mediated dilation in the OVX group was significantly impaired compared to the sham controls ($p = 0.0053$, **Figure 5**). However, our data also revealed that the Soy OVX group was able to restore endothelial function in response to insulin compared to the OVX group ($p = 0.0019$, **Figure 5**). Incubation with L-NAME responses were similar across groups ($p = 0{,}1858$, $p = 0.698$, $p = 0.1251$, **Figure 5).**

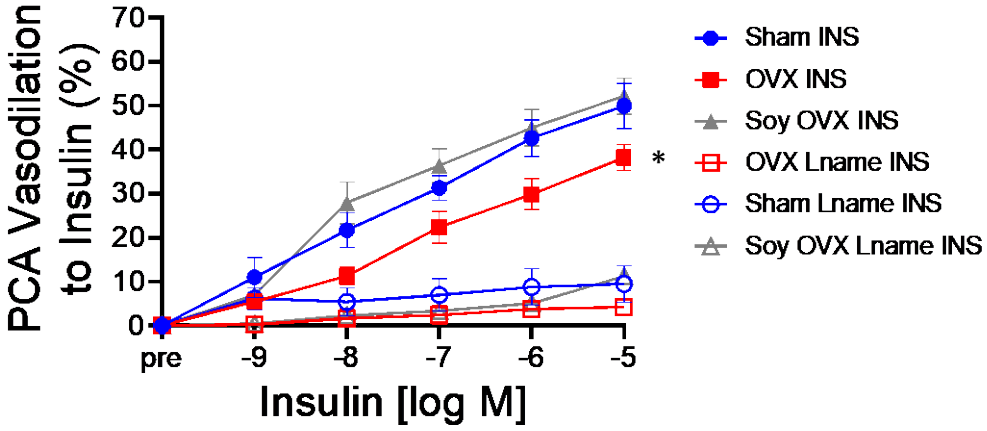

Figure 5: Dilation response to insulin in the absence of presence of L-NAME, the eNOS inhibitor in posterior cerebral arteries in Sham (n = 15), OVX (n = 13), and Soy OVX (n = 10) groups. *Indicates $p < 0.05$.

To determine if endothelium-independent dilation was affected by the loss of estrogen, we investigated the response of increasing doses of sodium nitroprusside (SNP), an NO donor, on the smooth muscle of the PCA's. There were no differences between groups in response to SNP ($p > 0.05$, **Figure 6**).

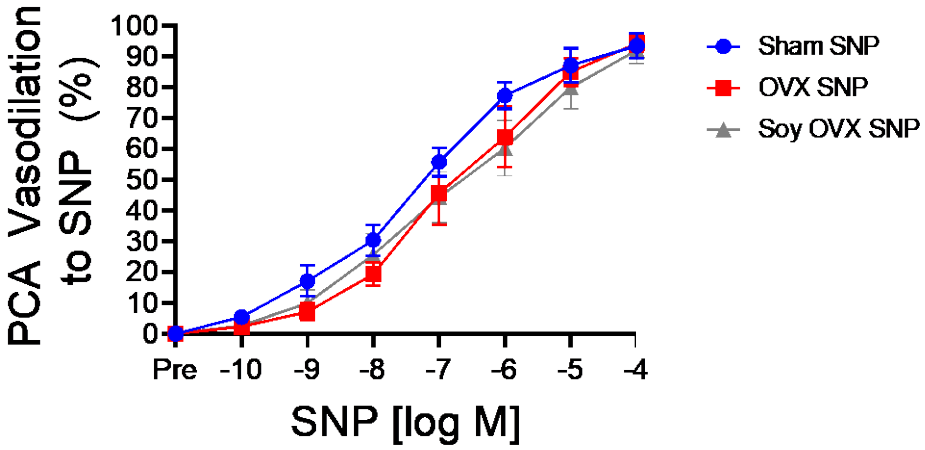

Figure 6: Endothelium-dependent dilation to sodium nitroprusside in posterior cerebral arteries in Sham (n = 15), OVX (n = 13), and Soy OVX (n = 10) groups.

Mesenteric Artery Gene Expression

We then investigated gene expression of ERα, GPR30, and SOD2 in mesenteric arteries. Expression of ERα was not significantly different between Sham and OVX groups ($p = 0.4082$, **Figure 7).**

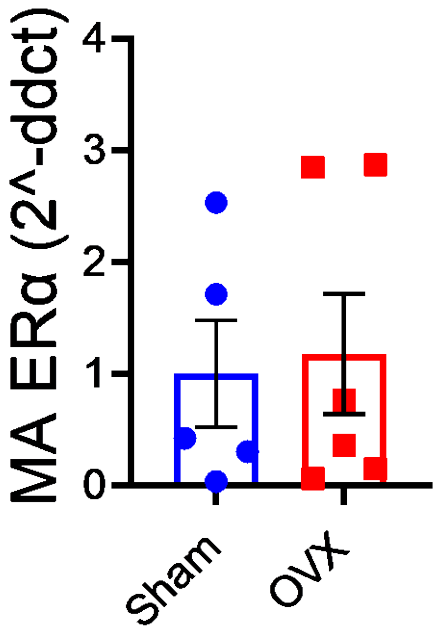

Figure 7: Expression of ERα in mesenteric arteries in sham (n = 5) and OVX groups (n = 6)

Expression of GPR30 was significantly different between Soy OVX and Sham groups ($p = 0.0132$, **Figure 8)** and OVX and Sham groups ($p = 0.0425$, **Figure 8).**

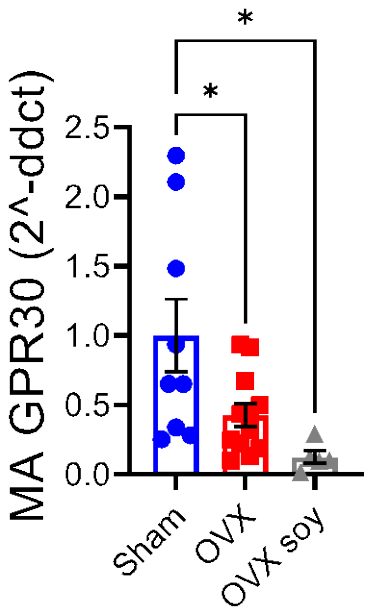

Figure 8: Expression of GPR30 in mesenteric arteries in Sham (n = 9), OVX (n = 12), and Soy OVX (n = 5) groups.

Finally, expression of SOD2 was not significantly different between all three groups (p = 0.5878, p = 0.0612, p = 0.2375, **Figure 9).**

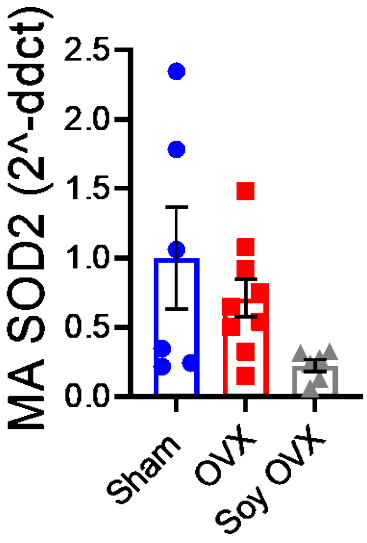

Figure 9: Expression of SOD2 in mesenteric arteries in Sham (n = 6), OVX (n = 9), and Soy OVX (n = 5) groups.

Discussion

Our study focused on examining the relationship between estrogen, soy, and the vascular system in mice in an effort to better understand the implications it may have on aging. Our results showed that soy plays a restorative role in instinctual behavior when there is a lack of estrogen, as seen through ovariectomies. Our data suggest that estrogen loss negatively impacts NO mediated dilation, but not large artery stiffness, and does not lead to any cognitive deficits when compared to the intact group. But soy returns NO mediated dilation to the same level as intact females and improves cognitive markers. Post-menopausal females are at higher risk for developing AD and CVD, and loss of estrogen post menopause is a key factor. Soy isoflavones, high in phytoestrogens have shown importance restorative role when studying aging in postmenopausal women. Studying estrogen in conjunction with the vascular system highlights the importance they both play in aging and age-related diseases.

Instinctual behavior showed improved with soy diet

Nest building is a measure of instinctual behavior and potential motor deficits. Soy diet showed improved cognition when compared to both OVX and sham groups on a soy free diet. This indicates that soy plays a restorative role in cognition in the absence of estrogen. Soy isoflavones have been seen to improve certain types of memory such as recall and sustained attention (Duffy et al., 2003). Studies have suggested that this improvement in cognition can be due to soy acting as estrogen agonists to mimic the effect of estrogens in the brain, or even an increase in cerebral blood flow (File et al., 2001; Lee et al., 2005).

Rotarod, used to assess motor coordination, did not show any differences in results between groups. This indicates that estrogen and soy status did not impact motor coordination in the mice. This is in accordance with past research that shows varying estrogen levels did not impact the ability of a mouse to stay on the Rotarod (Meziane et al., 2007). Soy implementation did not enhance mice's ability to stay on the rod either. Together, this data signifies that motor coordination is not impacted by varying levels of estrogen or soy.

Large artery stiffness did not change with soy or OVX

PVW, a measure of large artery stiffness, showed no differences between sham, OVX, and Soy OVX group. The absence of group differences between OVX and sham groups is contrary to what we originally hypothesized. Previous literature indicates that OVX mice develop greater increases in pulse pressure and arterial stiffness post menopause as measured through PWV (DuPont et al., 2019; Wong et al., 2020). Furthermore, our finding that soy did not impact PWV aligns with previous research that soy diet supplementation did not impact PWV in postmenopausal females (Pase et al., 2011). This could be due to the duration of, or amount of soy diet given to mice, not being enough to exert protective effects on arterial stiffness seen in mice.

Nitric oxide mediated endothelial dilation improves with soy

Through our ex-vivo pressure myograph, NO mediated endothelial function was significantly impaired in the OVX groups when compared to the other two groups in

response to both ACh and insulin. In response to increasing doses of ACh, a potent vasodilator, preconstricted PCA's of the OVX group dilated significantly less when compared to sham and OVX and soy groups. ACh works through a NO dependent mechanism. ACh can activate eNOS, which is responsible for the creation of NO in the endothelium (Katz et al., 1993; Kellogg et al., 2005). We saw that incubating vessels with L-NAME, an eNOS inhibitor, did not induce any significant changes between groups. This suggests that the lower vasodilation can be attributed to decreased NO bioavailability in the OVX group and that soy restores the NO availability to a similar level as in intact females. This data is corroborated by the Kellogg group who conducted vasodilatory responses to Ach and L-NAME to better understand NO mediated mechanisms and dilation (Kellogg et al., 2005). From this, we can gather that intact female mice have preserved NO mediated dilation compared to ovariectomized mice, and that soy was not able to restore this NO function in sham mice. These findings are important because they highlight the importance of estrogen in NO-mediation dilation as the loss of estrogen mitigated dilation.

In response to increasing doses of insulin, similar results were seen. After preconstriction, the PCAs of the OVX groups dilated significantly less when compared to sham and Soy OVX groups. The insulin pathway is important in production of NO and eNOS and thus endothelial dysfunction (Muniyappa & Sowers, 2013; Petrie et al., 1996). The insulin pathway is important to examine due to females shifting towards a more insulin resistant state post-menopause, thus increasing the likelihood of developing metabolic disease (Lin et al., 2006; Muniyappa & Sowers, 2013). Previous studies done in rats have shown that insulin induced vasodilation is dependent on the

endothelium and the NO/eNOS pathway and that this dilation response is impaired in aging and metabolic diseases (Katakam et al., 2009; Muniyappa & Sowers, 2013). Our results agree with another study in which estrogen loss is associated with an impaired vasodilation response (Duncan et al., 2008) thus highlighting the importance estrogen as a protective factor against age-related diseases.

Expression of GPR30 decreases with soy and OVX while ERα and SOD2 remain unchanged

For estrogen receptors, our results found that between OVX and sham groups expression of ERα remained the same, while expression of GPR30 was significantly decreased in the Soy OVX group and OVX group when compared to the sham group. This indicates that the loss of estrogen caused by OVX decreases the expression of GPR30 and that the soy diet does not recover this expression. This also implies that soy's protective effects are exerted through mechanisms other than GPR30 since expression of that gene did not increase with the soy diet. This aligns with previous research in which expression of ERα remained unchanged when measuring systemic response to OVX (Mohamed & Abdel-Rahman, 2000). Since estrogen acts on GPR30, loss of estrogen correlates with lower expression of GPR30 in the OVX group (Prossnitz et al., 2007, p. 30).

SOD2, a marker of antioxidant defenses, was not significantly different amongst groups, although the data was trending towards a decrease in SOD2 expression in the Soy OVX group. This indicates that estrogen loss and soy level do not affect markers

for oxidative stress. Past research indicates that SOD2 is upregulated in the presence of estrogen (Liu et al., 2014) which follows the trend shown in our data. Since our data doesn't show significance, this does contradict previous findings.

Limitations and Future Directions

Our study also contains some proposed limitations. First, we did not test any direct mechanistic effects of soy and estrogen loss. Furthermore, soy supplementation was given ad libitum, meaning it was not possible to measure the exact amounts of supplementary diet the mice were consuming. Thus, it is unknown how much soy is necessary to induce these effects and whether acute or long-term soy supplementation is necessary. Some of our proposed results are not in accordance with the current literature, for example our finding of no differences in arterial stiffness across the groups. The difference in these results could be due to equipment and measurement error. It may have been due to the time frame chosen for this study, given that our animals were of young age and there were only two months between ovariectomy surgeries and testing. Finally, our study analyzed data from mice at a young age, not old, to better understand the role of estrogen but these data does not look at differences between young and old mice and effects of estrogen and soy.

Further directions for this study would be to conduct western blot analysis to quantify protein expression. While RT-PCR tells us which genes can be transcribed, conducting western blot analysis will quantify the proteins translated. Proteins that could be beneficial to analyze would be eNOS, phosphorylated eNOS, and estrogen receptors.

Additionally, looking at the direct effect of estrogen on cerebral artery endothelial function by way of incubating arteries with estrogen and calculating their response would be beneficial to our understanding of the immediate effects of estrogen on endothelial function.

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