The Effects of Estrogen, Grape Pomace, and Resveratrol Supplementation on Glucose Tolerance and Insulin Signaling in Ovariectomized Rats.

By

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Abstract

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Eóin Anderson
University of Guelph, 2017

Advisor: David J. Dyck

Estrogen (E2) loss results in increased visceral adiposity and impaired glucose tolerance. Estrogen replacement may restore insulin sensitivity if given shortly after menopause, but may be associated with side effects such as ovarian cancer. Other alternatives should be explored. Natural compounds such as resveratrol (RESV) and grape pomace (GP) have potential insulin sensitizing effects. To our knowledge, the efficacy of RESV and GP in restoring glucose tolerance in ovariectomized (OVX) rats has not been explored. Diet was administered ad libitum to sham control (SHAM) and OVX female rats until the onset of glucose intolerance in OVX rats as determined by an intraperitoneal glucose tolerance test (IPGTT). Subsequently, OVX animals were divided into treatment and control groups. The treatment groups received daily, one of i) a physiological oral dose of E2, ii) RESV, or iii) GP supplemented in the diet. Treatment continued for 6 wks followed by IPGTT and insulin tolerance tests. 2-3 d later, terminal surgeries were performed during which red and white gastrocnemius muscles, visceral adipose tissue (VAT) and liver were sampled to assess Akt response to insulin injection. VAT was stained to determine adipocyte size. None of the treatments restored normal glucose tolerance. Insulin tolerance was not worsened in OVX rats, and was unaffected with treatment. No difference in phosphorylation of the insulin signaling protein, Akt, was found after insulin injection in any tissue between SHAM and OVX rats. Adipocyte size was significantly increased in the OVX-E2 and OVX-RESV groups compared to SHAM. Delayed E2 replacement, RESV and GP were not effective in restoring normal glucose tolerance in OVX rats.
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AAC</td>
<td>Area above the curve</td>
</tr>
<tr>
<td>AMPK</td>
<td>5’AMP activated protein kinase</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ARKO</td>
<td>Aromatase knockout</td>
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<tr>
<td>ATGL</td>
<td>Adipose triglyceride lipase</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>β-LGND</td>
<td>Estrogen receptor beta isoform-selective estrogen receptor ligand</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>CoA</td>
<td>Acetyl-coenzyme A</td>
</tr>
<tr>
<td>CON</td>
<td>Control</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-related kinase</td>
</tr>
<tr>
<td>E2</td>
<td>17Beta-estradiol</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>GP</td>
<td>Grape pomace</td>
</tr>
<tr>
<td>GPER</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>GSOE</td>
<td>Grape seed oil extract</td>
</tr>
<tr>
<td>GSPE</td>
<td>Grape seed procyanidin extract</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose tolerance test</td>
</tr>
<tr>
<td>HFD</td>
<td>High fat diet</td>
</tr>
<tr>
<td>HOMA</td>
<td>Homeostatic model assessment</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone sensitive lipase</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>IPGTT</td>
<td>Intraperitoneal glucose tolerance test</td>
</tr>
<tr>
<td>IPITT</td>
<td>Intraperitoneal insulin tolerance test</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate 1</td>
</tr>
<tr>
<td>IRS-2</td>
<td>Insulin receptor substrate 2</td>
</tr>
<tr>
<td>ITT</td>
<td>Insulin tolerance test</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LERKO</td>
<td>Liver-specific estrogen receptor alpha knockout</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomy</td>
</tr>
<tr>
<td>p-AMPK</td>
<td>Phospho-5’AMP activated protein kinase</td>
</tr>
<tr>
<td>p-IRS1</td>
<td>Phospho-insulin receptor substrate 1</td>
</tr>
<tr>
<td>pAkt</td>
<td>phosphor-Akt</td>
</tr>
<tr>
<td>PGC1α</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator 1-alpha</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethane sulfonyl fluoride</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator activated receptor-gamma</td>
</tr>
<tr>
<td>RESV</td>
<td>Resveratrol</td>
</tr>
<tr>
<td>RG</td>
<td>Red gastrocnemius</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>Ser473</td>
<td>Serine residue 473</td>
</tr>
<tr>
<td>SF1</td>
<td>Steroidogenic factor-1</td>
</tr>
<tr>
<td>SFO</td>
<td>Sunflower seed oil</td>
</tr>
<tr>
<td>SIRT-1</td>
<td>Sirtuin-1</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>Sterol regulatory element-binding protein 1c</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 Diabetes</td>
</tr>
<tr>
<td>tAkt</td>
<td>Total Akt</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with tween</td>
</tr>
<tr>
<td>Thr308</td>
<td>Threonine residue 308</td>
</tr>
<tr>
<td>TLR-2</td>
<td>Toll-like receptor 2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UCP-1</td>
<td>Uncoupling protein-1</td>
</tr>
<tr>
<td>VAT</td>
<td>Visceral adipose tissue</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WG</td>
<td>White gastrocnemius</td>
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</table>
Chapter 1 Literature Review

1.1 Introduction

As obesity levels and incidence of Type 2 Diabetes (T2D) continue to rise, an effective treatment for impaired glucose homeostasis is needed. A variety of medicines currently exist which improve blood glucose regulation, but these treatments do not cure T2D, but only prolong the progressive decline of health in these populations. These anti-diabetic medicines also often cause unwanted side effects. For these reasons, research into alternative treatments to improve glucose homeostasis and insulin action is necessary. The loss of estrogen in postmenopausal women is associated with a number of detrimental health effects, including an increased risk of developing T2D due to impaired insulin signaling and glucose uptake into skeletal muscle. Estrogen plays a number of protective roles in maintaining energy homeostasis, including improving insulin sensitivity, inhibiting lipid synthesis, and promoting a more anti-inflammatory environment in adipose tissue as well as improving satiety signals and increasing resting energy expenditure. Thus, a thorough understanding of estrogen’s effects on whole body metabolism, as well as estrogen’s direct effects in different tissues is required in order to develop an effective treatment for postmenopausal women to reduce risk of mortality and morbidity. Hormone Replacement Therapy (HRT) is partially successful at rescuing metabolic function in postmenopausal women; however there has been an association between estrogen replacement therapy and increased risk of comorbidities such as cardiac events and breast cancer. Although
some have questioned the validity of these claims, the fact remains that physicians are more cautious when prescribing estrogen replacement therapy \(^2\).

Resveratrol (RESV), a polyphenol found in the skins and seeds of grapes as well as in wine, has been the subject of extensive research, as it has been implicated as a potential anti-aging compound \(^9\). This increased research into RESV and its molecular mechanisms has led way for current research investigating RESV’s ability to act on multiple pathways, including the insulin signaling pathway \(^10\). Resveratrol may therefore be a potential therapy to improve insulin resistance, although current research is limited and conflicting. Although there have been a number of promising animal studies, RESV’s efficacy in human populations is difficult to ascertain as it is quickly metabolized by the liver \(^11\). Grape pomace (GP), which is a byproduct of the wine making process, is largely disposed of as a waste product \(^12\). This too, may function to improve insulin sensitivity as it is a potent antioxidant and also contains RESV \(^13\). Grape pomace has been theorized to improve insulin sensitivity by directly increasing insulin secretion, as well as indirectly by promoting a more anti-inflammatory environment throughout the body \(^14\). Animal studies have found GP provides protection against high fructose diets, and cell studies have found GP stimulates glucose uptake \(^15,16\). While these results are encouraging, human trials have found conflicting results \(^17,18\).

This review will explore estrogen’s functions in metabolism throughout major tissues including, skeletal muscle, adipose tissue, the liver, pancreas, and the brain. The potential for RESV and GP to improve glucose homeostasis and insulin action will be evaluated through a discussion of the current state of literature in cell, animal, and human studies.
2. Estrogen Signaling

2.1 Estrogen Background Information

Estrogen exists in different forms; however 17β-estradiol (E2) is the main circulating form of the hormone and is thought to be responsible for the majority of nuclear and genomic signaling. Estrone is aromatized from androstenedione in the ovaries and can be converted to E2. This is compromised in postmenopausal women due to follicular loss which causes the ovaries to become unresponsive to follicle-stimulating hormone and unable to synthesize E2. In premenopausal women, E2 mediates most of its effects through binding to a nuclear estrogen receptor (ER) which alters gene transcription and expression. Two types of nuclear ERs exist, ERα and ERβ. Estrogen may also bind to membrane bound ER which are mostly G-protein coupled receptors (GPER). The genomic mechanism of ER action occurs when E2 binds to ER causing it to dissociate from a chaperone heat-shock protein. ER is then free to bind directly to an estrogen response element at a target gene promoter or it may bind to an activator protein-1 response element through protein tethering to DNA. ERs become dimers during the activation process and ERα- ERα dimers, ERβ- ERβ dimers, and ERα-ERβ dimers exist, with ERα being dominant in the heterodimer group. ER dimers interact with cofactors to regulate gene expression through activation or suppression of target genes. ERα and ERβ exert different effects throughout the body. For example, ERα stimulation increases skeletal muscle GLUT4 translocation and ERβ stimulation in adipose tissue leads to fat loss. Estrogen also can exert non-genomic effects i.e. cytosolic signaling. This can happen very rapidly, in seconds to minutes and cause an influx of calcium, nitric oxide release, or activation of different cellular signaling pathways.
Estrogen has metabolic actions in numerous tissues, including skeletal muscle, adipose tissue, liver, and the pancreas; however its mechanism of action seems to be different in each tissue \(^{22,25–28}\). Estrogen also has an important role in the brain, by regulating appetite as well as increasing resting energy expenditure \(^{2,25}\). In the absence of E2 there is generally weight gain, increased food intake, and reduced insulin action \(^{24}\). The metabolic effects of E2 are not completely understood; however tissue specific ER knockout studies in skeletal muscle, adipose, and liver have demonstrated the role of E2 in preserving insulin signaling and glucose homeostasis as knockout results in increased blood glucose levels and diminished insulin signaling in those tissues. \(^{22,29,30}\). Interestingly, whole body E2 deficient models seem to result in similar metabolic alterations regardless of the method of removal of E2 action whether it be whole body ER knockout, aromatase reductions, or complete ovariectomy \(^{2}\). This suggests that E2 is indeed the active molecule responsible for whole body metabolic changes in these animals and that E2 depletion in postmenopausal women is responsible, at least in part, for the metabolic changes that occur in this stage of life.

### 2.2 Estrogen and the Brain

Estrogen is able to cross the blood brain barrier and bind to receptors on the endothelial cells \(^{31}\). In the rat brain, E2 has been shown to regulate energy homeostasis by decreasing food intake while increasing resting energy expenditure \(^{25}\). ER\(\alpha\) and ER\(\beta\) are present in varied concentrations throughout the brain, although ER\(\alpha\) is believed to be the primary metabolic regulator in this tissue \(^{25,32}\). ER\(\alpha\) has been shown to have distinct actions on metabolic function depending on its location \(^{25,32}\). For example, knocking out the ER\(\alpha\) in hypothalamic steroidogenic factor-1 (SF1)
neurons vs. pro-opiomelanocortin (POMC) neurons, have strikingly different effects on metabolism. Mice with SF1 specific ERαKO become obese and exhibit decreased energy (heat) expenditure, although their food intake is similar to controls. POMC ERαKO animals on the other hand, have similar levels of heat production compared to control, but exhibit hyperphagia. POMC neurons release α-melanocyte-stimulating hormone which decreases food intake and increases energy expenditure. Estrogen has been shown to activate POMC neurons, so while it was expected that ERαKO animals would consume more food, it was surprising that they also had similar levels of energy expenditure. These results depict the important role E2 plays in the brain which affects whole body metabolism and body composition.

2.3 Estrogen and Adipose Tissue

Increased adiposity is a major risk factor for the development of T2D. The loss of E2 is associated with increases in adipocyte size and number, particularly visceral fat. Estrogen is believed to promote reduced visceral adiposity and instead increase propensity for subcutaneous adipose tissue, which is less metabolically active and has been proposed to play a protective role in metabolism. Estrogen is also protective in males, as men with an aromatase deficiency and therefore negligible E2 levels, have increased abdominal adiposity compared to males with normal aromatase activity and higher E2 levels. The exact mechanisms underlying E2’s direct effects on adiposity and lipogenesis in human adipose tissue are currently not fully understood as trials have been limited and conflicting. Some studies have postulated that a greater ratio of
ERβ:ERα may contribute to the obese phenotype, but others have found no difference in this ratio in men, pre- or postmenopausal women.

Recently, ERα, ERβ and GPER have all been identified in adipose tissue suggesting that E2 plays both genomic and non-genomic signaling roles in adipose tissue metabolism enacting both rapid changes to metabolism as well as slower transcriptional changes. Further, ERα and ERβ isoforms are present in varying concentrations in different adipose depots. Adipose tissue-specific ERαKO models have increased adipocyte hypertrophy and hyperplasia, as well as decreased glucose tolerance. Adipose ERβ research has been equivocal. ERβ has been implicated to negatively impact metabolism by inhibiting peroxisome proliferator activated receptor-γ (PPARγ). PPARγ is an important regulator of insulin sensitivity, and elicits its effects, at least in part, through the regulation of adipokine release and adipogenesis. Conversely, ERβ stimulation may also reduce weight gain and increase adipose GLUT4 content. In rat OVX models, lipolysis and lipogenesis are perturbed resulting in increased circulating FFAs as well as triglyceride accumulation in muscle, pancreas, and liver. Estrogen treatment leads to suppression of many important promoters of lipogenesis such as acetyl-coenzyme A (CoA), sterol regulatory element-binding protein 1c (SREBP-1), lipoprotein lipase (LPL), and fatty acid synthase (FAS). The downregulation of these genes results in reduced WAT and liver triglyceride accumulation which prevents weight gain and insulin resistance with high fat diets (HFD).

While it is known that E2 treatment beneficially alters adipose tissue metabolism, it remains unclear whether E2 works through direct or indirect mechanisms. For example, D’Eon et al. found that E2-treated OVX animals had lower in vitro basal lipolytic rates in periovarian adipocytes compared to untreated OVX animals. Conversely, E2-treated animals had a 2-fold
greater increase in lipolysis in response to catecholamines compared to untreated OVX animals, assessed by measuring glycerol release of each adipocyte into media over a three hour period. This increase in lipolytic response to catecholamines was likely due to a 3-fold increase in protein expression of perilipin, a regulator of lipolysis. Perilipin is believed to decrease basal lipolysis, but increase the response to adrenergic stimulation, so this seems to be a likely factor effecting lipolysis in E2-treated animals. Interestingly, researchers found that E2 did not stimulate hormone sensitive lipase (HSL). A shortcoming of this study is the absence of a SHAM control group, so it is difficult to extrapolate the effectiveness of E2 in restoring lipolytic function to wildtype levels. In contrast to these findings, a recent study by MacDonald et al. found that E2 did not directly affect lipolysis. In this study, OVX animals and OVX animals receiving E2 were pair fed to SHAM controls for two weeks and indices of lipolysis were measured in vivo as well as in vitro in adipocytes. In vivo, under basal and adrenergic stimulated conditions, OVX and OVX E2 had comparable rates of lipolysis to SHAM controls. Ex vivo, the β-adrenergic agonist CL 316 stimulated lipolysis to similar levels in WAT isolated from OVX and SHAM animals regardless of whether E2 was present or not in the incubation medium. These results suggest that E2 does not directly affect adipose tissue lipolysis as OVX, OVX E2, and SHAM animals had similar lipolytic rates in both basal and stimulated conditions. The difference in the findings between these studies may be due to the different fat depots used to measure lipolysis. D’Eon et al. assessed lipolysis in periovarian adipocytes, whereas MacDonald et al. examined inguinal and retroperitoneal adipocytes. Another important factor to consider is that D’Eon et al. only measured lipolysis in vitro and did not assess in vivo lipolysis. It is possible that lipolysis may be increased in periovarian adipocytes in response to catecholamine stimulation, but may not affect the whole body response. Although it is known
that E2 promotes subcutaneous adiposity and reduces visceral adiposity, the exact mechanisms underlying these changes are not fully understood, and the actions of E2 in different subcutaneous depots have yet to be explored. Therefore, it is critical that more research focuses on E2s effects in adipose tissue to develop an effective therapy for postmenopausal women.

ERα is believed to be responsible for many of the beneficial effects that E2 has on adipose tissue metabolism. ERαKO rodent models have increased fat mass, adipocyte hypertrophy and hyperplasia, and decreased glucose disposal. ERα content decrease in response to HFD, and this is believed to be one mechanism that contributes to increased WAT in OVX animals. Further, adipose-specific ERαKO results in increased inflammation and fibrosis in adipose tissue, and E2 treatment does not rescue tissue inflammation in these animals.

While a role for ERα in preserving adipose metabolic function has been established, there is still much to be learned about ERβ stimulation. A recent study by Yepuru et al. utilized treatment with the ERβ isoform-selective estrogen receptor ligand (β-LGND) in OVX mice which resulted in many of the same protective effects on metabolism that are seen with E2 treatment, including protection from weight gain and reduced gene expression of promoters of lipogenesis such as LPL, FAS, and SREBP1. β-LGNDs also protected male mice from weight gain on a HFD compared to control HFD mice. This makes β-LGNDs an attractive new class of drugs which may have the potential to not only prevent, but also treat obesity in both males and females. β-LGNDs may exert their beneficial effects on energy metabolism in rodents through increases in brown adipose tissue (BAT) mass and increased expression of uncoupling genes known to release excess energy in the form of heat. β-LGND treated animals demonstrated a seven-fold increase in the expression and protein content of uncoupling protein-1.
(UCP-1) in BAT which would contribute to a large increase in energy expenditure in these animals\textsuperscript{37}. βLGNDs therefore inhibit lipogenesis in WAT while simultaneously increasing energy expenditure in BAT\textsuperscript{37}.

While the use of specific βLGNDs appears to have a favorable effect on reducing fat mass, the effect on insulin action has not been directly assessed. This is of concern as a study from Foryst-Ludwig \textit{et al.}\textsuperscript{23} concluded that although fat mass was reduced, ERβ stimulation had a pro-diabetogenic effect i.e. insulin sensitivity was reduced. In this study, researchers demonstrated that ERβKO increased activation of PPARγ in adipocytes leading to an increase in adipogenesis and weight gain, but also improved insulin sensitivity\textsuperscript{23}. These changes were reversed with PPARγ inhibition by a PPARγ antisense oligonucleotide\textsuperscript{23}. Similar results were reported by Tomicek \textit{et al.} who found E2 deficient aged rats had increased adipose ERβ concentrations as well as decreased PPARγ levels\textsuperscript{40}. Further complicating the ERβ story, research by Rüegg \textit{et al.}\textsuperscript{42} suggests that ERβ increases GLUT4 expression in adipocytes. In this study, adipocytes from mice that lacked ERβ had decreased expression of GLUT4 compared to SHAM or ERαKO cells, and that reintroduction of ERβ partially rescued GLUT4 expression. This would suggest ERβ improves adipose tissue glucose uptake, but this is contrasting to the study from Foryst-Ludwig \textit{et al.}\textsuperscript{23}. As ERs have varying roles in different tissues, an adipose tissue specific βLGND agonist may be beneficial, although this has yet to be confirmed. It is difficult to ascertain ERβ effects in adipose tissue as studies investigating ERβs direct effects in adipose tissue have significant methodological limitations, such as not having a control or not measuring adipose specific ERβ activity\textsuperscript{23,37}.

In contrast, it is well accepted that ERα is associated with the prevention of obesity and perseveration of insulin sensitivity. A number of studies have shown that ERαKO rat and mice
models have significant increases in fat mass and greatly reduced insulin sensitivity as well as lower energy expenditure. Pedersen et al. demonstrated that ERα specific agonist treatment increased α2A-adrenergic receptor concentration in human subcutaneous but not visceral adipocytes. This increase in α2A-adrenergic receptor was associated with anti-lipolytic effects in response to epinephrine. Thus, adipose ERα stimulation may represent a potential alternative therapy to HRT.

Considering E2’s significant effects in adipose tissue, it is interesting that there is little research into the effects of E2 on adipose triglyceride lipase (ATGL) and HSL, the rate-limiting enzymes that catalyze the release of the first and second FFAs respectively, in lipolysis. OVX has been demonstrated to increase ATGL expression in visceral adipose tissue in rats, which is thought to contribute to the increase in plasma FFA seen in E2 deficient animals. However, the OVX rats had unchanged visceral HSL activity. Further research regarding HSL has been equivocal. One study found that application of E2 onto isolated human adipocytes increased expression and activity of HSL in a dose dependent fashion. In contrast, a human study found no effect of E2 treatment in postmenopausal women on HSL in abdominal subcutaneous adipose tissue. Further research into ATGL and HSL is recommended to determine if E2 does in fact increase or decrease these enzymes expression in vivo.

2.4 Estrogen and Skeletal Muscle Metabolism

Skeletal muscle is an important tissue in regards to energy homeostasis, and has been estimated to be responsible for approximately 20-30% of total energy expenditure at rest. Similar to adipose tissue, skeletal muscle expresses ERα, ERβ, and GPER and these receptors
have been shown to enact varying effects $^{58}$. Estrogen has been demonstrated to have a regulating effect on the expression and translocation of GLUT4 to plasma membranes $^{58}$. ERα and ERβ have been proposed to play opposing roles in regulating GLUT4 in skeletal muscle however evidence for this is limited and conflicting $^{42,58}$.

Barros et al. $^{58}$ has shown that ERα stimulation in skeletal muscle enhances insulin stimulated GLUT4 translocation to the plasma membrane, and that ERβ stimulation decreases GLUT4 translocation $^{58}$. This is in contrast to the finding that ERβ stimulation increases GLUT4 expression in adipocytes $^{42}$. Similarly, the loss of ERα content in skeletal muscle results in a worsening of insulin sensitivity $^{46}$. The limited research in regards to ERs actions in skeletal muscle makes it difficult to ascertain ERα and ERβ direct mechanisms in improving glucose uptake, though studies generally agree that E2 depletion results in reduced skeletal muscle GLUT4 content and an overall decrease in skeletal muscle glucose uptake $^{22,58-60}$. Another interesting finding is that ERβKO animals have a heightened inflammatory response after injury $^{61}$, and that OVX animals treated with an ERβ, but not ERα, agonist, had a smaller increase in the pro-inflammatory protein TNF-1α in response to injury. Inflammation is associated with generation of reactive oxygen species as well as impairments in insulin sensitivity $^{61}$. This suggests that ERβ is important in the resolution of muscle inflammation in response to injury and may have implications for a role of ERβ in maintaining insulin action in response to muscle injury, although this has yet to be determined.

Estrogen has also been implicated in altering skeletal muscle mitochondrial biogenesis and function $^{59}$, which likely plays a role in improving whole body glucose and lipid metabolism. Isolated soleus and white gastrocnemius muscles from OVX animals oxidize less palmitoyl-carnitine and glycerol-phosphate compared to control animals, suggesting OVX animals have
impaired oxidative metabolism\textsuperscript{59,62}. This is mirrored by decreases in skeletal muscle expression of the so-called "master regulator", peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), as well as carnitine palmitoyltransferase-1 and glycerol-3-phosphate dehydrogenase 2, two important enzymes involved in lipid metabolism\textsuperscript{59}. Further, OVX animals have been demonstrated to have lower activity levels of β-3-hydroxyacyl-CoA dehydrogenase, which is involved in the β-oxidation pathway and reflects the muscles ability to oxidize fatty acids\textsuperscript{63}. OVX animals also have lower muscle mitochondrial content by eight weeks after removal of the ovaries compared to SHAM animals\textsuperscript{59}. The inability of mitochondria to oxidize lipids effectively may contribute to the hyperlipidemia and reduced insulin sensitivity observed in OVX animals\textsuperscript{59}. These changes were reversed with E2 treatment in OVX animals\textsuperscript{59} and interestingly another study\textsuperscript{64} demonstrated that short term E2 supplementation can also increase muscle PGC1α content in healthy men which may increase β-oxidation capacity. Therefore, skeletal muscle mitochondria seem to have a decreased ability to utilize fatty acids in response to E2 depletion which may be a factor influencing fat mass deposition in OVX animals\textsuperscript{59,63}.

2.5 E2 and the Liver

The liver acts as a storage reservoir for glucose (glycogen) and will release glucose into circulation as required through the processes of glycogenolysis and gluconeogenesis, which are the breakdown of glycogen into glucose and the synthesis of new glucose from non-glucose precursors, respectively\textsuperscript{26}. Insulin plays a large role in hepatic energy homeostasis as it will stimulate glycogen synthase to form new glycogen and inhibit glycogenolysis and
gluconeogenesis. ERs are found throughout the liver and their activation results in increases in catabolic processes (lipolysis and glycogenolysis), while decreasing anabolic processes (lipogenesis, and gluconeogenesis). Hepatic ERα expression increases with age, but is not difference between sexes or influenced by weight gain. Although both ERβ and GPER isoforms are present in the liver, differences in their expression after menopause are currently unknown. This is a possible direction for future research, as ERβ may play a significant role in liver energy homeostasis, although current literature seems to be divided in this regard. Paradoxically, ERβKO models as well as ERβ stimulation in intact female rats have been reported to lower liver TG levels, although the mechanism through which this happens is yet unknown.

While it is still not clear how E2 enacts its effects in the liver, E2 is known to be protective in hepatic metabolism. Estrogen depletion results in increased fat deposition in liver and decreased hepatic triglyceride export. Further, aromatase knockout (ARKO) models have impaired hepatic β-oxidation and increased FAS activity resulting in hepatic steatosis. These have been attributed mainly to a loss of ERα action, as treatment with ERα agonists decrease hepatic TG accumulation in ARKO mice. Whole body ERαKO has also been shown to impair hepatic insulin sensitivity and increase lipid transport. Estrogen has been demonstrated to inhibit expression of lipogenic enzymes such as LPL in the liver while also promoting lipolytic enzymes like HSL.

Liver-specific ERα knockout (LERKO) in animals has generated mixed results. A recent study by Zhu et al. found that male LERKO mice on a HFD had an impaired ability to stimulate FOX01. FOX01 inhibits hepatic adipogenesis by inhibiting PPARγ, and promotes gluconeogenesis by increasing transcription of phosphoenolpyruvate carboxykinase. These
KO animals displayed increased hepatic TG accumulation and insulin failed to suppress hepatic glucose production. Whole body insulin sensitivity was also decreased as determined by a hyperinsulinemic euglycemic clamp. These results suggest that hepatic ERα is important for maintaining normal insulin signaling in the liver, and ultimately, at the level of the whole body. However, in another study, LERKO mice displayed similar blood glucose and insulin levels, and response to a glucose challenge as control animals, which suggests the existence of a compensatory mechanism for the loss of liver ERα. However, the expression of ERβ and GPER in the liver was unaffected, suggesting that these ERs were not responsible for the compensatory actions. Therefore, it seems that other E2 responsive tissues may compensate for the loss of ERα in the liver. It is not obvious why these studies had different outcomes, but one difference is the inclusion of female mice in the study by Matic et al. It is possible that hepatic ERα is more important in males who do not have as much E2 or ERs present throughout the body as females. Females have ERs more ubiquitously expressed and may be better at compensating for impaired ER action in one tissue. However, more research is needed in this area. Interestingly, an earlier study by Zhu et al. used a female LERKO mouse model and found increased liver accumulation of TG content, but unfortunately did not measure insulin sensitivity in these animals.

2.6 Estrogen and the Pancreas

Pancreatic β-cells produce and release insulin into the circulation in response to an increase in plasma glucose. Estrogen plays direct and indirect roles in preserving pancreatic β-cell insulin secretion. Indirectly, E2 preserves insulin secretion by improving whole body glucose
homeostasis and tissue sensitivity to insulin, thereby reducing the amount of insulin required to be produced \(^71\). Directly, E2 has been demonstrated to protect β-cells from oxidative damage and cytotoxic insults \(^28\). Further, ERα, ERβ, and GPER are all present in the pancreas and work mainly through extra-nuclear receptors as opposed to the nuclear receptors that enact the majority of E2s activity throughout the rest of the body \(^71\).

One of the metabolic complications as a result of increased adiposity is the accumulation of lipids in the pancreas which can cause lipotoxicity and kill β-cells \(^72\). This is thought to be due at least in part to increased inflammation and activation of pro-apoptotic pathways, as well as the generation of superoxide species from mitochondrial β-oxidation which results in greater oxidative stress \(^73\). This makes it increasingly difficult for the pancreas to produce a sufficient amount of insulin to combat the elevated plasma glucose associated with obesity and insulin resistance \(^72\). Part of E2’s protective effect on β-cell function is mediated through ERα. ERα is believed to prevent apoptosis of these cells in response to oxidative insult \(^74\). Le May et al. \(^74\) generated ERαKO mice and found that β-cells in these animals were more susceptible to oxidative damage induced from acute streptozotocin treatment. However, ARKO animals which did not have any E2 were even more susceptible to oxidative damage than ERαKO, suggesting that other receptors also mediate E2's protective effects \(^74\). Stimulation of ERα increases β-cell differentiation in both embryonic mice, as well as adult mice with severe pancreatic injury while ERαKO mice demonstrate significantly less β-cell differentiation and impaired regeneration in response to injury \(^75\). Further supporting its protective role in the pancreas, E2 treatment in OVX animals results in increased concentrations of superoxide dismutase, catalase, and glutathione peroxidase in the pancreas of these animals \(^37,71\). These enzymes prevent lipid peroxidation and scavenge reactive oxygen species, preventing oxidative damage \(^71\). Other studies have found that
E2 protects β-cells from pro-apoptotic insults and prevents them from undergoing early cell death from oxidative stress via rapid extra nuclear signaling that may be mediated by ERα and ERβ, although the exact signaling mechanisms are currently unknown. Finally, E2 has been shown to directly stimulate pancreatic β-cells to release insulin via rapid extra nuclear signaling, and that this action was specifically due to the interaction with GPER. Collectively, these studies demonstrate the important roles that E2 plays in the pancreas and the protective function E2 has on pancreatic β-cell survival.

### 2.7 Concluding Remarks on Estrogen

The major consequences of E2 depletion in terms of energy metabolism are hyperphagia (brain) and decreased energy expenditure (multiple tissues), increased susceptibility of pancreatic β-cells to oxidative injury and apoptosis, and impaired glucose uptake into skeletal muscle and adipose tissue. These metabolic changes are responsible for the increased risk of T2D in postmenopausal women. Estrogen therapy is beneficial in restoring many of these metabolic complications. ERα, ERβ, and GPER are expressed throughout the body and may represent potential therapeutic sites for tissue specific agonists. Estrogen depletion results in reduced oxidative metabolism in skeletal muscle due to mitochondrial reduction and dysfunction, which impairs lipid metabolism and may promote lipid accumulation in tissues such as skeletal muscle. Whole body ERαKO animals have increased fat mass, glucose intolerance, and increased body weight compared to control animals, depicting a clear role for ERα in maintaining metabolic function. Therefore, stimulating skeletal muscle ERα may be a viable pharmacological target to improve glucose uptake. Stimulation of ERβ in adipose tissue attenuates weight gain, but whole body ERβ stimulation may be associated with insulin...
resistance, therefore adipose specific ERβ stimulation may be an effective therapy, although this has yet to be investigated \(^{23,37}\). Estrogen can directly stimulate insulin release from pancreatic β-cells through GPER; therefore, pancreatic GPERs may also represent a potential targeted therapy site for T2D \(^{77}\). Hepatic ER deletion results in increased hepatic TG accumulation and reduced insulin sensitivity in both males and females, but the mechanism of action is unknown \(^{29,43}\). Current research is divided as ERαKO has been found to both negatively impact hepatic insulin sensitivity as well as have no effect \(^{43,68}\). ERβKO has generated similar conflicting results \(^{23,78}\). Further research investigating the effects of ERs in the liver is needed.

3. Resveratrol

3.1 Resveratrol – Introduction and Background

Resveratrol (RESV) is a polyphenolic compound that is found in various concentrations in peanuts, cranberries, blueberries, and particularly, grapes and red wine \(^{79}\). This compound has been proposed to have numerous and broad beneficial effects on health including anticancer activity, neuroprotective effects, and antiaging effects \(^{79}\). An examination of these effects is beyond the scope of this review. More importantly for this thesis, RESV has been suggested to have a role in the maintenance of blood glucose homeostasis and insulin action \(^{80,81}\). Current long term studies (≥12 weeks) examining the efficacy of RESV in promoting insulin action in humans are limited, but some animal studies have shown promise \(^{80–82}\). One of the major limitations of RESV treatment is that while readily absorbed in the gut, RESV is rapidly metabolized in the liver and it is unclear how much RESV actually passes into the systemic circulation, although its metabolites (dihydroresveratrol glucuronide, dihydroresveratrol sulfate, \(trans\)-resveratrol-3-O-sulfate, and \(cis\)-resveratrol-3-O-sulfate) are absorbed \(^{11}\). To overcome this limitation, methylated polyphenol analogs of RESV have been created which improve the bioavailability of RESV in
rat models. Interestingly, RESV has been proposed to protect cells from oxidative stress by a mechanism that requires ERβ. Numerous studies have suggested that RESV may improve insulin action though sirtuin-1 (SIRT-1) dependent and independent pathways. Resveratrol may improve mitochondrial function through SIRT-1 activation causing an increase in PGC1α activation and increasing mitochondrial biogenesis, or independently increase AMPK activation, leading to an increase in PGC1α activation and increased mitochondrial biogenesis.

3.2 – Resveratrol and Insulin Signaling- Cell Studies

Studies using cell lines have begun to elucidate RESV’s mechanism of action, although there have been conflicting results which suggest that, much like E2, RESV may have tissue specific effects. Varshney and Dey have shown that application of RESV to mouse derived neuronal cells leads to decreased Akt activation and a worsening of insulin stimulated GLUT4 translocation and glucose uptake. This was one of the few cell studies which have found negative effects of RESV treatment. Breen et al. have shown that RESV stimulation of L6 myotubes significantly increased glucose uptake due to AMPK and SIRT-1 activation, as inhibition of either SIRT-1 or AMPK abolished the increased glucose uptake. A third cell study, found that high-dose RESV treatment of human adipocytes resulted in reduced GLUT4 translocation due to reduced Akt activation. Interestingly, in cells of the same lineage, long term low dose RESV increased Akt activation and insulin stimulated glucose uptake, highlighting a need to determine optimal RESV doses. It is possible that, similar to E2, RESV enacts the most beneficial effects within a tight concentration range. Finally, a fourth cell study demonstrated that direct application of RESV to human pancreatic islet cells resulted in greater glucose uptake and a
potentiation of insulin signaling. This effect appears to be SIRT-1 dependent, as SIRT-1 inhibition by expression of inactive genes, or the compound EX-527, completely abolished this response. Due to the differences in RESV doses and the different cell-types investigated, conclusions are difficult to make based on cell studies.

3.3 Resveratrol and Insulin Signaling – Animal Studies

Supplementation of RESV in rodents has led to promising results in regards to improving fasting blood glucose and pancreatic β-cell survival, reducing oxidative stress, and maintaining and restoring insulin action. Zhang et al. investigated the effects of long term (24 weeks, 400mg/kg bw/day) RESV treatment in mice on a HFD and found RESV improved glucose homeostasis, oxidative stress, and β-cell mass, which appeared to be due at least in part to increased islet SIRT-1 activation. Another study found that addition of very high dose (2-4g/kg bw/day) RESV in diabetic KKA mice for 12 weeks resulted in improved whole body glucose homeostasis and was accompanied by increases in SIRT1, p-AMPK, p-IRS1, and p-AKT content in liver and soleus muscle. It is important to note that the dose of RESV used in this study is supra-physiological and would not be realistically attainable with supplementation in humans. Other animal studies have shown that RESV treatment improves insulin signaling, but are likely independent of SIRT-1 expression. In healthy animals, Andersen et al. found that 300 mg/kg bw/day of RESV significantly lowered fasting blood glucose and insulin and improved insulin sensitivity as assessed by HOMA-index. However, there was no increase in hepatic SIRT-1 expression; no other tissues were quantified for SIRT-1 content. Interestingly, direct stimulation of isolated hepatic cells with 10 times the concentration of RESV did result in an increase in hepatic SIRT-1 expression. These results seem to be at odds with the finding
that high dose RESV impairs adipocyte insulin signaling. A final study found low dose RESV (2.5mg/kg bw/day) resulted in improved hepatic insulin signaling and whole body glucose homeostasis, but specific SIRT-1 overexpression did not. Overall, findings suggest that SIRT-1 is likely only increased in the presence of high concentrations of RESV.

### 3.4 Resveratrol and Insulin Signaling – Human Studies

There have been several human studies investigating the effects of RESV on insulin action and signaling with equivocal findings. While some studies suggest that RESV may improve insulin signaling in diabetic patients, others did not. Again, a striking difference between intervention studies is the dosages of RESV ranging from 5 mg/day to 1 g/day. However, even trials using similar dosages have found different results. Timmers et al. found that 150 mg/day of RESV supplementation for 4 weeks in obese, but healthy men, resulted in improvement of a wide number of metabolic measures including increased skeletal muscle SIRT-1, PGC1α, pAkt, and insulin sensitivity as assessed by HOMA-index. Interestingly, a similar study that also recruited obese, but otherwise healthy males found a dose of RESV of 150 mg/day did not result in any metabolic changes after 4 weeks of treatment. It is surprising that these two studies found such different results, although a potential reason for this discrepancy may be the crossover study design (vs. randomized control trial) and low participant numbers (11 participants vs. 24) by Timmers et al. However, a more recent study by Timmers et al. with a slightly larger sample size (n=17) also did not find any improvements in metabolic measures in patients with well controlled T2D in response to 30 days of RESV treatment at 150mg/day. One possibility for this is that the anti-hyperglycemic medication the T2Ds were
taking had a more pronounced effect on glucose homeostasis than RESV, masking any potential benefit \(^9^5\). However, a similar study \(^9^7\) that also used subjects with T2D on anti-hyperglycemics found that RESV supplementation (1000 mg/day) for 45 days did result in significant improvements in fasting blood glucose and insulin action assessed by HOMA-IR. A major difference between these two studies is the dosage of RESV; Timmers et al. \(^9^5\) utilized 150 mg of RESV/day compared to the second study \(^9^7\) which used the much higher dose of 1000 mg/day. Finally, a 12 week human trial investigating the effects of RESV supplementation on markers of glucose homeostasis in non-obese, insulin tolerant, post-menopausal women did not result in any differences compared to controls \(^9^8\). Clearly there is significant disagreement between human studies and the effect of RESV on insulin action currently remains unclear.

### 3.5 Concluding Remarks on Resveratrol

Cell studies have begun to determine possible mechanisms by which RESV increases glucose uptake and preserves insulin function; however they have also revealed potential downsides to high dose RESV supplementation as high dose RESV reduces glucose uptake in adipocytes \(^9^1\). Resveratrol induces different effects in different cell types, highlighting the potential for RESV targeted therapy as low dose RESV increased glucose uptake in skeletal muscle \(^9^0\) and adipocytes \(^9^1\), but negatively impacted glucose uptake in neuronal cells \(^9^2\). Future cell studies should investigate the variable effects of a standard dose of RESV on different tissue types. Rodent studies have depicted a more clear benefit of RESV, in which doses between 2.5 mg/kg bw/day and 4g/kg bw/day have resulted in improved skeletal muscle glucose uptake \(^8^7\), protection from oxidative stress in the pancreas \(^8^5\), and improved hepatic insulin signaling \(^8^8\). Resveratrol may
work through SIRT-1 dependent and independent pathways, an area of focus for future animal studies. Unfortunately, despite the promising cell and animal studies, human studies have not found a consistent effect of RESV supplementation. Due to the relatively small number of trials, it is recommended that future human studies are conducted with diabetic participants who receive a standard dose of RESV to determine the efficacy of treatment. Given that two human studies\textsuperscript{94,97} showed improvements in insulin signaling and glucose homeostasis following RESV supplementation, it is an area that should be further explored.

4. Grape Pomace

4.1 Grape Pomace Background

Grape pomace (GP) is a by-product of the wine-making process and contains the remnant skins, stems and seeds of grapes\textsuperscript{12}. Currently, this is largely deemed a waste product, but it may represent a potential nutraceutical. Grapes contain the largest natural concentration of RESV of all foods, and the majority of RESV is found in the grape skin\textsuperscript{13}. Grape pomace composition varies greatly depending on the manufacturer and which grape species make up the composition of the product, so RESV content can vary significantly\textsuperscript{12}. However, GP is also rich in anthocyanins, such as cyandin and petunidin\textsuperscript{99} which have been demonstrated to have numerous beneficial health effects such as inducing apoptosis in defective cells, as well as anti-oxidant and anti-inflammatory properties\textsuperscript{13}. Further, GP is believed to function as an α-glucosidase inhibitor, which results in less breakdown and absorption of complex carbohydrates from the diet after a meal\textsuperscript{100}. For these reasons, GP may improve insulin action and blood glucose levels in the body\textsuperscript{12}. Cell studies and animal studies have found promising results\textsuperscript{81,90,101}. However, there have been very few human studies and it is unclear if GP improves glucose homeostasis and insulin action\textsuperscript{17,18,102–104}. 
4.2 Grape Pomace and Insulin Signaling – Cell Studies

Cell studies have begun to elucidate possible signaling pathways that may be affected by GP. While most cell studies have investigated GP effects on human 3T3-L1 adipocytes, there have also been trials investigating its effects on L6 myotubes as well as INS-1E pancreatic β-cells \textsuperscript{14,16,105,106}. Elucidating GP effects on metabolism is difficult because many studies don’t utilize GP as a whole, but rather use constituents of GP to different degrees. Two common treatments are grape seed oil extract (GSOE) and grape seed procyanidin extract (GSPE). One difficulty that this presents is that concentrations of procyanidins and grape seed oil vary between studies, as well as between different species of grapes \textsuperscript{13}. As GP is a potent anti-oxidant, it may enact insulin sensitizing effects through reduced whole body inflammation \textsuperscript{106}. Direct application of GP to human adipocytes has been demonstrated to decrease expression of pro-inflammatory genes such as TNF-α, monocyte chemoattractant protein-1 (MCP-1) and toll-like receptor 2 (TLR-2). This translates to reduced activation of inflammatory pathways, including extracellular signal-related kinase (ERK) and c-Jun NH2-terminal kinase \textsuperscript{106}. Activation of these pathways ultimately prevents insulin from binding to IRS-1, impairing insulin stimulated glucose uptake \textsuperscript{106}. Montagut \textit{et al.} \textsuperscript{14} found that application of GSPE to 3T3-L1 adipocytes causes auto-phosphorylation of the insulin receptor and increased glucose uptake. GSPE required phosphorylation of IRS-1 to stimulate glucose uptake suggesting that GSPE stimulated glucose uptake is initiated similarly to insulin stimulated uptake, but differences exist in downstream signaling \textsuperscript{14}. For example, GSPE was found to stimulate the Thr308 residue of Akt to a lesser extent than insulin, but Ser473 residue to a greater extent \textsuperscript{14}. Similar results have been observed in other studies using 3T3-L1 adipocytes as well as L6 myotubes, in which GSPE stimulated glucose uptake and this uptake was inhibited in response to wortmannin, which is a well-known...
inhibitor of PI3-kinase and insulin-stimulated glucose uptake\textsuperscript{16}. These results suggest that GSPE improves glucose homeostasis through activation of the insulin signaling pathway. Finally, GSPE has been shown to decrease β-cell lipid synthesis and triglyceride accumulation\textsuperscript{107}. Interestingly, GSPE may only be beneficial in pancreatic β-cells in hyperlipidemia conditions as it reduces lipid accumulation, but also negatively impacts insulin secretion, which may be due to increased apoptosis\textsuperscript{105}. In totality, these studies suggest GSPE may play both beneficial and harmful roles in pancreatic β-cells, while positively increasing glucose uptake through the insulin signaling pathway in adipocytes and muscle cells.

4.3 Grape Pomace and Insulin Signaling – Animal Studies

Numerous animal studies investigating the effect of GP on glucose homeostasis have shown beneficial results\textsuperscript{15,16,100,108–111}. Supplementation of GP into the diet of high fructose fed rats prevents insulin resistance and increased liver triglyceride content, which is thought to be due, at least in part, to improved Akt activation and attenuated increases in ERK\textsuperscript{15,108,109}. Further, these animals had lower hepatic lipid peroxidation suggesting reduced hepatic oxidative stress due to greater superoxide dismutase levels\textsuperscript{109}. Grape pomace, at 1% of the diet, increases muscle GLUT4 expression and content as well as glycogen synthase activity in rats resulting in greater glycogen accumulation\textsuperscript{108}. In diabetic mice, GP significantly reduced α-glucosidase activity resulting in reduced breakdown and digestion of complex carbs reducing postprandial hyperglycemia by 35\%\textsuperscript{100}. Finally, high dose GSOE (4g/kg body weight/day) has been effective in preventing a number of deleterious effects of HFD on the pancreas\textsuperscript{112}. Notably, it significantly reduced oxidative stress and lipid peroxidation preserving β-cell mass and
protecting the pancreas from lipotoxicity \textsuperscript{112}. While promising, none of these studies used GP to treat metabolic complications of hypercaloric diets i.e. GP was always used preventatively. More animal studies are needed to determine if GP can treat and improve glucose uptake and insulin action in already metabolically compromised models, such as T2D.

4.4 Grape Pomace and Insulin Signaling – Human Studies

Of five human trials \textsuperscript{17,18,102–104}, only two \textsuperscript{17,102} have found any improvements in metabolic measures, such as fasting blood glucose and HOMA-IR following GP supplementation. However, in each of these two studies, there are confounding issues which temper the interpretation of the data. One study \textsuperscript{17} found slight improvements in HOMA-IR in obese women following GSOE supplementation, but the GSOE was supplemented at a dose of 15\% of total caloric intake, which would not be attainable for the general population. Further, while researchers claimed that GSOE was effective at reducing inflammation and insulin resistance compared to their control sunflower seed oil (SFO) group, there was large variation in their data and although differences between baseline and post treatment HOMA-IR scores were slightly greater in the GSOE group, the final HOMA-IR was almost identical between groups (3.05 ± 1.32 GSOE vs. 3.07 ± 1.41 SFO) \textsuperscript{17}. Another confounding factor in this study was that participants receiving GSOE consumed 25\% less calories by the end of the study \textsuperscript{17}. The second study observed improvements in fasting blood glucose in obese men following 20 g/day of GP for 16 weeks \textsuperscript{102}. Interestingly, postprandial insulin was improved in subjects given GP, but postprandial glycaemia was not. It should be noted that fasting glucose was lowered in both the control and treated groups to almost the same extent. Insulin sensitivity as assessed by HOMA-IR did was not different between groups at any point \textsuperscript{102}. Other human trials utilized smaller
doses of GP between 300-600 mg/day\textsuperscript{18,103,104} and none of these studies found any changes in insulin sensitivity or serum lipid or glucose levels. However, there were reductions in cholesterol and C-reactive protein levels suggesting a possible role for GP in people with increased cardiovascular risk \textsuperscript{18,103,104}. The discrepancies between studies may be due to the difference in dosages, as the two studies which found improvements in markers of insulin resistance used much larger doses compared to the other studies.

\textbf{4.5 Concluding Remarks on Grape Pomace}

Similar to RESV, cell and animal studies have demonstrated an effect of GP in improving glucose uptake in muscle \textsuperscript{16} and adipose \textsuperscript{14,15}, but human studies have been less conclusive. Cell studies have determined GP stimulates glucose uptake through the insulin signaling pathway in muscle \textsuperscript{16} and adipose cells \textsuperscript{14} and protects pancreatic β-cells from lipotoxicity, but may negatively impact insulin secretion from the pancreas \textsuperscript{107}. Grape pomace also decreases the expression of pro-inflammatory genes which may contribute to improved insulin signaling in adipose \textsuperscript{106}. Animal studies have found that GP supplemented into high fructose and HFDs can prevent negative effects of these diets, such as decreased GLUT4 expression and impaired adipose \textsuperscript{108} and hepatic insulin signaling \textsuperscript{109}. Additionally, GP may be beneficial in reducing carbohydrate absorption when taken with a meal, as it inhibits α-glucosidase, decreasing the rise in glucose following a meal \textsuperscript{100}. Only 2 human studies \textsuperscript{17,102} have found beneficial effects of GP on blood glucose homeostasis and insulin signaling, although GP has been noted to improve C-reactive protein levels and blood pressure \textsuperscript{103,104}. The two studies \textsuperscript{17,102} used very high dosages of GP \textsuperscript{17,102}. Future cell studies should investigate GP’s potential at increasing apoptosis in
pancreatic β-cells. Overall, more research is recommended before using GP to improve markers of the metabolic syndrome.
Chapter 2 Objective of the Thesis

Estradiol (E2) is known to be protective in metabolism through mechanisms such as improving adipose lipolysis, skeletal muscle glucose uptake and mitochondrial content and function. Estrogen loss, as occurs during menopause, results in increases in visceral fat mass, decreases in insulin sensitivity, and an overall increased risk of developing T2D. While significant research has determined that E2 supplementation shortly after ovariectomy (1-3 weeks) is effective at restoring many of the metabolic dysfunctions that occur with E2 loss, there is little research that has investigated the effectiveness of delayed E2 supplementation. Investigating the effect of delayed E2 supplementation on metabolic measures is important information for physicians to have as it will aid in knowing when to prescribe HRT. This is valuable information as HRT has been associated with adverse side effects such as stroke, cardiac events, and breast cancer and as such, HRT should only be prescribed when necessary.

Previous research from our laboratory has determined exercise is a more effective alternative than E2 in restoring glucose tolerance in an OVX rat model; however other alternatives should be explored for those unwilling or unable to exercise. As an extension of that research, the present study investigated whether RESV and GP powder could restore glucose tolerance to an equal or greater degree than E2 supplementation. Previous animal studies have determined that RESV in conjunction with a HFD can prevent the insulin resistance associated with a HFD; however there are no studies that have investigated its potential as a treatment to animals that have already developed glucose intolerance. Further, the present study is the first to investigate the effects of RESV in an OVX rat model.

A final objective of this thesis was to determine if a GP supplement from Ontario, which has limited RESV concentration, but substantial amounts of anthocyanins and quercetins, could also
function as a potential nutraceutical in improving glucose tolerance in an intolerant model. Research has shown that anthocyanins can prevent decreases in insulin sensitivity in response to a HFD in animal models. Similar research has been seen in quercetin animal models as well. However, no research has investigated the effectiveness of GP in restoring glucose tolerance in an already intolerant OVX rat model.
Chapter 3 Grape Powder and Resveratrol Do Not Restore Glucose Tolerance in the Ovariectomized Rat.

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Abstract.

Background: Estrogen (E2) loss, as occurs during menopause, results in increased visceral adiposity and insulin resistance and impaired glucose tolerance. Estrogen replacement may restore insulin sensitivity if given shortly after menopause, but may be associated with adverse side effects such as stroke and ovarian cancer. Previous results from our laboratory have found exercise is an effective alternative to E2 replacement, but other alternatives should be explored for those unwilling or unable to exercise. Natural compounds such as resveratrol (RESV) have potential insulin sensitizing effects. Grape pomace/powder (GP) is a byproduct of winemaking, and contains RESV and other antioxidants. To our knowledge, the efficacy of RESV and GP in restoring glucose tolerance in ovariectomized (OVX) rats has not been explored.

Methods: Phyto-estrogen free diet was administered ad libitum to sham control (SHAM) and OVX female Sprague-Dawley rats until the onset of glucose intolerance in OVX rats (12 wks) as determined by an intraperitoneal glucose tolerance test (IPGTT). Subsequently, OVX animals were divided into treatment and control groups (n=10 each). The treatment groups received daily one of i) a physiological oral dose of E2 (28µg/kg body mass), ii) RESV (5mg/kg body mass), or iii) GP supplemented at 1.5% by weight of the diet. Treatment continued for 6 wks followed by intraperitoneal glucose and insulin tolerance tests. 2-3 d after the tolerance tests, terminal surgeries were performed during which red (RG) and white gastrocnemius (WG) muscles, visceral adipose tissue and liver were sampled to assess Akt response to insulin injection. Visceral adipose was preserved and stained to determine adipocyte size.

Results: OVX animals were significantly heavier than control animals at the onset of glucose intolerance and this did not change throughout the treatment period. None of the treatments restored normal glucose tolerance during the treatment. Insulin tolerance was not worsened in
OVX rats, and was unaffected with treatment. No difference in phosphorylation of the insulin signaling protein, Akt, was found after insulin injection in RG, WG, visceral adipose tissue, or liver between SHAM and OVX animals. Adipocyte size was significantly increased in the OVX-E2 and OVX-RESV groups compared to SHAM.

Conclusion: Delayed E2 replacement, RESV and GP were not effective in restoring normal glucose tolerance in OVX rats.
**Introduction**

Premenopausal women have been demonstrated to be partially protected against insulin resistance and type 2 diabetes (T2D) compared to age matched men, but this protection disappears after menopause. The protective effect is believed to be conferred by circulating estrogen, specifically 17β-estradiol (E2). Estrogen has been demonstrated to play various protective roles including improving insulin sensitivity, inhibiting lipid synthesis, and promoting a more anti-inflammatory environment in adipose tissue as well as improving satiety signals and increasing resting energy expenditure. Estrogen hormone replacement therapy (HRT) has shown promise in rescuing metabolic function in many animal studies when giving shortly after ovariectomy. However, E2 supplementation has become controversial due to the potential/perceived risk of adverse side effects. The Women’s Health Initiative led large scale studies investigating HRT, but halted their trials due to increased risk of comorbidities such as cardiac events and breast cancer. Further, recent research has suggested there is a critical time period between perimenopause and post menopause where E2 supplementation is effective and E2 treatment outside this window may be detrimental. Alternative treatments to HRT to restore metabolic function in E2-deficient females are needed. A recent study from our laboratory demonstrated that treadmill-based endurance exercise was an effective treatment in ovariectomized female rats, conferring similar if not better improvement in oral glucose tolerance compared to E2 treatment. However, alternatives are still needed for those that are unwilling or unable to exercise.

Resveratrol (RESV) is a polyphenolic compound found in different foods, particularly grape skins. Several studies have investigated the effects of RESV on glucose homeostasis in humans with mixed, although largely negative findings. In obese, but otherwise healthy
subjects, the outcomes of RESV supplementation have largely found no improvement\(^95,96,98\), although at least one study has shown improved insulin sensitivity\(^94\). Some studies suggest that RESV may improve insulin response in diabetic patients\(^97\), but others do not\(^95\). Rodent studies have depicted a more clear benefit of RESV, with evidence for improved skeletal muscle glucose uptake\(^87\), protection from oxidative stress in the pancreas\(^85\), and improved hepatic insulin signaling\(^88\). Interestingly, RESV has been proposed to protect cells from oxidative stress by a mechanism that requires the ER\(\beta\) receptor\(^84\). To our knowledge, the efficacy of RESV in restoring glucose tolerance in an E2-deficient animal has not been explored.

Grape pomace (GP) is a byproduct of the wine making process and is largely disposed of as a waste product. Grape pomace is composed of grape skins, seeds, and stems and contains considerable amounts of RESV\(^13\). Grape pomace has been shown to be a potent antioxidant\(^12\) and is believed to function as an \(\alpha\)-glucosidase inhibitor, which results in less breakdown and therefore lower absorption of complex carbohydrates from the diet\(^100\). Grape pomace also contains substantial concentrations of the phenolic compounds, anthocyanins, which have been shown to improve inflammation and insulin sensitivity in diabetics\(^118\). Quercetin, which is also present in significant concentrations, has been shown to reduce inflammation and insulin resistance in human adipocytes, myotubes, and ob/ob obese mice\(^119,120\). To our knowledge the efficacy of GP in restoring glucose homeostasis in OVX animals has not been explored.

Therefore, the aim of the current study was to determine whether RESV would be effective in restoring glucose tolerance in the ovariectomized, glucose intolerant rat. Furthermore, we also wished to examine the effect of GP, a natural food product containing RESV and antioxidants, in restoring glucose tolerance in this model.
Methods.

Materials and reagents. Reagents, molecular weight markers, and nitrocellulose membranes were purchased from BioRad (Mississauga, ON, Canada). Western Lightning Plus enhanced chemiluminescence (ECL) was purchased from PerkinElmer (NEL105001EA). The following primary antibodies were purchased from Cell Signaling: phospho-Akt Ser^473 (cat. # 9271) and Akt (cat. #9272). NP40 cell lysis buffer was acquired from Life Technologies and phenylmethane sulfonyl fluoride (PMSF) and protease inhibitor cocktail were obtained from Sigma (cat. #78830 and 9599). Insulin (Humulin, rDNA origin) was purchased from Eli Lilly (Toronto, ON, Canada). 17β-Estradiol Molecular Assay Kit (Cat. #ab108667) was purchased from Abcam (Toronto, ON, Canada). Resveratrol was purchased from Cayan Chemical Company (Michigan, USA) and Bioflavia grape pomace powder was kindly supplied by Southbrook Vineyards (Ontario, Canada).

Animals. All procedures were approved by the Animal Care Committee at the University of Guelph and followed Canadian Council of Animal Care guidelines. Female Sprague Dawley rats were purchased from Charles River Laboratories at 2 months of age (body mass, 207.8 g ± 1.2g). Two days prior to arrival, all rats underwent either bilateral ovariectomy (OVX; n=40) or SHAM surgery (SHAM; n=10) by Charles River technicians. Two flank incisions were made on the dorsal side, ovaries were identified, and were either removed by cauterization (OVX) or left intact (SHAM). OVX surgeries were verified following terminal experiments by assessment of plasma E2 content.

Two to three animals were grouped in cages of either SHAM or OVX for the first 12 weeks, with no further stratifications. Rats were housed in a temperature controlled room (25°C) with a 12:12-h standard light–dark cycle and were allowed ad libitum access to food and water. All
animals were fed a phytoestrogen-purified, soy protein-free diet (Harlan 2020X) which provided 60% of calories from carbohydrates, 24% from protein and 16% from fat. A pre-weighed, excess amount of food was placed in each hopper and the amount leftover per cage was weighed and recorded every 2–3 days. Body mass was recorded weekly throughout the study. Animals were anesthetized prior to terminal surgeries by an intraperitoneal injection of pentobarbital sodium (60mg/kg body weight). After surgeries and while still anesthetized, animals were sacrificed by intracardiac injection of sodium pentobarbital.

Initial Glucose Tolerance Tests. At the end of the initial 12 weeks, the establishment of glucose intolerance in the OVX rats was confirmed by an intraperitoneal glucose tolerance test (IPGTT). Animals were fasted for approximately 8 hours prior to the GTT. A bolus of glucose (2.0g/kg body mass) was injected into the intraperitoneal cavity of each animal and blood glucose was measured from the tail vein at 15, 30, 45, 60, 90 and 120 min with a handheld glucometer (FreeStyle Lite). Area under the curve for the 120 min duration for the glucose response was calculated to compare glucose tolerances between SHAM and OVX animals.

Treatment Period. At the end of 12 weeks, following the induction of glucose intolerance, OVX animals were divided into 3 treatment (OVX-E2, OVX-RESV and OVX-GP) and control (no treatment, OVX-CON) groups of n=10 each. The OVX animals were randomly distributed into these groups and it was confirmed that the average glucose tolerance i.e. glucose AUC of each group was statistically similar. A SHAM group was also included. The OVX-E2 group received a daily physiological dose of powdered E2 (28µg/kg body mass) via a Nutella cream solution.
(5µL sesame oil/1g Nutella/kg body mass) as previously described\textsuperscript{117,121}. The OVX-RESV group received a daily dose of powdered RESV (5mg/kg body mass) mixed into the Nutella cream solution. Finally, the OVX-GP group received powdered GP in their diet at 1.5% by weight, similar to other studies\textsuperscript{100,110}. The composition of the powdered GP is shown in Table 1, and provides approximately 0.02mg/kg body mass / day of RESV, in addition to the antioxidants anthocyanin and quercetin. SHAM, OVX-CON, and OVX-GP groups also received a daily dose of untreated Nutella cream solution. Animals were allowed ad libitum access to food, and treatment continued for six weeks. The phytoestrogen-free diet (pellet form) was powdered to ensure even mixing of GP into the diets. To eliminate the powdering of the diet as a confounding variable, all groups received the powdered pellets as the base to their diet. During this time food intake was measured 3 times weekly and animal mass was recorded weekly.

*Final GTT and Insulin Tolerance Test.* After six weeks, animals were given another IPGTT (2.0g glucose/kg body mass). Blood glucose was measured at 0, 15, 30, 45, 60, 90, and 120 minutes post injection. Two days later, an insulin tolerance test was performed. A bolus of insulin (0.5 U/kg body mass) was injected into the intraperitoneal cavity of each animal approximately 3 hours after removal of food i.e. non-fasted state and blood glucose was measured in 15 minute intervals for 1 hour. Area above the curve was determined for each animal and used to assess insulin tolerance between groups.

*Terminal Surgeries.* Two to three days after completion of the tolerance tests, terminal surgeries were performed during which skeletal muscle (red and white gastrocnemius) and adipose (subcutaneous, inguinal and visceral, retroperitoneal) tissue were harvested before and 10
minutes after insulin injection (1 U/kg body mass). A piece of the medial lobe of the liver was sampled post insulin injection only i.e. not pre injection, in order to avoid potential bleeding issues. All tissues were immediately frozen in liquid nitrogen and stored at -80°C. Animals were anesthetized prior to all surgical procedures with an intraperitoneal injection of sodium pentobarbital (60mg/kg body mass). Animals were sacrificed with an intracardiac injection of pentobarbital.

*Tissue Homogenization.* Frozen tissue was chipped in liquid nitrogen and samples were placed in homogenization tubes with lysis beads. Tubes were then placed back in liquid nitrogen to ensure samples did not thaw. Cell lysis buffer was supplemented with protease inhibitor and PMSF and added at a volume to weight ratio of 10x for muscle, 5x for adipose tissue, and 20x for liver. Samples were homogenized for 60 seconds and then centrifuged for 15 minutes at 4°C at 1500 rcf. Supernatant was transferred to a new eppendorf tube, snap frozen in liquid nitrogen and stored at -80°C. Blood samples were centrifuged and the separated plasma stored -80°C.

*Total Protein Quantification by Bicinchoninic Acid (BCA) assay.* Homogenized samples were diluted with distilled H2O and kept on ice. 10 µL of each standard and sample were pipetted into a 96 well microplate and run in triplicate. 200 µL of BCA solution was added into each well and plates were incubated at 37°C for 30 minutes. Plates were read using SoftMax program v5.2 to determine fluorescence of each sample.

*Western Blot.* 20µg of samples were loaded onto 15% gels. Gels were placed in a running tank for 20-30 minutes at 100V until sample had left the stacking gel at which point voltage was
increased to 145V for ~1 hour. Protein was then transferred to a nitrocellulose membrane by cold-wet transfer at 0.2A for 1 hour. At this point the membrane was blocked in a 5% TBST skim milk solution for one hour at room temperature. Primary antibodies for phospho-Akt(Ser473) and total Akt (tAkt) were diluted at a ratio of 1:1000 in 15mL of a TBST 5% BSA solution. The membrane was incubated in this solution on a shaker table in a 4°C cold room overnight. The next day membranes were washed twice with TBST at room temperature. Next, membranes were incubated in secondary anti-rabbit antibody solution. This solution was composed of secondary antibody at a ratio of 1:2000 in 0.15g skim milk powder plus 15mL TBST. Membranes were incubated at room temperature for one hour. Following incubation, membranes were washed twice in TBST and once in TBS solution before detection. Bands were visualized using enhanced chemiluminescence and quantified using densitometry with Alpha Innotech Imaging software v3.4.0.0.

Adipocyte Size. Average adipocyte size was calculated using AdipoSoft software v. 1.13 (University of Navarra, Pamplona, Spain). Adipose tissue samples from each animal (n=8 from each of the 5 groups) was stained with modified Harris hematoxylin and eosin with phloxine. Images were captured using an Olympus FSX 100 light microscope, Olympus, Tokyo, Japan). Images containing approximately 300 cells were used to assess cross sectional diameter of the cells.

Estradiol Concentration. E2 concentration was determined using an estradiol specific enzyme-linked immunosorbent assay (ELISA) kit. 25 µL of sample and standard were pipetted into a 96
well anti-17 beta estradiol IgG coated microplate. 200 µL of E2-HRP conjugate was then added to each well and the plate was incubated at 37°C for two hours. The wells were then aspirated and washed 3 times with 300 µL of a diluted washing solution. After washing, 100 µL of TMB substrate solution was then added into each well. The plate was then incubated at room temperature in the dark for 30 minutes. Finally, 100 µL of stop solution was added into all wells and the plate was read in a microplate reader at an absorbance wavelength of 450 nm.

Statistics. All statistics were analyzed using GraphPad Prism 6. Data are expressed as mean ± standard error (SE). A one way analysis of variance (ANOVA) was used to compare mean values between treatment groups. In the instances of repeated time points, or pre and post-insulin treatment, a repeated measures ANOVA was utilized. A Tukey's posthoc test was used to determine significant differences between groups if there was a significant main effect or interaction revealed by the ANOVA. In all tables and figures, letters are used to denote statistical significance, such that means sharing a letter are not significantly different from one another. Significance was accepted at p ≤ 0.05.
Results

Ovariectomy results in hyperphagia and increased body mass. Ovariectomy resulted in significant hyperphagia from the start of the introductory period (p<0.001; Table 1), but tapered off after 5 weeks, similar to the findings of others. The body mass of all OVX groups was significantly higher than SHAM controls by the second week of the induction period (p<0.0001; Table 1) and this continued until the end of the treatment period. None of the treatments resulted in significant changes in body mass in the OVX rats.

Plasma E2 concentration is significantly reduced following OVX but is restored with E2 supplementation. Plasma E2 was significantly reduced by ~4-fold (p=0.02) in OVX animals that did not receive E2 compared to SHAMs (Figure 1). Estrogen supplementation successfully raised plasma E2 concentrations such that no difference existed between OVX E2 and SHAM rats.

Glucose tolerance is impaired by 12 weeks following OVX animals. Calculated incremental glucose AUC was significantly increased (p=0.03) in OVX animals compared to SHAMs by 12 weeks post ovariectomy, indicating a worsening of glucose tolerance (Figure 2). This impaired glucose tolerance still remained after 6 weeks of treatment, regardless of the type of treatment (E2, RESV or GP), with none of the treatment groups showing a significant reduction i.e. improvement in glucose AUC.
Insulin tolerance is not different between groups. Next, to more directly determine whether the impaired glucose tolerance in the OVX animals might be due to a loss of insulin action, an intraperitoneal ITT was conducted. Insulin tolerance, as determined by the calculated glucose area above the curve, was not significantly different between SHAM and any of the OVX groups (Figure 3). Therefore, changes in insulin tolerance cannot explain the worsening of glucose tolerance observed in OVX versus SHAM animals.

Adipocyte size is significantly larger in OVX E2 and OVX RESV rats. OVX-E2 and OVX-RESV rats had significantly larger visceral adipocyte size compared to SHAM controls (Figure 4). Compared to SHAM animals, OVX-E2 and OVX-RESV had 38% (p=0.0552) and 46% (p=0.0121) larger adipocyte size. OVX-CON and OVX-GP rats were not statistically different from SHAMs, although there was still a trend towards larger adipocytes with 23.3% (p=0.4225) and 27.2% (p=0.2712) larger adipocytes, respectively, compared to SHAM controls.

Total Akt content and phosphorylation is not altered by ovariectomy or subsequent treatment. There were no differences in total Akt content of red and white gastronemius muscle, or visceral adipose tissue, between groups, or between basal and insulin stimulated conditions (Figure 5). Liver total Akt content (assessed only after insulin stimulation) was not significantly different between OVX treatment groups and SHAM controls. To determine whether OVX animals had inhibited activation of downstream insulin signaling targets in response to insulin, pAkt(Ser473) was assessed in pre (basal) and post insulin injection samples of red and white gastrocnemius muscle and visceral adipose tissue (Figure 6). Liver pAkt(Ser473) content was assessed only in
the insulin stimulated state. As expected, insulin stimulation significantly increased pAkt(Ser473) content approximately 4-fold compared to the basal state in red and white gastrocnemius, and approximately 2-fold in visceral adipose (p<0.001). However, there were no differences in pAkt(Ser473) content between treatments under either basal or insulin conditions. Hepatic insulin stimulated pAkt(Ser473) content was also not significantly affected by ovariectomy or treatment. Overall, neither ovariectomy nor treatment with E2, RESV or GP had any effect on total or pAkt(Ser473) content in muscle, adipose or liver tissues.
### Tables and Figures

**Table 1. Body Mass and Food Intake.**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>SHAM</th>
<th>OVX-CON</th>
<th>OVX-E2</th>
<th>OVX-RESV</th>
<th>OVX-GP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Mass (g)</td>
<td>235.4 ± 4.2</td>
<td>268.5 ± 3.8</td>
<td>269.9 ± 3.8</td>
<td>266.3 ± 4.5</td>
<td>266.4 ± 4.4</td>
</tr>
<tr>
<td>Mass at Treatment (g)</td>
<td>349.6 ± 12.4</td>
<td>422.9 ± 16.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>428.9 ± 14.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>437.1 ± 16.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>429.4 ± 16.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Final Mass (g)</td>
<td>364.9 ± 12.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>432.8 ± 16.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>437.1 ± 18.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>451.2 ± 18.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>429.6 ± 14.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean Food Intake Prior to Treatment (g/wk)</td>
<td>129.1 ± 2.4</td>
<td>141.3 ± 4.4</td>
<td>141.9 ± 4.1</td>
<td>147.6 ± 4.1</td>
<td>146.1 ± 4.2</td>
</tr>
<tr>
<td>Mean Food Intake During Treatment (g/wk)</td>
<td>127.0 ± 4.6</td>
<td>111.2 ± 4.8</td>
<td>109.6 ± 4.8</td>
<td>120.7 ± 4.4</td>
<td>113.3 ± 3.4</td>
</tr>
</tbody>
</table>

OVX animals were significantly heavier than SHAM by 3 weeks and this didn’t change throughout the treatment period. Food intake was not significantly different between treatments after 6 weeks. Data are presented as mean ± standard error, \( n = 10 \) for all groups. Groups which share a letter are not statistically different. Statistical significance accepted at \( P < 0.05 \).
### Table 2. Grape Pomace Powder Composition

<table>
<thead>
<tr>
<th>Grape Pomace Composition As % of Whole</th>
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<tbody>
<tr>
<td><strong>Vitis Vinifera</strong> Grape Skin Powder</td>
</tr>
<tr>
<td><strong>Vitis Vinifera</strong> Grape Powder</td>
</tr>
<tr>
<td>Vitamin C (Ascorbic Acid)</td>
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<tr>
<td>Fibre</td>
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#### Bioactive Components

<p>| | |</p>
<table>
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<tbody>
<tr>
<td>Anthocyanins</td>
<td>2.1%</td>
</tr>
<tr>
<td>Quercetins</td>
<td>0.013%</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>0.003%</td>
</tr>
</tbody>
</table>

Supplemented into diet at 1.5% weight/weight. This provides a dose of RESV of about 0.02 mg/kg/day.
Figure 1. Plasma estrogen content.

Estradiol Concentration between SHAM (n=8), OVX CON (n=23), and OVX E2 (n=7). OVX resulted in significantly diminished plasma E2 concentration and this was recovered in E2 treated animals. Estrogen plasma content presented as pg/ml. Data are presented in mean ± standard error. P<0.05 accepted as significant.
Figure 2. (A) Glucose tolerance test and (B) calculated area under the curve prior to, and after treatment in OVX animals.

OVX animals were significantly glucose intolerant by 12 weeks, and not treatment restored glucose tolerance. Data are presented as mean ± standard error, n = 10 for all groups. Groups which share a letter are not statistically different. Statistical significance accepted at P < 0.05.
Figure 3. (A) Insulin tolerance test and (B) calculated area above the curve in OVX-treated animals.

Data are presented as mean ± standard error, n = 10 for all groups. No groups were significantly different at p<0.05.
Figure 4. Visceral adipocyte size (A) images and (B) diameter in OVX-treated animals.

Average adipocyte cell size of SHAM and OVX animals at terminal surgeries. OVX-E2 and OVX-RESV animals had a significantly increased adipocyte size. Cell size is expressed as the average diameter of a cell in µm. Data are presented in mean ± standard error, n=8 for all groups. Groups which share a letter are not statistically different. Statistical significance accepted at P<0.05.
Figure 5. Total Akt content in OVX-treated animals.

tAkt content is not different between treatment groups in (A) red gastrocnemius, (B) white gastrocnemius muscle, (C) liver, or (D) visceral adipose. tAkt content is not significantly different post insulin injection. Data are presented as mean ± standard error, n = 10 for all groups. Groups which share a letter are not statistically different. Statistical significance accepted at P < 0.05.
Figure 6. Phosphorylated Akt content in OVX-treated animals.

pAkt content is not different between treatment groups in (A) red gastrocnemius, (B) white gastrocnemius muscle, (C) visceral adipose, or (D) liver. pAkt content significantly increased post insulin injection, but this response does not differ between SHAM and OVX. Data are presented as mean ± standard error, n = 10 for all groups. Groups which share a letter are not statistically different. Statistical significance accepted at P < 0.05.
Discussion

Estrogen loss is associated with decreased insulin sensitivity and glucose clearance \(^2\). Estrogen replacement has been associated with adverse effects such as increased risk of ovarian cancer, stroke, and other cardiac events \(^8\). Furthermore, E2 replacement also varies in effectiveness based on how early repletion begins \(^{113}\). Therefore, alternatives to HRT are needed. The current research investigated the effects of RESV and GP in restoring glucose tolerance in ovariectomized Sprague-Dawley rats. The doses of RESV and GP were chosen to represent an amount realistically attainable through dietary supplementation. Our findings demonstrate that 6 weeks of oral E2, RESV, or GP did not improve the impaired glucose tolerance in ovariectomized animals. The observed impairment in glucose tolerance does not appear to be due to reduced insulin action as whole body insulin tolerance and maximal stimulated insulin signaling in major insulin responsive tissues (skeletal muscle, adipose and liver) was unaffected by ovariectomy or subsequent treatments. Our findings suggest that RESV and GP may not represent an effective nutraceutical supplement for improving glucose homeostasis in physiological states of reduced estrogen.

In the current study, no treatment restored glucose tolerance, including the oral administration of physiological doses of E2. Previous research from our laboratory which used the same dose and method of E2 delivery observed a trend in moderate improvement in glucose tolerance in OVX animals of 20\% (p=0.08); these animals received E2 treatment 10 weeks post ovariectomy.\(^{117}\). The animals in the present study had a relatively longer period of E2 depletion (12 weeks) before treatment began, compared to other studies which begin E2 repletion only 1-3 weeks post ovariectomy \(^{60,122}\). This suggests that longer periods of E2 deficiency lead to altered metabolism which becomes more difficult to correct. One study which investigated the
effectiveness of E2 replacement in women who were in early menopause (≤6 years since final menses) and who were in late menopause (≥10 years since final menses) found late E2 replacement was actually detrimental to insulin sensitivity. Other studies which have looked at hippocampal responsiveness to E2 or CVD risk have found E2 repletion is only effective shortly after E2 depletion.

Estrogen depletion has been shown by some to result in impaired insulin stimulated GLUT4 translocation and glucose transport in skeletal muscle, as well as impaired glucose uptake in adipose tissue, although this has not been seen by all. To determine whether impaired glucose uptake in insulin-sensitive tissues might be the cause of the observed glucose intolerance, an insulin tolerance test was performed. Interestingly, no OVX animals, including untreated OVX controls demonstrated a significant change in blood glucose clearance in response to an acute insulin injection. In addition to this, phospho-Akt(Ser473) response to a high concentration insulin bolus (1 U/mL) in muscle, adipose and liver was not different in SHAM and OVX animals, and not altered by any of the treatments. Considering these findings and that previous results from our laboratory did not find a difference in skeletal muscle GLUT4 content between OVX and E2 treated animals, it seems unlikely that insulin stimulated glucose uptake or impaired insulin signaling was responsible for the observed decreased glucose tolerance. However, it should be recognized that the insulin concentration used to determine tissue Akt response was supraphysiological in order to maximize the ability to detect changes and any potential differences amongst groups. The possibility exists that differences in glucose clearance in response to insulin and tissue Akt phosphorylation may have been revealed at lower insulin concentrations which would depict insulin sensitivity and not a maximal or near maximal response. Lastly, we cannot discount the possibility that pancreatic insulin release was impaired.
in OVX animals, as we were unable to assess plasma insulin concentrations during the glucose tolerance tests. Given that E2 protects pancreatic β-cells from oxidative damage and directly stimulates insulin release from β-cells\textsuperscript{28,77}, this should be considered a possibility.

Consistent with previous literature\textsuperscript{25,117}, ovariectomy resulted in significantly increased body mass compared with SHAM controls. Food intake was significantly greater immediately following ovariectomy, but this effect was essentially lost after 6 weeks. Similar food intake patterns have been seen in previous studies with rats\textsuperscript{117,124}. However, in spite of the fact that food intake eventually was similar in OVX and SHAM groups, the increase in body mass of OVX animals became significant by the 3\textsuperscript{rd} week post-surgery and remained greater throughout the treatment period, suggesting a change in metabolic efficiency. Visceral adipose was analyzed as OVX animals tend to have increases in visceral fat mass, which is more metabolically active and detrimental to whole body insulin action than subcutaneous tissue\textsuperscript{33}. This propensity to gain visceral adipose tissue led us to expect more pronounced changes in visceral adipose tissue. Visceral adipocyte size was generally increased in OVX animals, although this was statistically significant only in OVX-E2 and OVX-RESV groups (38% and 46% larger, respectively). OVX-CON and OVX-GP adipocytes were 23% and 27% larger than SHAM, respectively, but this did not reach significance. Previous studies have found that oral E2 administration results in increased fat mass\textsuperscript{125} which is in agreement with our observations in the OVX-E2 group. Previous research has suggested that increased adipocyte size can cause insulin resistance\textsuperscript{126,127}; however, changes in adipocyte size in our study were not associated with decreases in maximal insulin stimulated pAkt(Ser473) or tAkt content in adipose tissue of either OVX-E2 or OVX-RESV animals. However, glucose uptake into adipocytes was not directly assessed in the current study.
Supplementation of RESV into the diets of glucose intolerant rodents and humans have had mixed results. Studies using humans have found both positive \textsuperscript{94} and negligible \textsuperscript{96} improvements in glucose tolerance in response to RESV. While numerous animal studies have found improvements in glucose tolerance in response to RESV treatment \textsuperscript{85-88}, the magnitude of this effect has been quite variable, and does not appear to be correlated to the dosage given, which is also highly variable (2.5 mg/kg body mass to 4 g/kg body mass) \textsuperscript{88-86}. In the present study, we utilized a relatively low dosing regimen of 5 mg/kg body mass to represent an attainable dose in humans through commercially available supplements. To our knowledge, this is the first study that has investigated the effects of RESV on whole body glucose tolerance in an OVX animal model. One human study which investigated the effects of RESV supplementation in healthy, postmenopausal women also found no effect of treatment \textsuperscript{98}. Resveratrol’s effect on skeletal muscle glucose uptake has also been shown to be dependent on ERα stimulation \textsuperscript{128}, which suggests that the beneficial effect of RESV on glucose tolerance may require normal E2 signaling pathways.

Grape pomace has been shown to be effective at preserving insulin action and glucose tolerance in high-fructose fed animals \textsuperscript{108,109}. Most studies that have shown a beneficial role of GP on blood glucose levels have done so from the perspective of prevention i.e. that GP can prevent the deleterious consequences of a high fructose fed diet when supplemented simultaneously. To our knowledge, this is the first study to investigate GP’s ability to rescue the already impaired glucose tolerance in an E2-deficient model. Six weeks of GP treatment did not restore impaired glucose tolerance. As with RESV, it may be possible that GP is more effective when administered simultaneously to the induction of the ovariectomy as a means of prevention, although this remains to be determined. Another factor that is important to consider is the
variable composition of GP or powder. Resveratrol and antioxidant content varies significantly between different GP manufacturers and grape species. The GP utilized in this study was chosen as it is a commercially available product from a nearby vineyard. Our supplement has a lower amount of RESV compared to other animal studies (30 µg/g vs. 60 µg/g), but contains a significant concentration of other anthocyanins and quercetins which have been shown to be beneficial to whole body metabolism. Anthocyanins are potent antioxidants that have been shown to improve fasting insulin levels and insulin sensitivity as assessed by HOMA-IR in diabetic patients. Quercetin is another phenol found in fruits and vegetables which reduces inflammation in animal models and increases skeletal muscle GLUT4 content. One study found that quercetin reduced inflammation in human adipocytes to a greater degree than RESV. These bioactive compounds make GP a potentially useful nutraceutical. Unfortunately in the current study, GP did not rescue glucose tolerance in OVX animals.

Due to the gap in knowledge between preventive supplementation and treatment for the restoration of metabolic dysfunction of RESV and GP, future research should focus on investigating RESV and GP’s ability to restore glucose homeostasis in glucose intolerant, ovary intact animals. Further investigation into RESV’s ER signaling mechanisms would also be beneficial. Estrogen replacement seems to have a window of time when it is effective in restoring metabolic dysfunction. Investigating the timeline between when E2 depletion occurs and when E2 treatment remains effective would be a valuable tool for physicians deciding when to prescribe HRT to menopausal women.
Conclusion

In conclusion, this study is the first to investigate the effects of RESV and GP on glucose tolerance in an E2-deficient model. Low dose RESV and GP were not effective at rescuing impaired glucose tolerance caused by estrogen deficiency in female Sprague-Dawley rats. Impairments in glucose tolerance also did not appear to be due to impaired insulin stimulated glucose uptake, or Akt signaling in liver, red or white gastrocnemius, or visceral adipose in response to a high insulin dose. Resveratrol and GP supplementation are likely not effective alternatives to HRT to restore compromised glucose tolerance.
Brief Integrative Discussion

Prolonged E2 loss seems to confer permanent negative effects that cannot be resolved with E2 supplementation. For example, reductions in satellite cell proliferation and hippocampal proliferation have been observed in late (77-180 days) vs. early (6-14 days) E2 replacement. Only two studies have looked specifically at the effect of late E2 replacement on insulin sensitivity and surprisingly, E2 treatment in late menopause may cause detrimental effects. The animals in the current study did not receive treatment until 12 weeks post ovariectomy which is considerably longer than most studies which replace E2 within 1-3 weeks after surgery. Considering the rat life span (2-3 years), 12 weeks would represent a substantial amount of time in the E2 deficient state. This could explain the lack of effect of E2 treatment on glucose tolerance in our current study, as well as the relatively modest effect observed in a previous study from our laboratory, which also had a relatively prolonged period of E2 deficiency. However, other factors may have influenced the results as well. For example, a recent study has found that the sex of the animal handler can significantly affect research results. Male and female rats and mice are more stressed in response to male handlers compared to female handlers. Along with the increase in stress, hormones that negatively impact glucose tolerance would be released such as cortisol, which promotes hepatic glycogenolysis. While previous research from our laboratory showed a modest trend for increased glucose tolerance in response to delayed E2 treatment, E2 was not only replaced slightly earlier, but the experimenter was female compared to the current study which was led by a male scientist. The possibility exists that the rats were significantly more stressed during GTTs which would negatively impact glucose tolerance.

While there has been a substantial amount of RESV animal studies, it is difficult to extrapolate results from these studies as the doses utilized is variable (2.5 mg/kg body mass to 4
g/kg body mass) and the magnitude of effect does not seem to be correlated to dose size. For example, RESV doses of 2.5mg/kg bm/day and 2 g/kg bm/day have elicited similar improvements as indicated by reductions in plasma insulin levels. Further, the rodent model used is also variable, including IRS-2 deficient mice, KKA’y mice, and high-fat fed Sprague-Dawley rats. On top of this, these animal studies only investigate RESV’s effectiveness at preventing metabolic complications in response to HFDs, but do not investigate its ability to treat impaired glucose tolerance. Considering these factors together, more consistent research, with respect to dose and animal model, is needed to properly evaluate the efficacy of RESV treatment on metabolic indices like glucose tolerance and insulin sensitivity.

Interestingly, the only other study that has investigated the effects of RESV on metabolic function in rats with ovarian disturbances found that RESV was not effective in improving insulin sensitivity. In that study, the effect of RESV was investigated in rats with polycystic ovarian syndrome which typically presents with increased circulating levels of both androgens and estrogens, as well as glucose intolerance. The present findings suggest that RESV is not effective at improving glucose tolerance in E2-deficient models. Furthermore, one human study which investigated the effects of RESV supplementation in healthy, postmenopausal women also found no effect of treatment. Resveratrol’s effect on skeletal muscle glucose uptake has also been shown to be dependent on ERα stimulation, as ERα inhibition completely removed RESV’s improvements of both insulin dependent and independent skeletal muscle glucose uptake.

Taken together, considering that the study using rats with supraphysiological levels of E2 and the present study using rats with very low levels of E2 found no effect of RESV treatment, this may suggest RESV’s effects on glucose tolerance are dependent on normal E2 signaling.
Similar to RESV, while there has been a considerable amount of research investigating the effects of GP on metabolic indices like glucose tolerance and insulin signaling, there is also a lack of consistency. Grape pomace is actually quite a broad definition as it includes anything that is made from the remnants of grapes from the wine making process and may contain any or all of the skins, seeds, and stems of grapes. Due to the variability of grape species which are used for grape pomace and the actually constituents of each powder, the composition of GP varies widely between studies. Some studies use grape seed oil while others use grape seed extract and others whole grape pomace. This makes it difficult to compare results between studies. Our grape pomace powder was chosen as it is a commercially available product from a vineyard in our region from Southwestern Ontario. We chose this as it would be directly available to consumers within ours and surrounding communities. Interestingly, our supplement had a lower concentration of RESV compared to other studies (30 µg/g vs. 60 µg/g). However, our GP did contain substantial amounts of anthocyanins and quercetin, which also reduce inflammation and possibly insulin resistance. This lack of consistency should be considered when interpreting the results of each study. Future studies into GP should focus on determining which grape species and at which dose is most effective at improving insulin action in a glucose intolerant, ovary intact animal.
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