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# Actions of Grape Seed Extract in Rodent Brain and Differences in Metabolism of its Polyphenols in a Rodent Model of Menopause

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# ACTIONS OF GRAPE SEED EXTRACT IN RODENT BRAIN AND DIFFERENCES IN METABOLISM OF ITS POLYPHENOLS IN A RODENT MODEL OF MENOPAUSE

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**A DISSERTATION**

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**PHARMACOLOGY AND TOXICOLOGY**

## ABSTRACT

Grape seed extract (GSE), a dietary supplement, has potential in the treatment and prevention of human chronic age-related diseases including cancers, cardiovascular diseases, and neurodegenerative diseases. GSE and adult hippocampal neurogenesis, independently, have enhanced learning and memory in rodents. We hypothesized that GSE enhances learning and memory, at least partially, by enhancing hippocampal neurogenesis. However, adult mice given GSE did not exhibit increased number of progenitor cells or new neurons, established markers of neurogenesis, in the dentate gyrus (DG). Also, 26-day-old pups whose mother was given GSE only while nursing had fewer new neurons in the DG compared to control pups. These results suggest that the beneficial actions of GSE on learning and memory may be independent of enhancement of hippocampal neurogenesis. Lowered estrogen that accompanies menopause has been associated with impaired cognitive function in women. Ovariectomized (OVX) rats are used to model menopause and GSE enhanced cognition in young OVX rats. We hypothesized that GSE attenuates cognitive impairment in an older rodent model of menopause that more closely relates to human menopause. Rats were OVX at 6 months of age, given GSE for 6 months, and tested for cognitive function at 12 and 16 months of age. Unexpectedly, there was no measurable cognitive impairment due to OVX, nor was there a measurable effect of GSE on OVX or sham-OVX rats. 17β-Estradiol, which is

produced primarily in the ovaries, has been shown to reduce expression and activity of catechol-*O*-methyltransferase and uridine 5'-diphospho-glucuronosyltransferases. These enzymes are involved in the metabolism of catechin and epicatechin which, along with their metabolites, are thought to be the bioactive components of GSE. We hypothesized that OVX rats have increased methylated and/or glucuronidated forms of catechin and epicatechin. Urine from 18-week-old OVX rats given GSE for 4 days had increased glucuronidated catechin and epicatechin but no changes in methylated catechin and epicatechin compared to sham-OVX rats. These data are the first to show that in a rodent model of menopause a change in urinary catechin metabolites and suggest that postmenopausal women may experience increased metabolism of catechin containing dietary supplements.

## DEDICATION

To my wife Alicia and my children, Maggie and Jackson, I thank you for your love and support. To my parents, Ken and Patti, I thank you for giving me the desire to push myself and further my education.

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have joined our family during this time and have been such a joy and blessing in my life. They have constantly reminded me to have fun along the way. Last, I want to thank my wife Alicia, who has stood by my side throughout my entire graduate career. She has been supportive through the successes and frustrations that come as a graduate student. She has been understanding, especially during the writing of this dissertation, and has always seen the potential in me. I am truly grateful for her and my family.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AD | Alzheimer's disease |
| BFF | Benzofuran forming fission |
| BrdU | 5-bromo-2'-deoxyuridine |
| CA | *Cornu Ammonis* |
| COMT | Catechol-*O*-methyltransferase |
| CYP | Cytochrome P450 |
| DG | Dentate gyrus |
| EC | Entorhinal cortex |
| EGCG | Epigallocatechin-3-gallate |
| EGFP | Enhanced green fluorescent protein |
| ER | Estrogen receptor |
| ERE | Estrogen response element |
| GFP | Green fluorescent protein |
| GSE | Grape seed extract |
| HCl | Hydrochloric acid |
| HPLC | High performance liquid chromatography |
| HRP | Heterocyclic ring fission |
| Hsp | Heat shock protein |
| $K_d$ | Dissociation constant |
| KPBS | Potassium phosphate buffered saline |

| | |
|---|---|
| KPBST | Potassium phosphate buffered saline + Triton X-100 |
| LC | Liquid chromatography |
| LTP | Long-term potentiation |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| *m/z* | Mass to charge ratio |
| N | Normal |
| NaCl | Sodium chloride |
| NeuN | Neuronal nuclei |
| NGS | Normal goat serum |
| OVX | Ovariectomy/Ovariectomized |
| PBS | Phosphate buffered saline |
| PFA | Paraformaldehyde |
| POMC | Proopiomelanocortin |
| Q | Quadrupole |
| RBA | Relative binding affinity |
| RDA | retro-Diels-Alder |
| RT | Room temperature |
| SHBG | Sex hormone-binding globulin |
| SHR | Spontaneously hypertensive rat |
| SULT | Sulfotransferase |
| TBS | Tris-buffered saline |

| | |
|---|---|
| $t_R$ | Retention time |
| UGT | Uridine 5’-diphospho-glucuronosyltransferase |

# CHAPTER 1

# INTRODUCTION

## Botanical Dietary Supplements

In 1994 Congress passed the Dietary Supplements Health and Education Act. It defined dietary supplements as foods, and therefore not under the purview of the Food and Drug Administration. Since then, the use of botanically-derived dietary supplements including St John's wort, ginseng, resveratrol, green tea extract, and grape seed extract (GSE), has become wide spread for their potential in prevention and treatment of disease. These can be purchased over-the-counter at grocery and convenience stores like Walmart, Target, and CVS Pharmacy.

The mission of the National Institutes of Health and the National Center for Complementary and Alternative Medicine supported research is to define the usefulness and safety of complementary and alternative medicine interventions and their roles in improving health and health care. This mission has supported many studies to determine the health benefits of botanical dietary supplements. The purported health benefits of these botanical dietary supplements include the use of St John's wort as an anti-depressant (1), ginseng to treat type 2 diabetes (2, 3), resveratrol for cardioprotection (4-

6), and cranberry extract to reduce cell growth in a number of tumor cell lines (7). One of the more documented supplement compounds has been the soy isoflavones; their structural similarity with estrogens warranted study to determine to what extent these isoflavones mimicked the actions of estrogen. Soy isoflavones have been shown to improve cognition in postmenopausal women (8, 9) and attenuate oxidative stress and improved parameters related to aging and Alzheimer's disease (AD) (10). Genistein, one of the principal isoflavones in soy, also reduced blood pressure in a rat model of hypertension (11). When genistein is administered before puberty it protects against chemically-induced breast cancer in rodents (12).

Many botanical dietary supplements are comprised of polyphenols that fall into a group called flavonoids. Figure 1 shows some of the chemical structures of these flavonoids. Flavonoids containing botanical dietary supplements can act as antioxidants (13), tyrosine kinase inhibitors (14), and peroxisome proliferator-activated receptor agonists (15, 16). Flavonoids are enriched in a number of plant-based foods including grapes, cranberries, green tea, and chocolate, and have been studied extensively for their health benefits.

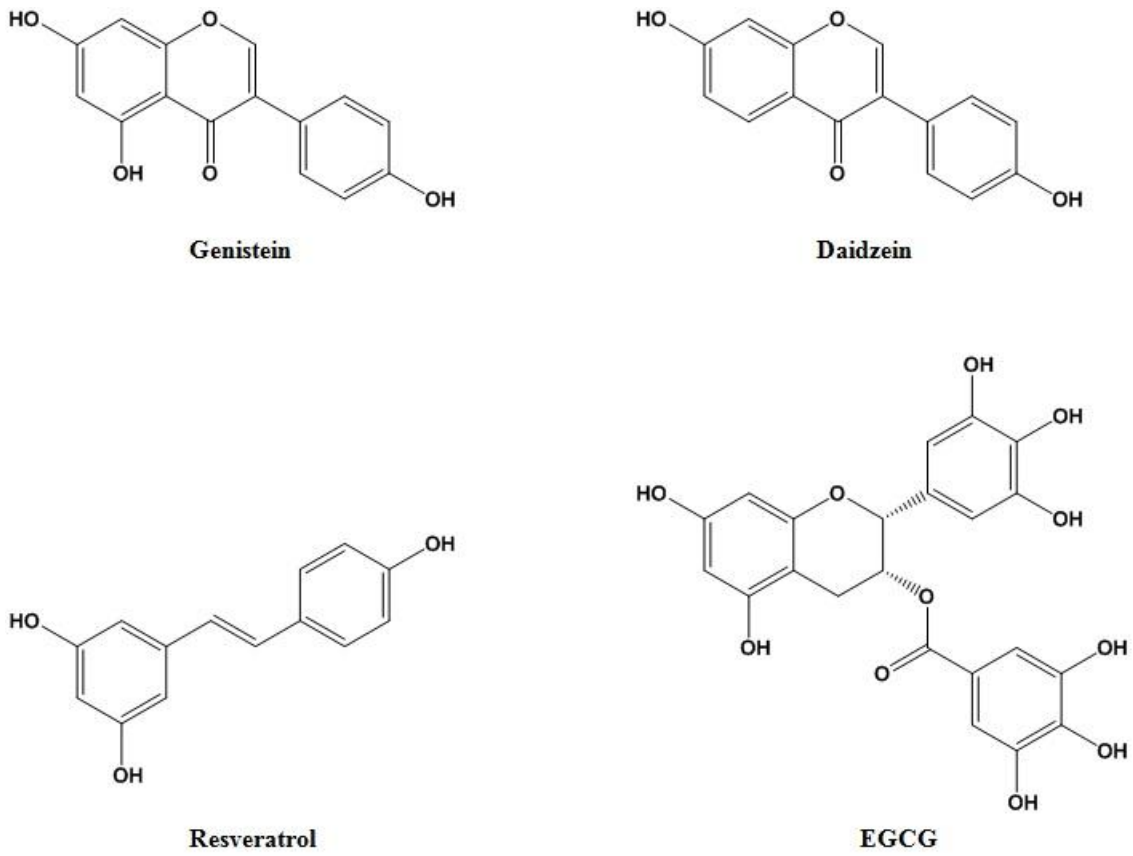



Figure 1. The chemical structures of several flavonoids that are found in botanical dietary supplements. Genistein and daidzein are isoflavones found in soy. Resveratrol is a stilbenoid found in the skin of red grapes and red wine. Epigallocatechin-3-gallate (EGCG) is a catechin found in green tea. Flavonoids are structurally similar as they are all polyphenols.

## Grape Seed Extract

GSE, as currently available, is a mixture of polyphenols extracted from grape seeds that is enriched in proanthocyanidins, oligomers of the monomeric flavan-3-ols (+)-catechin and (-)-epicatechin (Fig. 2). The preparation used in the studies described here was donated by Kikkoman Corporation. Yamakoshi *et al*. (Kikkoman) determined that GSE contains 89.3% proanthocyanidins (6.6% dimers, 5.0% trimers, 2.9% tetramers, and 74.8% pentamers or larger), 6.6% monomeric flavanols (2.5% [+]-catechin, 2.2% [-]-epicatechin, 1.4% [-]-epigallocatechin, and 0.5% [-]-epigallocatechin gallate), 2.24% moisture, 1.06% protein, and 0.8% ash (17). GSE contains less than 1% organic acids such as α-ketoglutarate, acetic acid, and malic acid. GSE does not contain related flavonoids such as kaempferol and quercetin. GSE may contain phenolic acids, like gallic acid, but in very low amounts (< 0.01%). As illustrated in Figure 2, catechin and epicatechin are epimers – they have identical chemical formulas and masses but vary in orientation of the hydroxyl group on carbon 3.

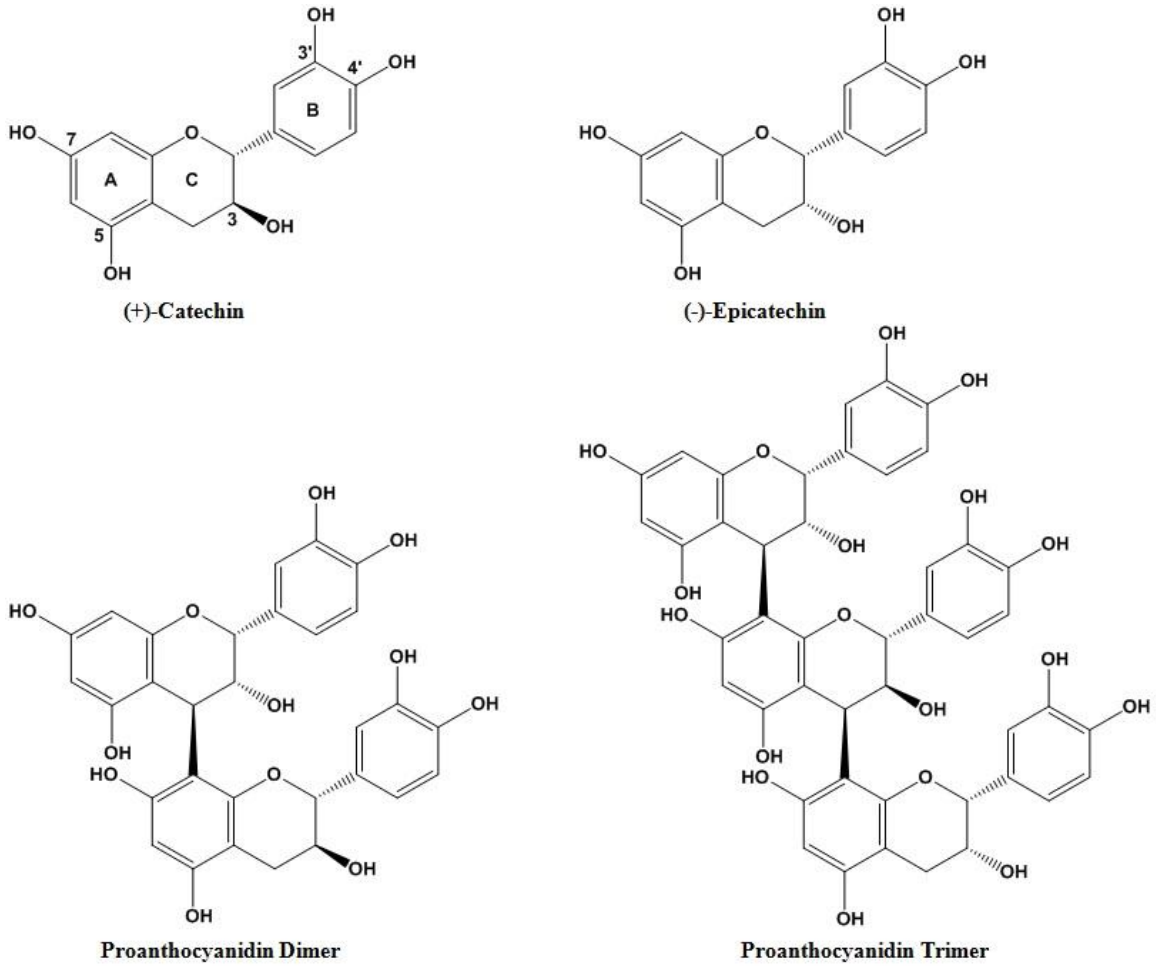



Figure 2. The chemical structures of (+)-catechin, (-)-epicatechin, a proanthocyanidin dimer, and a proanthocyanidin trimer. (+)-Catechin and (-)-epicatechin are epimers meaning they are structurally the same except with the orientation of the hydroxyl group on carbon 3 (C3). Proanthocyanidin dimers and trimers are comprised of catechin and epicatechin monomers that are connected via the C4 on the C ring and the C8 on the A ring of the catechin/epicatechin. Proanthocyanidins in GSE can range from dimers to decamers.

A number of health benefits have been shown for GSE in cell culture and animal models of disease. The health benefits include: inhibition of non-small cell lung tumor (18), induction of apoptosis of leukemia cells (19), inhibition of tumor growth and formation in chemically-induced skin cancer (20), chemoprevention of colorectal cancer (21, 22), reduction of blood pressure in hypertension (23), and protection against reperfusion-induced injury (24). Furthermore, dietary GSE attenuated the loss of cognitive function and reduced Aβ plaques in the brains of a transgenic mouse model of AD (25, 26). Healthy rats given GSE showed differences in a set of brain proteins with altered expression or charge that were quantitatively in the opposite direction from these same proteins in AD and mouse models of neurodegeneration which suggests GSE is neuroprotective (27). The concept of GSE having neuroprotective actions was corroborated by the demonstration that dietary intake of GSE enhanced hippocampal-dependent learning and memory in a young rat model of menopause (23).

Catechin and epicatechin comprise 4-8% of GSE by weight (17); nonetheless they, and/or their metabolites, may be the principal bioactive components of GSE. Wang *et al*. showed that the monomeric portion of GSE is responsible for increased spatial learning and memory and for reduced Aβ oligomers in the brain of a transgenic mouse model of AD (28). Schroeter *et al*. showed that ingestion of flavanol-rich cocoa increased nitric oxide levels and vasodilation and that epicatechin and its metabolite epicatechin-7-*O*-glucuornide are at least partially responsible for this benefit (29). van Praag *et al*. showed that epicatechin enhanced retention of spatial memory in mice (30).

Together these studies suggest that GSE plays a role in the prevention and treatment of diseases that are associated with aging; however, many of these studies do not take into account changes in hormone levels, such as estrogens, due to aging. Lowered levels of circulating estrogens in postmenopausal women is accompanied with increased risks for osteoporosis (31), coronary heart disease (32, 33), and AD (34-38). Whether GSE or other flavonoid containing dietary supplements have benefits in the postmenopausal brain and whether lower circulating levels of estrogen affect the metabolism of bioactive flavonoids, such as catechin and epicatechin, has not been addressed systematically.

This dissertation describes studies that addressed the following hypotheses: 1) that GSE enhances adult hippocampal neurogenesis, since enhanced hippocampal neurogenesis has been linked with improved hippocampal-dependent learning and memory (39); 2) that GSE enhances learning and memory in an older rodent model of menopause, as was detected in a young rodent model of menopause (23); and 3) that the metabolism of the GSE monomeric flavanols catechin and epicatechin is different in ovariectomized (OVX) rats, due to lowered estrogen.

## Hippocampal Neurogenesis

The hippocampus plays a critical role in short- and long-term memory as well as in spatial navigation. The hippocampus contains three primary regions that form a trisynaptic pathway for the flow of neural information: the dentate gyrus (DG), *Cornu*

*Ammonis* (CA) 3, and CA1. The hippocampus and the entorhinal cortex (EC) form a circuit that plays an important role in memory formation and consolidation, especially for spatial memories. Granule cells in the DG receive input from the EC. Pyramidal neurons in the CA3 receive input from the axons of the granule cells from the DG. Pyramidal neurons in the CA1 receive input from the axons of the pyramidal neurons from the CA3. The EC receives input from the axons from the pyramidal neurons in the CA1 completing the circuit (Fig. 3).

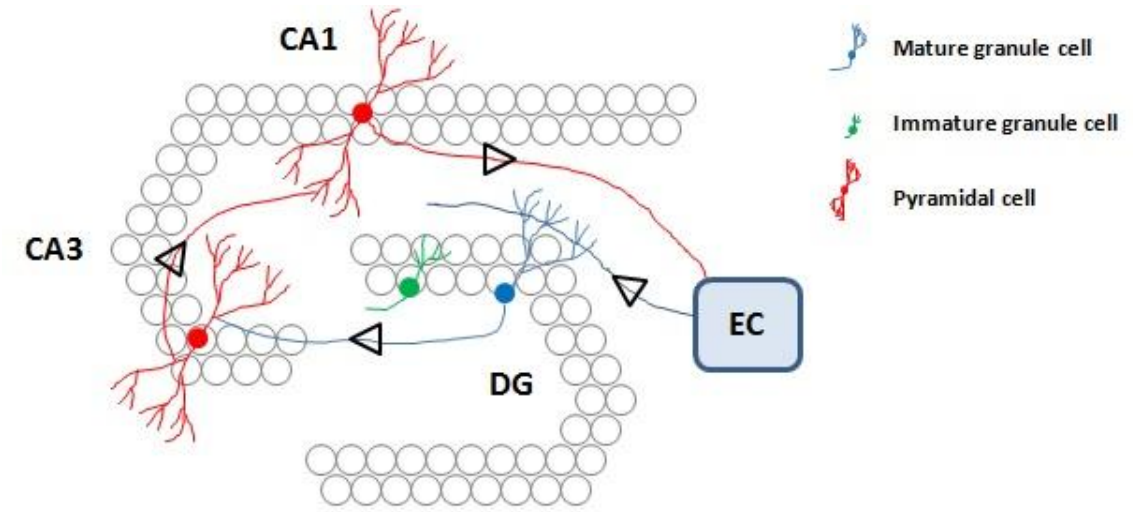



Figure 3. The circuitry of the hippocampus. The hippocampus has three main regions: DG, CA3, and CA1 that form a circuit with the EC and play a role in memory formation and consolidation. The EC passes information to the granule cells in the DG which pass information onto pyramidal cells in the CA3 and these onto pyramidal cells in the CA1 which then converge with the EC. The DG is where neurogenesis occurs in the adult brain and comprises of immature (new) and mature granule cells.

The hippocampus is unusual because it is the primary site of adult neurogenesis. Neurogenesis is the generation of new neurons from neural stem and progenitor cells. While it occurs mostly in the prenatal brain, there is significant neurogenesis in the early postnatal brain. As the brain continues into adulthood, neurogenesis is detected in two areas of the brain: the subventricular zone and the subgranular zone of the DG (40-42). Although hippocampal neurogenesis does continue into adulthood there is a significant decrease with age (43, 44). The study of hippocampal neurogenesis requires an understanding of how neurons in the DG mature. In the subgranular cell layer, neuronal progenitor cells proliferate and migrate to the granular cell layer where they differentiate into neurons. During the first 3 weeks, newborn neurons will sprout dendrites and an axon. The dendrites grow into the inner molecular layer; and with maturation, the dendrites and their branches continue to grow towards the outer molecular layer. After 4-8 weeks, immature neurons can be incorporated into the neuronal network (Fig. 4) (45). In young adult rats, neuronal progenitor cells produce over 9000 new cells each day (46); however, not all new neurons survive to become mature neurons (47, 48).

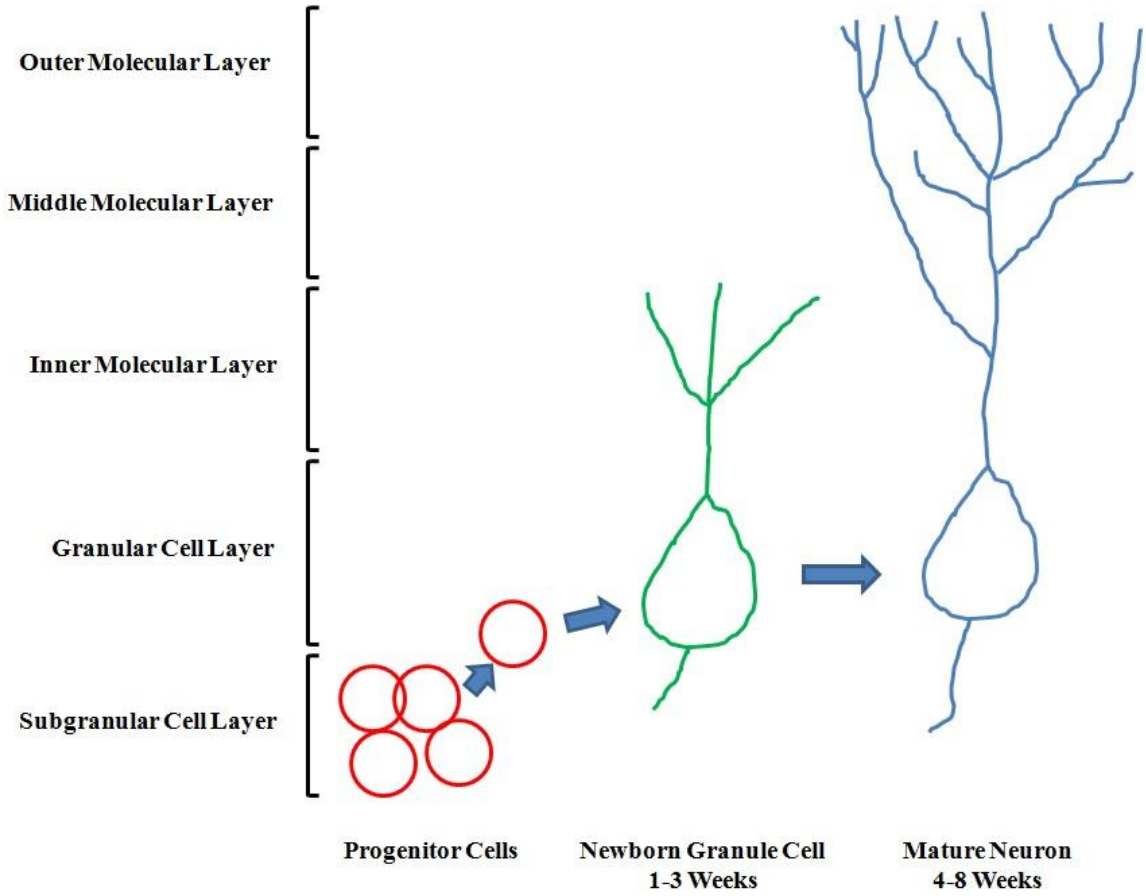



Figure 4. The maturation of granule cells in the DG. Neurogenesis continues into adulthood in the DG. Neuronal progenitor cells proliferate in the subgranular cell layer and migrate to the granule cell layer to differentiate into new neurons. During the first 3 weeks, new neurons sprout an axon and dendrites that grow into the inner molecular layer of the DG. As new neurons mature they can incorporate into the neuronal network, their dendrites continue to grow, reaching the outer molecular layer of the DG, and their dendrites begin to have many branches.

Hippocampal neurogenesis can be altered by a number of diseases. In human AD brains, there were increased markers of immature neurons in the hippocampus; suggesting an increase in hippocampal neurogenesis, possibly to replace neuronal loss (49). In a mouse model of epilepsy, the morphological and synaptic development of new neurons is increased in the DG (50). In a mouse model of brain ischemia, there is an increase in proliferation of neuronal precursor cells in the DG (51). Of interest, retinoic acid reduced hippocampal neurogenesis in a rat model of brain ischemia (52). Hormones can also affect neurogenesis. One study showed that repeated administration of estradiol benzoate in OVX rats slightly increased proliferation while decreasing the survival of new neurons; however, there was a decrease in overall cell death in the DG (53).

Many studies have sought to determine what physiological conditions and experiences regulate hippocampal neurogenesis. One factor studied is an enriched environment, for rodents this includes the addition of a nest, tunnels, toys, and a running wheel. Mice in an enriched environment had increased survival of new neurons compared to mice in a standard environment (54). Another factor studied that enhances neurogenesis is exercise. Exercise-induced mice were found to have an increase production of new neurons in the DG. This increase in production of new neurons was correlated by an increase in learning and memory when tested by the Morris water maze, a hippocampal-dependent spatial learning and memory task, and an increase in long-term potentiation (LTP), which is thought of as the cellular correlate of learning and memory, in the DG (39). Similar findings were detected in old (19 months) exercise-induced mice

(55). A third factor that affects neurogenesis is caloric restriction. Caloric restriction can also increase the survival of new neurons in the DG (56).

There are other factors which have been shown to impair hippocampal neurogenesis; such as, caffeine which depresses proliferation of neuronal stem cells in mice and rats (57, 58). Stress also decreases the number of proliferating progenitor cells in the DG (59).

To further understand the role of hippocampal neurogenesis on learning and memory, studies have been carried out where hippocampal neurogenesis is ablated. Ablation of hippocampal neurogenesis impaired LTP in the DG as well as learning and memory as tested by contextual fear conditioning. Interestingly, hippocampal-dependent spatial learning as tested by the Morris water maze was not affected (60). However, another experiment showed that ablation of hippocampal neurogenesis improved working memory, a form of short-term memory that involves the hippocampus and prefrontal cortex in spatial learning and memory (61). Ablation of hippocampal neurogenesis attenuated the conversion of recent learning to long-term memory (62). Also, ablation of hippocampal neurogenesis impaired the ability to distinguish small differences in spatial separation; however, the ability to distinguish large differences was not affected (63). It has been shown that an increase in hippocampal neurogenesis leads to an increase in discrimination between similar contexts (64). Further studies showed that new neurons in the DG are responsible for pattern separation, the ability to distinguish similar but different cues, and older neurons in the DG are responsible for pattern completion, the

process of retrieving more complete memories from partial cues (65). Together these studies suggest that the role of neurogenesis in hippocampal-dependent learning and memory is complex and that it has some role in enhancing memory, in particular the ability to discriminate similar contexts. Therefore, further research is of interest to understand what factors, intrinsic and extrinsic to the brain, affect and impact hippocampal neurogenesis.

There is evidence that dietary supplements play a role in regulating hippocampal neurogenesis. Mice that were fed a diet enriched in polyphenols and polyunsaturated fatty acids had increased neurogenesis in the DG (66). Aged rats (21 months) that were fed a diet supplemented with blueberries for two months had increased proliferation of precursor cells in the DG (67). Daidzein, a major soy isoflavone, increased cell proliferation in the subgranular layer of the DG (68). EGCG, a catechin enriched in green tea, given orally to mice was shown to increase proliferation of neurons as well as the number of new neurons in the DG (69, 70). The flavonoid oroxylin A increased cell proliferation and the number of new neurons in the DG (71). Thus, the goal of the first part of this dissertation was to assess whether enhancement of adult hippocampal neurogenesis is one mechanism that could contribute to GSE-induced learning and memory enhancement (23, 25, 28, 72).

## Estrogens

Estrogens are endogenous hormones that are principally produced in the ovaries and from dehydroepiandrosterone in the adrenal glands of women. In women estrogens

are involved in development, reproduction, and metabolism, including mineral, carbohydrate, protein, and lipid metabolism. The three major endogenous estrogens are: estrone, 17β-estradiol, and estriol. In premenopausal women 17β-estradiol is the most abundant estrogen. It is also the most potent form of estrogen with regards to affinity for the estrogen receptors (ERs). There are two types of ERs: ERα (73, 74) and ERβ (75, 76). ERα is expressed most abundantly in the uterus, vagina, ovaries, mammary gland, the hypothalamus, endothelial cells, and vascular smooth muscle. ERβ is expressed most abundantly in the prostate and ovaries. 17β-Estradiol binds with high affinity for ERα with a dissociation constant ($K_d$) of 0.1 nM and for ERβ with a $K_d$ of 0.4 nM (77). The relative binding affinity (RBA) for estrone compared to 17β-estradiol for ERα is 60 and for ERβ is 37 (77). The RBA for estriol compared to 17β-estradiol for ERα is 14 and for ERβ is 21 (77). The RBA is the ratio of concentrations of 17β-estradiol and estrone/estriol required to reduce 17β-estradiol binding by 50%.

Estrogens are synthesized from androgens (Fig. 5). Cholesterol is the precursor for all steroid synthesis. Through multiple steps, cholesterol can be converted to the androgen androstenedione. Androstenedione can interconvert into another androgen, testosterone by 17β-hydroxysteroid dehydrogenase. Through aromatase, androstenedione is converted to estrone and testosterone is converted to 17β-estradiol (78). Similar to androstenedione and testosterone, estrone and 17β-estradiol can interconvert between each other by 17β-hydroxysteroid dehydrogenase. The ovaries produce primarily 17β-estradiol whereas estrone and estriol can also be produced in the liver from 17β-estradiol or in other peripheral tissues such as adipose tissue, from androstenedione and other

androgens such as dehydroepiandrosterone, which can be produced in the adrenal gland (79). Thus, reduction in ovarian function primarily affects levels of 17β-estradiol.

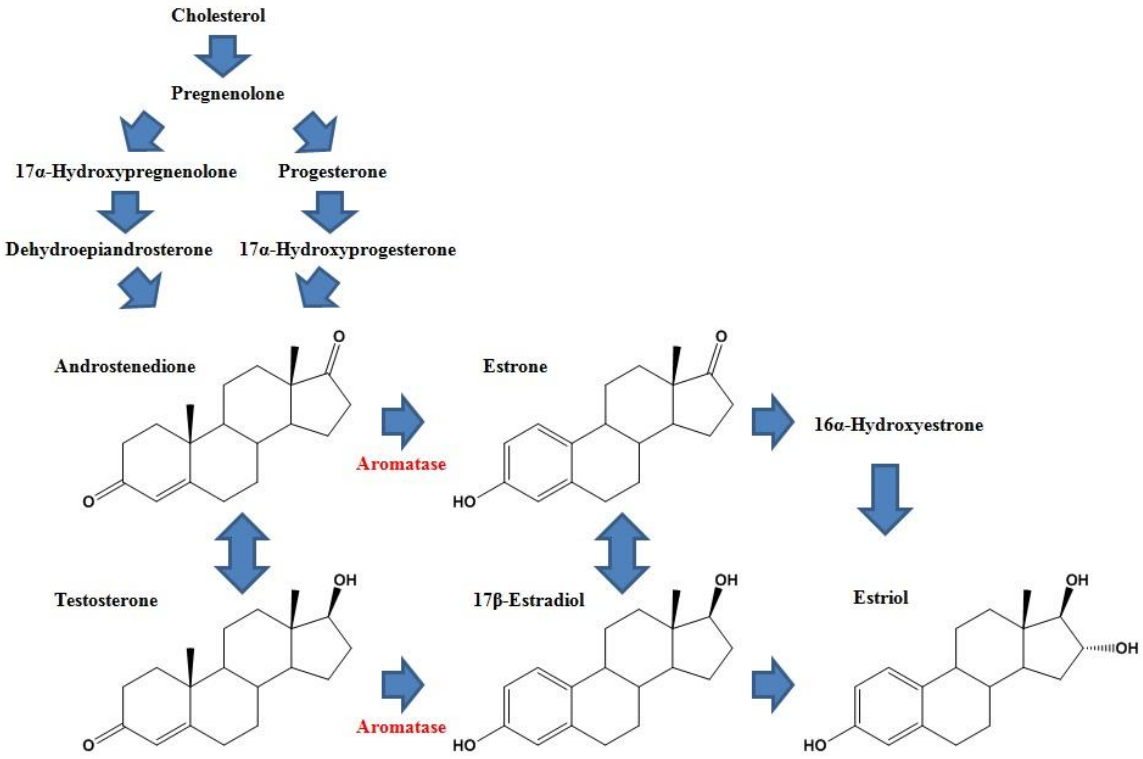



Figure 5. The synthesis of estrogens. The precursor to all steroid synthesis is cholesterol. Through multiple steps, cholesterol can be converted to the androgen androstenedione. Androstenedione can be converted into testosterone. The enzyme aromatase converts androstenedione to estrone and testosterone to 17β-estradiol. 17β-Hydroxysteroid dehydrogenase is responsible for the interconversion between androstenedione and testosterone and between estrone and 17β-estradiol.

Estrogens are mainly conjugated with glucuronic acid or sulfate with only a small fraction as unconjugated estrogens. Estrogens circulate in the blood mainly bound to sex hormone-binding globulin (SHBG) and to a lesser extent albumin. Estrogens enter cells by diffusing across the plasma membrane. ERs are nuclear receptors that are predominantly found in the nucleus where they are bound with heat shock proteins (Hsp). Estrogens bind to a single ER and once bound enables the ER to dimerize with another ER bound with estrogen (80). This complex binds with estrogen response elements (EREs) on the DNA and recruits other cofactors that are involved in the transcription of genes (81, 82) (Fig. 6).

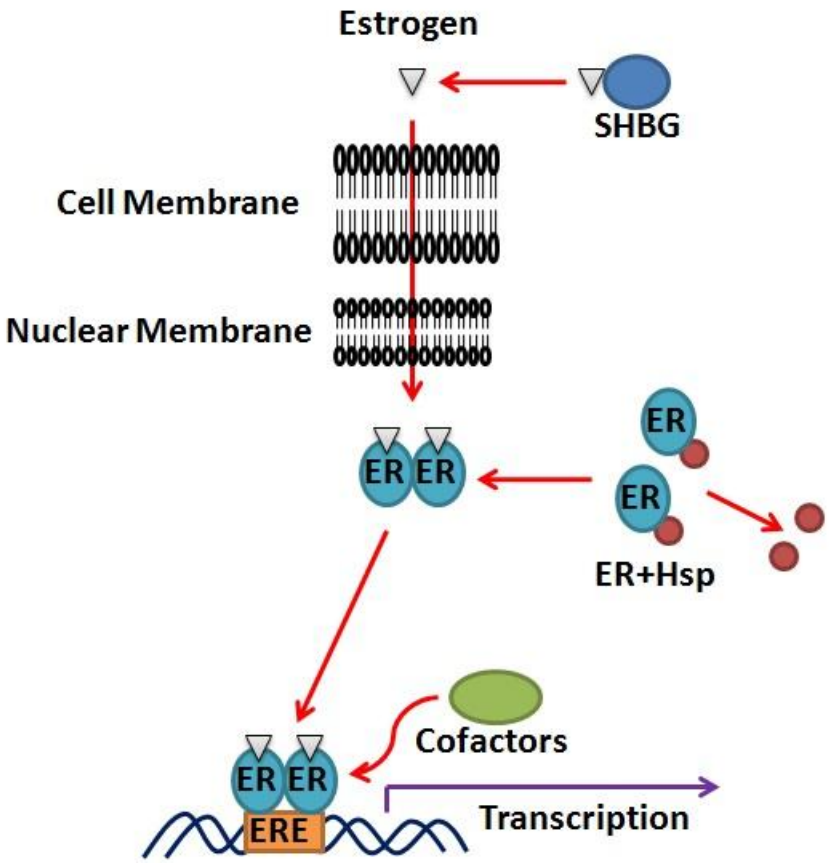



Figure 6. Classical estrogen signaling pathway. Estrogens are bound primarily to SHBG in the blood and interstitial fluid. Estrogens diffuse across the plasma membrane and into the nucleus. ERs are bound to Hsp and upon dissociating with Hsp can bind with estrogens. ERs bound with estrogens form dimers which bind with EREs on the DNA, recruit cofactors involved in transcription, and elicit gene transcription.

As women age they will eventually go through menopause, typically in their late 40s or early 50s. At menopause the ovaries no longer produce estrogens; this lowers the circulating levels of estrogens, especially 17β-estradiol. Premenopausal women will have fluctuating serum 17β-estradiol level depending on where they are in the menstrual cycle; this can range between 50-400 pg/ml, the highest being right before ovulation. Postmenopausal women will have serum 17β-estradiol levels ≤ 35 pg/ml. This decrease in estrogens accompanies an increased risk for osteoporosis (31), coronary heart disease (32, 33), AD (34-38), and cognitive decline (83-86). Because of these increased risks due to lowered estrogens, it is reasonable to propose that estrogens have a role in protecting against each of these conditions. Indeed, hormone therapy studies with estrogens have indicated that estrogens are protective against osteoporosis (87, 88), cardiovascular disease (89), and some hormone independent cancers (90, 91). Timing of the administration of estrogen therapy has become an important consideration, since those receiving estrogen therapies during the early period of lowered levels of estrogen fared better than those receiving estrogen therapies after a prolonged period of estrogen deprivation (92). Moreover, it has been shown that OVX in rats results in impaired learning and memory with time (93, 94). The OVX rat has been used as a model of menopause to study osteoporosis (95), hypertension (23, 96), and cognitive impairment (97, 98). The OVX rat does not always best represent natural menopause; at times it may only represent clinical menopause (99, 100).

## Effects of Estrogen and GSE on Learning and Memory

While hormone therapy with estrogens and with and without progestins can be beneficial, it may not be the long-term solution, and may have diverse effects, such as in hormone dependent cancers (101). Studies have shown the lowering of estrogen caused by ovariectomy in rodents impairs learning and memory in hippocampal-based behavioral tasks (98, 102-104) but when 17β-estradiol is given to OVX rats it restores learning and memory (104-106). While estrogen therapy may restore cognitive function, the use of estrogen therapy has raised concerns, especially with women who are dealing with or are at risk for hormone-dependent cancer. It has become important to identify alternatives to estrogen that can act in reducing the risk for cardiovascular and neurodegenerative diseases without the hazards of estrogen therapy. Botanical dietary supplements enriched in the polyphenols, including GSE, could be viable alternatives. Peng *et al*. showed that in rats OVX at 4 weeks of age and given GSE for 10 weeks had increased spatial learning and memory compared to OVX rats (23). Intake of dietary GSE by healthy rats caused changes in the brain proteome that were consistent with neuroprotection (27). Dietary intake of epicatechin, a monomeric catechin found in GSE, improved memory in wild type mice (30). A metabolite of epicatechin, 3'-*O*-methyl epicatechin glucuronide, enhanced LTP when added exogenously to hippocampal slices from a mouse model of AD (28). These data suggest that GSE, specifically the catechin monomers, plays a role in learning and memory. Thus, the goal of the second part of this dissertation was to assess whether GSE attenuates the cognitive loss in an older rodent model of menopause as was detected in a young rodent model of menopause (23).

## Xenobiotic Metabolism

Xenobiotics are foreign substances that our bodies absorb. A large portion of this occurs by oral intake and absorption in the intestines. Many of these xenobiotics, such as drugs and dietary supplements, are bioactive either in their native state or one of their metabolites. Xenobiotics tend to be lipophilic, which allows them to easily cross the plasma membrane, and one of the functions of metabolism is to make them more polar so they can be excreted. A function of metabolism is to inactivate xenobiotics. However, not all xenobiotics are inactivated when metabolized. In fact some are activated through metabolism (107, 108). Scientists have used metabolism to prolong the duration of certain drugs in the body by making prodrugs, drugs that get activated once they are metabolized. The primary way xenobiotics are excreted, which also terminates their biological activity, is by the kidney (109, 110).

The liver is the principal organ of metabolism; however, many of the enzymes for metabolism are expressed in other organs and tissues such as the gastrointestinal tract. Therefore, selective metabolism can occur in many organs and tissues in the body. Xenobiotics can be absorbed in the small intestines and enter the portal vein which carries them to the liver where they can undergo metabolism before entering the circulation. This is called the first-pass effect. If the intestinal lining contains metabolism enzymes for a specific xenobiotic, the small intestines can also contribute to the first-pass effect (109, 110). Xenobiotics can also be metabolized by microbacteria in the gut before being absorbed (111).

Metabolism is generally split into two phases: phase I and phase II metabolism. As drugs and xenobiotics are usually lipophilic, the role of phase I metabolism is to introduce or unmask polar functional groups such as hydroxyl, amine, or thiol groups. Phase I metabolism may be enough to excrete some xenobiotics. It also can inactivate some xenobiotics. In some cases phase I metabolism is not enough to have xenobiotics excreted and additional metabolism is needed. Phase II metabolism adds a bulky polar side group and helps make xenobiotics more polar to be excreted. In general, phase II metabolites are inactive. It is not essential for xenobiotics to go through phase I metabolism before phase II metabolism (112-114). Some xenobiotics might have a functional hydroxyl, amine, or thiol group and are already good substrates for phase II metabolism (109, 110).

A group of enzymes called the cytochrome P450s (CYPs) play a role in phase I metabolism (115). CYPs contain a heme group, which binds and activates oxygen, and work in connection with NADPH-cytochrome P450 reductase which transfers electrons from NADPH to the CYPs. CYPs are found on the cytosolic side of the endoplasmic reticulum and can dealkylate, remove functional groups, or add oxygen such as hydroxyl, epoxide, and carbonyl groups to xenobiotics. The liver contains many CYP isoforms. CYPs have a large number of substrates due to being potent oxidizing enzymes. The most common feature of CYP substrates is high lipid solubility (109, 110). In the human liver the CYP isoforms that are responsible for a large portion of drug and xenobiotic metabolism are CYP1A2, 2A6, 2C9, 2D6, 2E1, and 3A4 (116). CYP3A4 metabolizes a large portion of prescribed drugs (116, 117). Xenobiotics can induce CYPs by enhancing

their rate of synthesis or decreasing their rate of degradation (118). Some xenobiotics induce CYPs responsible for their own metabolism. Drugs and xenobiotics can also be inhibitors of CYPs, binding reversibly or irreversibly to the enzyme (119).

Phase II enzymes are made up of transferases that conjugate drugs and xenobiotics by adding bulky polar side groups such as glucuronic acid, sulfonic acid, acetic acid, glutathione, methyl group, or an amino acid (120). The addition of these groups leads to excretion of phase II metabolites and often inactivates them. Enzymes such as catechol-*O*-methyltransferase (COMT, adds a methyl group) and sulfotransferases (SULTs, adds sulfonic acid) are found in the cytosol; uridine 5'-diphospho-glucuronosyltransferase (UGTs, adds glucuronic acid) are found on the lumen side of the endoplasmic reticulum (109, 110).

Xenobiotic metabolites are eliminated from the body in the urine, bile, and feces. The kidney plays a primary role in the excretion of drugs and xenobiotics. Kidney excretion involves glomerular filtration, active tubular absorption, and passive tubular reabsorption. The glomerulus filters unbound xenobiotics and their metabolites into the urine. Active transporters can also move xenobiotics from the blood to the urine. Small hydrophobic xenobiotics filtered by the glomerulus can be reabsorbed in the proximal tubules back into the blood by diffusion (109, 110). The biliary system also plays a role in excreting xenobiotics and their metabolites (121). Bile is excreted into the gastrointestinal tract and the xenobiotic β-glucuronides and sulfates esters are poorly absorbed from the small intestine. Xenobiotic metabolites can interact with the

microbacteria in the gut and these microbacteria may remove the side groups of some of these xenobiotic metabolites allowing for their reabsorption. The removal of metabolites in the bile and the re-uptake in the intestines after removal of conjugates by microbacteria is referred to as enterohepatic recycling and can prolong the presence of xenobiotics in the body. Interaction with colonic bacteria also produces reductive metabolites such as S-equol, a bacterial product of the isoflavone daidzein (122).

## Effects of Hormones on Xenobiotic Metabolism

Endogenous hormones are capable of regulating enzymes involved in xenobiotic metabolism and therefore changes in hormone levels can affect the extent of metabolism of xenobiotics. This becomes of interest as large changes in circulating steroidal hormones occur during pregnancy and at menopause in women. In the case of menopause it is unknown what lower levels of circulating estrogens have on the metabolism of flavonoids in dietary supplements such as the catechins in GSE.

It has been shown that progesterone and 17β-estradiol have effects on various phase I and II enzymes. In primary human hepatocytes treated with progesterone or 17β-estradiol there was an increase in CYP2A6 and CYP3A4 mRNA levels (123). In this same study, 17β-estradiol also increased the enzyme activities of CYP2C9 and CYP2E1 without affecting mRNA levels. An *in vitro* study showed that 17β-estradiol induces CYP 1B1, an enzyme involved in its own metabolism, through a mechanism involving ERα (124). In adult female rats 17β-estradiol has been shown to suppress CYP2C19 and

CYP3A2, which are male specific CYP isoforms (125). The expression of CYP2C19, a drug-metabolizing enzyme, is down-regulated by17β-estradiol through a mechanism involving ERα (126).

One study showed that progesterone induces COMT in the uterus of female rats (127). Another study confirmed that progesterone upregulates protein expression of COMT (128). On the other hand, 17β-estradiol at physiological concentrations has been shown *in vitro* to lower mRNA levels of COMT (129) leading to lower levels of COMT protein (130) through an ER dependent mechanism. These findings were consistent with what was seen in rats. 17β-Estradiol lowered protein levels of COMT in the prefrontal cortex and the kidney whereas tamoxifen, an estrogen antagonist, up-regulated protein expression of COMT (131). In this same study, tamoxifen slightly increased activity of COMT but overall the addition or removal (by OVX) of 17β-estradiol did not affect the activity of COMT only the expression.

An *in vitro* study showed that progesterone induces UGT1A1 mediated by pregnane X receptor (132). OVX female rats were shown to have higher levels of UGT1A1 mRNA levels compared to sham-operated rats; whereas OVX female rats given 17β-estradiol had lower levels of UGT1A1 mRNA compared to sham-operated and OVX rats (133). An *in vitro* study showed that 17β-estradiol reduced the activity of UGT1A1 and UGT1A9 (134). Androgens and estrogens, including 17β-estradiol, were shown to reduce the activity of the UGTs that are involved in the glucuronidation of dihydrotestosterone and androsterone (135).

It has been shown that 17α-ethinylestradiol, an estrogen found in oral contraceptives, inhibits SULT1A1 (136) and that 16α-hydroxyestrone inhibits SULT1E1 (137).

As the studies presented in this dissertation used rats to analyze the metabolism of catechin and epicatechin, it is important to remember that there are metabolism differences between rats and humans. As phase II enzymes are the focus of these studies, it is important to know of any differences between rat and human metabolism. Such as, it has been shown that epicatechin, which is found in GSE, was not glucuronidated by human liver and small intestinal microsomes, but was glucuronidated by rat liver microsomes (138). Additionally, epicatechin was efficiently sulfonated by human liver cytosol whereas the sulfation was less in rat liver cytosol (138). Also, epicatechin glucuronides and sulfates are detected in the plasma of humans who have ingested epicatechin (139).

## Catechin Metabolism

Catechin and epicatechin are good substrates for phase II metabolism and are known to be metabolized by COMT, UGTs, and SULTs (138-143) (Fig. 7). The methylated forms of catechin and epicatechin can also be glucuronidated or sulfonated. Catechin and epicatechin can be methylated on the hydroxyl groups at carbon 3' or carbon 4' (143, 144). Catechin and epicatechin can be conjugated with glucuronic acid or sulfonic acid on the hydroxyl groups at carbon 3', carbon 5, or carbon 7 and

methylated catechin and epicatechin can be conjugated with glucuronic acid or sulfonic acid on the hydroxyl groups at carbon 5 or carbon 7 (139). Orally administered catechin and epicatechin are absorbed in the small intestine. They are metabolized in the small intestine and liver to methylated, glucuronidated, and sulfonated forms (139, 143, 145, 146) (Fig. 8). Catechin, epicatechin, and their metabolites have been detected in the blood, urine, bile, and brain after ingestion of catechin, epicatechin, or GSE (28, 144-150). Catechin and epicatechin are not only metabolized to conjugated forms, but there is also evidence that catechin and epicatechin are further broken down to phenolic acids (151). Catechin, epicatechin, and their metabolites can be excreted in the urine, and catechin and epicatechin metabolites, such as the β-glucuronides, can be excreted in the bile.

GSE comprises roughly 90% catechin oligomers, but these are poorly absorbed in the small intestine (152, 153). Catechin and epicatechin comprise only 4-8% of GSE (17) yet they and/or their metabolites are thought to be the bioactive components of GSE (28-30). It is important to understand how these compounds are metabolized once ingested and whether lowered estrogen, as in menopause, impacts their metabolism. Thus, the goal of the third part of this dissertation was to assess whether the metabolism of the GSE monomeric flavanols catechin and epicatechin is affected due to lower levels of estrogen in a rodent model of menopause.

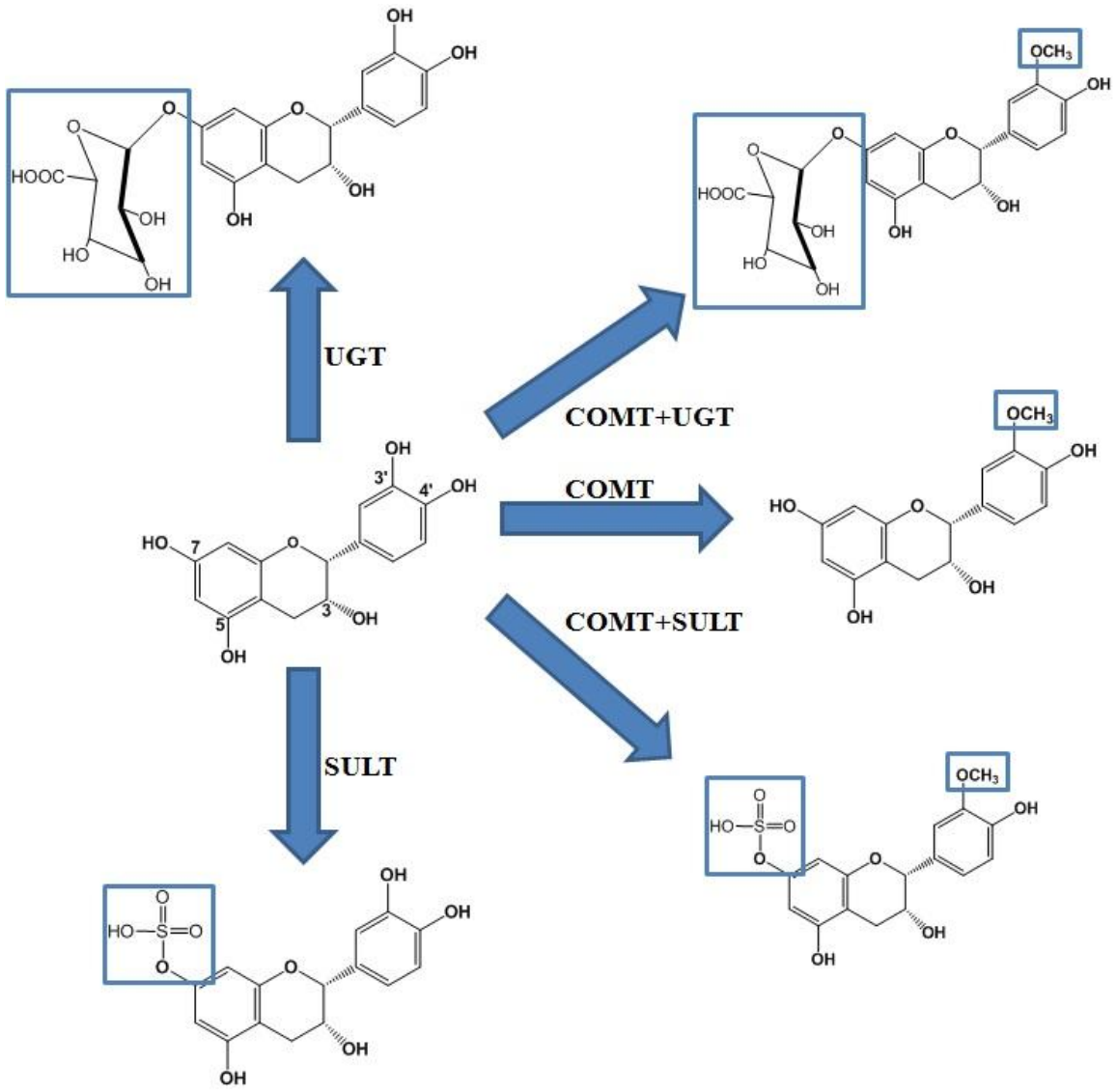



Figure 7. The chemical structures of catechin and epicatechin metabolites. Catechin and epicatechin can be methylated by COMT, glucuronidated by UGTs, or sulfonated by SULTs. Methylated catechin and epicatechin can also be glucuronidated or sulfonated. Catechin and epicatechin can be methylated on the hydroxyl group at carbon 3' or carbon 4'. Catechin and epicatechin can be glucuronidated or sulfonated on the hydroxyl group at carbon 3', carbon 5, or carbon 7. Methylated catechin and epicatechin can be glucuronidated or sulfonated on the hydroxyl group at carbon 5 or carbon 7.

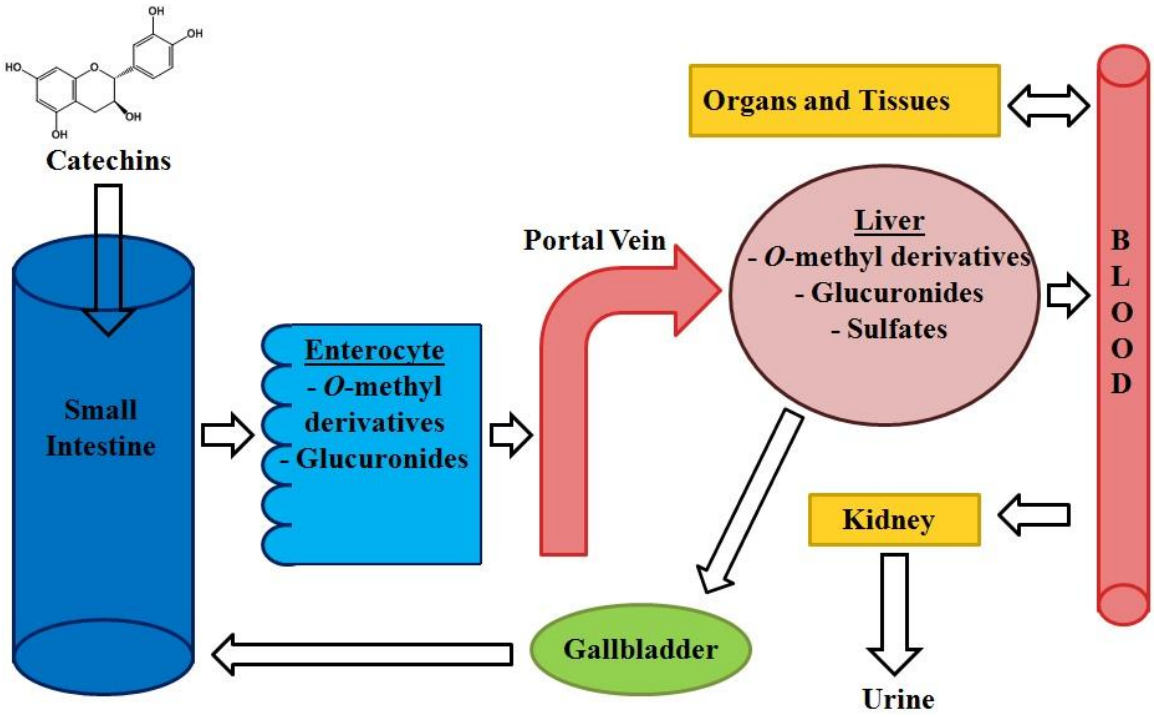



Figure 8. The absorption, distribution, metabolism, and excretion of catechins. Catechin and epicatechin are absorbed in the small intestine and enter the portal vein which carries them to the liver. After passing through the liver, they enter into circulation where they can interact with organs and tissues or be excreted in the urine by the kidney. Catechin and epicatechin can be metabolized to *O*-methyl derivatives and glucuronides in enterocytes in the intestinal wall or metabolized to *O*-methyl derivatives, glucuronides, and sulfates in the liver. Catechin and epicatechin metabolites can also enter the bile and be excreted back into the intestines.

## Mass Spectrometry and Catechin Fragmentation

Mass spectrometry (MS) was used in this study to detect and quantify catechin, epicatechin, and their metabolites. MS was performed on a triple quadrupole mass spectrometer (Fig. 9). A triple quadrupole mass spectrometer analyzes compounds in the gas phase and accelerates them through the mass spectrometer by electrical charge. Therefore, the first step of MS is to vaporize a compound and give it a charge; this is done by electrospray ionization. The compound that has ionized has a mass-to-charge ratio (*m/z*). Compounds can be positively or negatively charged. Small compounds are singly charged; therefore, compounds such as catechin will have an *m/z* of their molecular weight +1 proton (positively charged) or -1 proton (negatively charged). In the case of catechin, with a molecular weight of 290, its positively charged molecular (precursor) ion is *m/z* 291; the corresponding negatively charged molecular (precursor) ion is *m/z* 289. A potential gradient then accelerates the precursor ion into the mass spectrometer and following ion focusing it enters the first quadrupole (Q1) of the mass spectrometer. Q1 is a mass filter; this means for the precursor ion *m/z* 291, only ions with an *m/z* of 291 (0.7 *m/z* band pass) will enter the second quadrupole (Q2) where it encounters an inert gas ($N_2$). The resulting heating fragments the precursor into product ions which then enter the third quadrupole (Q3) which is like Q1 as it is also a mass filter. It is not always necessary to filter ions in Q3. Q3 can allow all product ions from the precursor ion to pass on to the detector. This is useful to characterize the compound under study or to compare it to a known standard with similar fragmentation. Where Q3 becomes powerful is when performing multiple reaction monitoring (MRM). In MRM, Q3 can select one of the product ions resulting from the precursor ion to further enhance

the specificity and sensitivity in detecting the compound while minimizing the background. In the experiments described in this dissertation, one of catechins product ions has an *m/z* of 139 in positive ion mode; therefore, the mass transition of 291/139 can be used to detect catechin. This becomes important as there may be several compounds in samples with a similar precursor ion mass. After exiting Q3, the ions hit a detector. The detector is an electron multiplier which produces a peak in a spectrum whose intensity is based on how many ions hit the detector at a certain *m/z*. Standards can be used to create standard curves and quantification can be performed. In some cases, even with the enhanced specificity and sensitivity of MRM, it is not possible to separate two compounds. This is the case for catechin and epicatechin as they are epimers and fragment the same. To separate catechin and epicatechin liquid chromatography (LC) is necessary. A reverse phase column, which separates them based on hydrophobicity, was used in our experiments. LC separates catechin and epicatechin by ~1 minute. LC and MS are used in combination (LC-MS). Samples containing catechin, epicatechin and their metabolites were analyzed by LC-MRM MS.

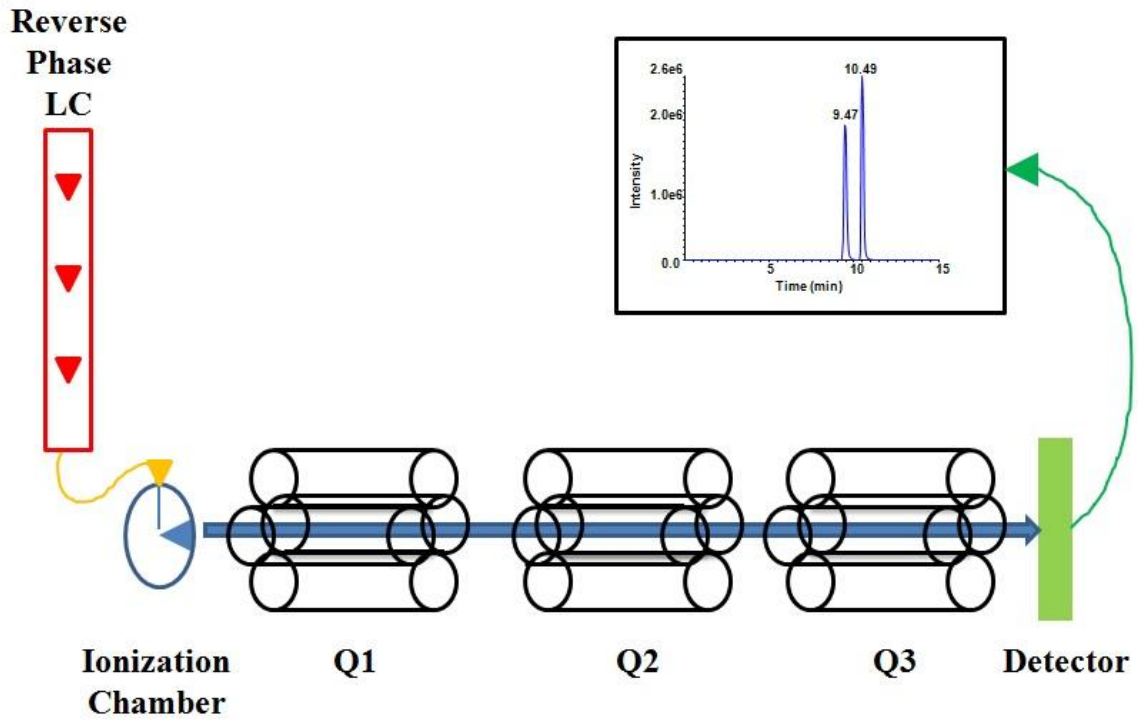



Figure 9. LC-MRM MS on a triple quadrupole mass spectrometer. A sample containing catechin and epicatechin is injected onto a reverse phase column and separated by LC. The sample is vaporized and ionized in the ionization chamber. Q1 selects for the *m/z* of 291, the *m/z* of catechin and epicatechin in positive ion mode. Q2 fragments all of *m/z* 291 in the injected sample by collision with nitrogen. Q3 selects the *m/z* of 139 the most abundant catechin and epicatechin product ion in positive ion mode. The ions of *m/z* 139 hit the detector. This generates an ion chromatogram for catechin and epicatechin.

To detect and perform quantitative analysis on catechin and epicatechin by liquid chromatography-tandem mass spectrometry (LC-MS/MS), it is necessary to know how catechin and epicatechin fragment in the mass spectrometer. Catechin and epicatechin have the same fragmentation pattern (Fig. 10). Cren-Olive *et al*. (154) and Li and Deinzer (155) have fragmented catechin and epicatechin and shown that the most abundant product ions detected are *m/z* 123, 139, 147, and 165 in positive ion mode. In the present experiments, LC-MS/MS of *m/z* of 291 in positive ion mode was used to confirm the presence of catechin and epicatechin in GSE, urine, and serum. We also detected the product ions *m/z* 123, 139, 147, and 165 which were consistent with those detected with catechin and epicatechin standards and with Cren-Olive *et al*. and Li and Deinzer. The most abundant product ion we detected in positive ion mode was *m/z* 139; therefore, this was used when creating a MRM mass transition for catechin and epicatechin.

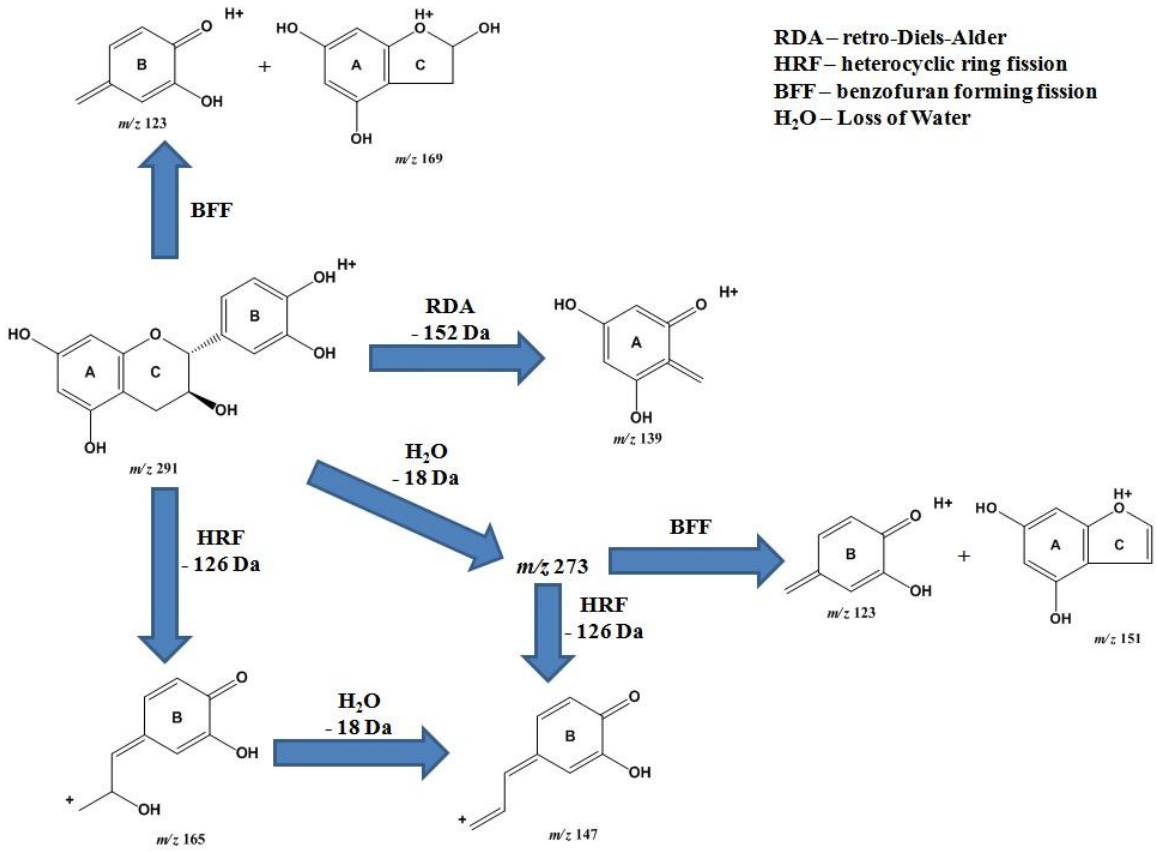



Figure 10. Catechin and epicatechin fragmentation. The most abundant product ions from catechin and epicatechin detected by MS are *m/z* 123, 139, 147, and 165 in positive ion mode. The formation of these product ions occur through various mechanisms including retro-Diels-Alder (RDA), heterocyclic ring fission (HRF), benzofuran forming fission (BFF), and the loss of water.

## Specific Aims

This dissertation describes studies that addressed the following hypotheses: 1) that GSE enhances adult hippocampal neurogenesis; 2) that GSE enhances learning and/or memory in an older rodent model of menopause; and 3) that the metabolism of the GSE monomeric flavanols, catechin and epicatechin, is different in OVX rats due to lowered estrogens.

The specific aims to address these hypotheses are:

1. Determine whether dietary GSE enhances hippocampal neurogenesis in the DG of POMC-EGFP mice.
2. Determine whether dietary GSE attenuates impaired hippocampal-dependent learning and memory in OVX SHRs.
3. Determine whether there is an increase in methylation and/or glucuronidation of catechin and epicatechin in OVX SHRs given GSE.

# CHAPTER 2

# ACTIONS OF GRAPE SEED EXTRACT ON HIPPOCAMPAL NEUROGENESIS

## INTRODUCTION

Neurogenesis, the generation of new neurons, continues into adulthood in the hippocampus and the olfactory bulb. Hippocampal neurogenesis occurs in the DG and continues into adulthood but it does decrease with age (43, 44). Hippocampal neurogenesis can be induced by enriched environment (54), exercise (39, 55), and caloric restriction (56). Hippocampal neurogenesis plays a role in learning and memory (60, 62) and the ability to distinguish similar contexts (63-65). Increased hippocampal neurogenesis has been shown to improve learning and memory in the Morris water maze and enhance LTP in the DG of mice who exercise (39). These studies suggest that hippocampal neurogenesis has a role in enhancing learning and memory.

GSE is comprised of catechin monomers and oligomers and has been reported to be consistent with neuroprotection in healthy rat brains (27). Consumption of GSE improved learning and memory in the Morris water maze in a transgenic mouse model of AD (25) and improved performance in the eight-arm-radial maze in young OVX rats (23). Both the Morris water maze and the eight-arm-radial maze are hippocampal-dependent tasks. Consumption of epicatechin, which is found in GSE, improves memory

in the Morris water maze of mice (30) and a metabolite of GSE, 3'-*O*-methyl epicatechin glucuronide, enhances LTP in hippocampal slices of a mouse model of AD (28). These studies suggest that GSE, like neurogenesis, has a role in enhancing learning and memory.

Dietary supplements such as soy (68), blueberries (67), and green tea (69, 70) induce hippocampal neurogenesis. These data suggest that dietary supplements play a role in regulating hippocampal neurogenesis. As both hippocampal neurogenesis and GSE have been shown to enhance learning and memory we hypothesized that GSE enhances hippocampal neurogenesis which leads to enhanced learning and memory.

The soy isoflavones, genistein and daidzein, were found in the stomach milk of 7-day-old rats whose mothers were given diets supplemented with genistein and daidzein while pregnant and nursing (156, 157). Genistein and daidzein are structurally similar to catechins, which are found in GSE, and it is probable that catechins pass from mother to pup through the milk. We therefore hypothesized that GSE or components of GSE pass through the milk of mothers given GSE to nursing pup enhancing hippocampal neurogenesis in the pups.

These hypotheses were tested in proopiomelanocortin-enhanced green fluorescent protein (POMC-EGFP) transgenic mice where newborn granule cells express GFP 1-2 weeks postmitotic in the DG. Experiments were carried out to determine whether GSE enhanced neurogenesis in the POMC-EGFP mouse brain in 5- and 12-month-old POMC-

EGFP mice and in 26-day-old POMC-EGFP mice whose mother was given GSE only while nursing. Changes in neurogenesis were determined by changes in the number of proliferating progenitor cells, new neurons, and/or mature neurons as well as whether changes occurred in the morphology of dendrites in new neurons.

# MATERIALS AND METHODS

## Control and GSE Supplemented Diet

Powdered AIN-76A diet was purchased from TestDiet (Richmond, IN) and powdered GSE was provided by Kikkoman Corporation (Chiba, Japan). All diets in these studies were given in powdered form. AIN-76A and GSE were mixed together to obtain a 5% mixture of GSE by weight (5 g of GSE and 95 g of AIN-76A). Mice were fed a control diet of AIN-76A or a diet of AIN-76A supplemented with 5% GSE. AIN-76A was used because it is soy free. Soy isoflavones are structurally similar to catechins which make up a large portion of GSE and we wanted to ensure that any differences detected were caused by GSE and not soy. Each animal was caged separately and given 15 g of powdered control or GSE diet every 24 h. Due to the dispersal of the food within the cage by the animals, the amount of food remaining at the end of day was not determined prior to giving fresh diet.

## Animals, GSE Administration, and Sample Collection

Proopiomelanocortin-enhanced green fluorescent protein (POMC-EGFP) transgenic mice with C57BL/6J genetic background were used to study the effects of dietary GSE on hippocampal neurogenesis. GFP expression is driven by the POMC

promoter and is expressed transiently in immature granule cells 1-2 weeks postmitotic in the DG (44). Male 10- to 11-month-old POMC-EGFP mice were fed either a control diet (n=4) or a GSE diet (n=4) for 31 days. 5-bromo-2'-deoxyuridine (BrdU) was injected (50 mg/kg of body weight) 2 h before mice were euthanized, which is an established protocol used to quantify the number of proliferating cells (39, 43). Isoflurane was inhaled and 0.8 mL 2% Avertin (2,2,2-tribromoethanol) was injected subcutaneously to anesthetize the mice. Organs were flushed with 30 mL of saline solution (0.9% NaCl) followed by 30 mL of 4% paraformaldehyde (PFA) by intracardiac perfusion. Brains were removed and stored in 4% PFA at $4^{o}$C for 24 h before sectioning. Brains were placed in PBS and sectioned in the horizontal plane every 50 µm using a vibratome. Sections were collected once the hippocampus was detected. Approximately 48 sections were collected from each brain. Sections were stored in antifreeze at $-20^{o}$C until analysis. All experimental procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

To further investigate the effects of dietary GSE on hippocampal neurogenesis, a mixture of 4-month-old male and female POMC-EGFP mice were fed either a control diet (n=3) or a GSE diet (n=3) for 28 days. BrdU was injected (50 mg/kg of body weight) 4 times every 2 h in the mice given the control diet. BrdU was injected (50 mg/kg of body weight) 3 times every 2 h in the mice given the GSE diet. The first injection was 24 h before the mice were euthanized. The mice were euthanized, their brains were taken, and sections were generated and saved using the same procedure as the 10- to 11-month-old mice.

To study the effects on hippocampal neurogenesis in weaned POMC-EGFP mice whose mother was given GSE while nursing, two pregnant POMC-EGFP mice were maintained on standard rodent chow until they gave birth; at which time they were switched to a control diet or a GSE diet. At postnatal day 26, the POMC-EGFP pups were weaned and euthanized. The brains were taken and sections generated and saved as with the 10- to 11-month-old mice with the exception of flushing the organs with 15 mL of saline solution followed by 15 mL of 4% PFA versus flushing the organs with 30 mL of saline solution followed by 30 mL of 4% PFA.

## Antibodies of Markers for Neurogenesis

BrdU and Anti-BrdU polyclonal IgG were purchased from abcam (Cambridge, MA). Anti-green fluorescent protein (GFP) rabbit IgG fraction Alexa Fluor 488 conjugate, streptavidin secondary antibody against anti-BrdU Alexa Fluor 594 conjugate, polyclonal rabbit anti-Ki-67, goat anti-rabbit alexa 568, and goat anti-rabbit alexa 594 were purchased from Invitrogen (Grand Island, NY). Monoclonal anti-Neuronal Nuclei (NeuN) were purchased from Millipore (Billerica, MA).

## Detection of Proliferating Progenitor Cells, New Neurons, and Mature Neurons in the DG by Immunohistochemistry

Detection of new neurons in the DG of 5- and 12-month-old POMC-EGFP mice was performed using antibodies for endogenous GFP produced by new neurons in the

DG of the POMC-EGFP transgenic mice. Detection of proliferating progenitor cells in the DG of 5- and 12-month-old POMC-EGFP mice was performed using antibodies for BrdU which would label cells that had recently undergone division. Fixed brain sections were washed with potassium phosphate buffered saline (KPBS) twice for 10 min at room temperature (RT) and incubated in 2 Normal (N) hydrochloric acid (HCl)/KPBS for 30 min at 37$^{o}$C. Sections were washed with KPBS (pH 8.5) followed by KPBS + 0.4% Triton X-100 (KPBST) for 10 min each at RT. Sections were blocked with KPBST + 5% normal goat serum (NGS) for 60 min at RT. Sections were incubated with anti-BrdU (1:500) in KPBST + 5% NGS overnight at 4$^{o}$C. Sections were washed with KPBS 3 times for 10 min at RT. Sections were incubated with streptavidin secondary against BrdU (1:200) in KPBST for 2 hours at RT. Sections were washed with KPBS 2 times for 10 min at RT. Sections were incubated with rabbit anti-GFP (1:1000) in KPBST overnight at 4$^{o}$C. Sections were washed with KPBS 2 times for 10 min at RT. Sections were mounted on slides and dried in the dark. A drop of antifade (Vectashield Mounting Medium) was placed on each section and covered with a cover slip. Slides were stored at 4$^{o}$C.

Detection of new neurons in the DG of weaned POMC-EGFP mice was performed using antibodies for endogenous GFP produced by new neurons in the DG of the POMC-EGFP transgenic mice. Detection of proliferating progenitor cells in the DG of weaned POMC-EGFP mice was performed using antibodies for Ki-67, a nuclear protein found in cells in late G1, S, G2, and M phases of the cell cycle. Fixed brain sections were washed with Tris-buffered saline (TBS) 3 times for 10 min at RT. Sections

were blocked with TBS + 10% NGS for 90 min at RT. Sections were incubated with rabbit anti-Ki67 (1:300) in TBS + 10% NGS overnight at 4°C. Sections were washed with TBS 3 times for 10 min at RT. Sections were incubated with secondary goat anti-rabbit alexa 568 (1:200) for 2 h at RT. Sections were washed with TBS 3 times for 10 min at RT. Sections were incubated with rabbit anti-GFP (1:1000) overnight at 4°C. Sections were washed with TBS 3 times for 10 min at RT. Sections were mounted on slides and dried in the dark. A drop of antifade was placed on each section and covered with a cover slip. Slides were stored at 4°C.

Detection of mature neurons in the DG of weaned POMC-EGFP mice was performed using an antibody for NeuN, a DNA-binding nuclear protein found in mature neurons. Fixed brain sections were washed with TBS 3 times for 10 min at RT. Sections were blocked with TBS + 10% NGS for 90 min at RT. Sections were incubated with mouse anti-NeuN (1:10,000) in TBS + 10% NGS overnight at 4°C. Sections were washed with TBS 3 times for 10 min at RT. Sections were incubated with secondary goat anti-rabbit alexa 594 (1:400) for 2 h at RT. Sections were washed with TBS 3 times for 10 min at RT. Sections were mounted on slides and dried in the dark. A drop of antifade was placed on each section and covered with a cover slip. Slides were stored at 4°C.

**Analysis of Proliferating Progenitor Cells, New Neurons, and Mature Neurons in the DG by Stereology**

Stereology was used to determine the number of proliferating progenitor cells, new neurons, and mature neurons in the DG of 5-month-old and 26-day-old POMC-EGFP mice. Stereology is unbiased measurements of the number or shape of objects. In the case of counting cells, stereology estimates the total targeted cells within a tissue. The software program StereoInvestigator (MBF Bioscience, Williston, VT) using the Optical Fractionator workflow was used to count GFP, Ki-67, and NeuN positive cells using an Olympus BX51 fluorescence microscope. Fluorescence was used to trace the outline of the right and left DG at 10x magnification. GFP, Ki-67, and NeuN positive cells were counted at 60x oil magnification. The guard zones and dissector height are parameters in StereoInvestigator that make up the thickness of the brain section and help prevent counting areas of the section that cells may have been damaged or removed due to slicing of the brain. The guard zones were 2.5 μm and the disector height was 25 μm for 5-month-old mice. The guard zones were 5 μm and the disector height was 35 μm for 26-day-old mice. The counting box is StereoInvestigator's way of not counting a cell twice within a section. The counting box's dimensions were 40 μm x 40 μm. GFP, Ki-67, and NeuN positive cells were counted if they were within the disector height and the counting box (Fig. 11). Estimated Total Mean Measured Thickness, which calculates the cell number based on the average thickness of the tissue, was used to estimate the total number of GFP, Ki-67, and NeuN positive cells in the DG. Every 3$^{rd}$ section, 9 total, for GFP positive cells were counted in 5-month-old mice. StereoInvestigator was not used to

count BrdU positive cells. The $4^{th}$, $5^{th}$, and $6^{th}$ brain sections of the GFP/BrdU slides were counted for BrdU positive cells in 5-month-old mice. Every $6^{th}$ section, 8 total, for GFP and Ki-67 positive cells and every $6^{th}$ section, 5 total, for NeuN positive cells were counted in 26-day-old mice.

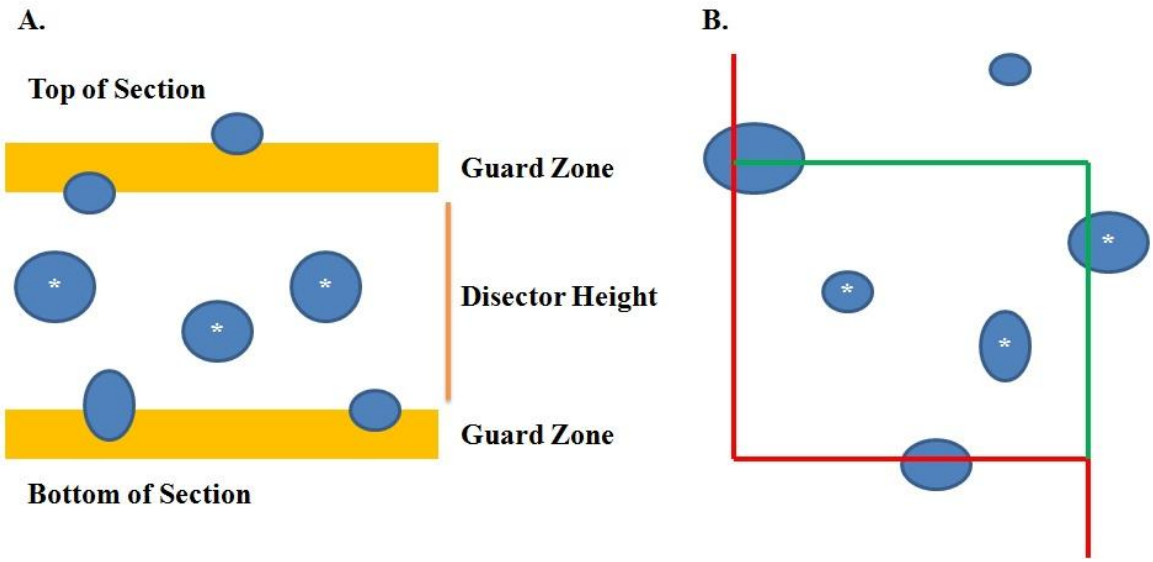



Figure 11. Stereological counts of GFP, Ki-67, and NeuN positive cells using guard zones, disector height, and the counting box parameters. (A) A representative brain section with guard zones and disector height. Cells that fall within the disector height and are not touching the guard zones were counted. In this picture, 3 cells (blue circles with asterisks) fall within the disector height while not touching the guard zones and therefore would be counted. (B) A representative counting box. Cells that fall within the counting box were counted. If a cell touched a green line it was counted. If a cell touched a red line it was not counted even if it was also touching a green line. In this picture, 3 cells (blue circles with asterisks) would be counted.

Stereology was used to determine dendrite morphology of new neurons by confocal microscopy using the software program NeuroLucida (MBF Bioscience). NeuroLucida draws concentric circles emanating out from the cell body of a neuron equidistant from each other. The distance between the concentric circles in this study was 10 μm. Fluorescence from GFP of new neurons was used to trace the cell body and its dendrites. The dendrite parameters measured were total dendrite length, number of branch points, and number of intersections (the place a dendrite crosses one of the concentric circles). The total dendrite length is the sum of the length in μm of all dendrites from one neuron. The number of branch points is the number of times dendrites split from one neuron. The number of intersections is the total number of times dendrites cross the concentric circles. The length of dendrites, the number of branch points between concentric circles, and the number of intersections that crossed each concentric circle were all determined (Fig. 12). Sholl analysis was used to analyze dendrite morphology.

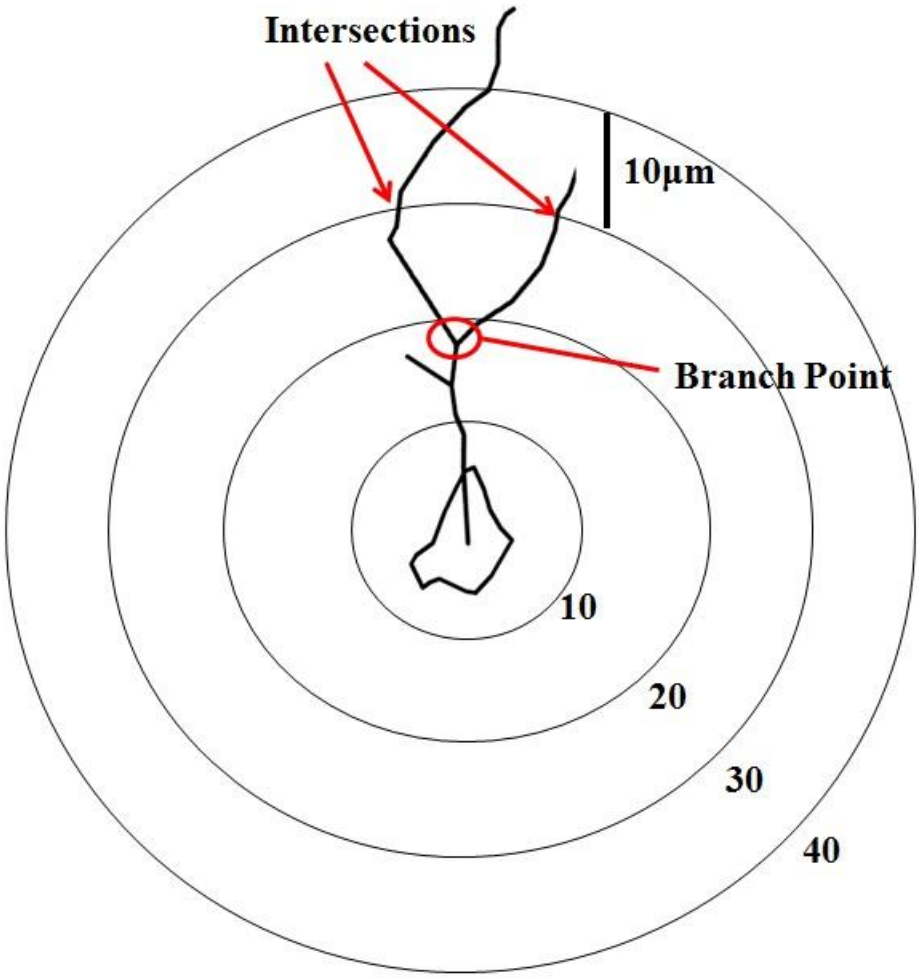



Figure 12. The quantification of total dendrite length, number of branch points, and number of intersections in new neurons by Sholl analysis. NeuroLucida draws concentric circles emanating out from the cell body of a neuron equidistant (10 µm) from each other. Dendrites of new neurons were traced and the three parameters were quantitatively examined. The new neuron represented here contains two branch points both between 10 µm and 20 µm and 6 intersections, 1 at 10 µm, 2 at 20 µm, 2 at 30 µm, and 1 at 40 µm.

## Statistical Analysis

A two-sided equal variance Student's t-test was used to determine statistical differences in the numbers of new neurons, proliferating progenitor cells, and dendrite morphology between 5-month-old POMC-EGFP mice given control or GSE diet. A two-sided equal variance Student's t-test was used to determine statistical differences in the numbers of new neurons, proliferating progenitor cells, and mature neurons between 26-day-old POMC-EGFP mice whose mothers were given control or GSE diet while nursing. A p-value of $\leq 0.05$ was considered to be statistically significant. Data are presented as mean ± standard error of the mean (SEM).

# RESULTS

## Actions of GSE on Adult Hippocampal Neurogenesis in the POMC-EGFP Mouse

To examine the effects of dietary GSE on adult neurogenesis, 12-month-old POMC-EGFP mice were used to visualize changes in new neurons in the DG following administration of GSE. Control diet (AIN-76A, n=4) or GSE diet (AIN-76A supplemented with 5% GSE, n=4) were given to 10- to 11-month-old POMC-EGFP mice for 31 days. BrdU, which incorporates into DNA in place of thymidine in dividing cells, was injected (50 mg/kg body weight) 2 h before mice were euthanized to determine the number of proliferating neural progenitor cells. It was determined after examination of the hippocampal sections that there were too few GFP positive cells and BrdU positive cells in the DG to perform stereological counts and therefore determine differences in proliferating progenitor cells and new neurons. As this analysis was done in 12-month-old POMC-EGFP mice, an age with low levels of neurogenesis, it was determined these animals were too old to perform quantitative analysis on the number of new neurons and proliferating progenitor cells.

Body weights were measured at the beginning and the end of the study. Table 1 shows that mice given the GSE diet did not gain body weight during the course of the

study whereas mice given the control diet gained 20% of their starting body weight. Both groups had similar body weights at the beginning of the study ($p=0.777$) whereas by the end of the study the control group had higher body weights compared to the GSE group ($p=0.058$). Mice from both groups appeared healthy and due to the dispersal of the food within the cage it is not known whether the mice given the GSE diet had a lower intake of food.

Table 1.

*Effect of GSE on body weight in 10- to 11-month-old POMC-EGFP mice*

| Group (n) | Starting Weight (g) | Final Weight (g) | Difference in Weight (%) |
|---|---|---|---|
| Control Diet (4) | 32.4 ± 3.4 | 38.8 ± 1.6 | 20 |
| GSE Diet (4) | 33.7 ± 2.8 | 32.7 ± 2.0 | -3 |

Difference in weight is the percent body weight gained or lost from starting weight to final weight in each group. Data are expressed as the mean ± SEM.

The potential actions of dietary GSE on adult neurogenesis were next examined in 5-month-old POMC-EGFP mice. Control diet (n=3) or GSE diet (n=3) were given to 4-month-old POMC-EGFP mice for 28 days. BrdU was injected (50 mg/kg of body weight) 4 times every 2 h in the mice given control diet and 3 times every 2 h in the mice given GSE diet. The first injection was 24 h before mice were euthanized. The number of new neurons was determined by counting GFP positive cells in 9 brain sections, 100 μm apart, spanning the DG (Fig. 13). The software StereoInvestigator was used to determine the total number of new neurons in the DG. The number of new neurons in the DG of mice given the control diet was 3329 ± 271 and in the mice given the GSE diet was 2918 ± 233 (Fig. 14A). There was no statistical difference between the number of new neurons in mice given the control diet compared to mice given the GSE diet ($p=0.314$).

The number of proliferating progenitor cells was determined by counting BrdU positive cells in 3 sections spanning the DG. All BrdU positive cells in these sections were counted. The mean number of BrdU positive cells per section in the mice given the control diet was 81 ± 9 per section whereas the mean number of BrdU positive cells per section in the mice given the GSE diet was 76 ± 4 per section (Fig. 14B). There was no statistical difference between the number of proliferating progenitor cells in mice given the control diet compared to mice given the GSE diet ($p=0.689$). These data suggest that dietary GSE does not affect proliferation of neural progenitor cells or the number of new neurons in the DG of 5-month-old POMC-EGFP mice.

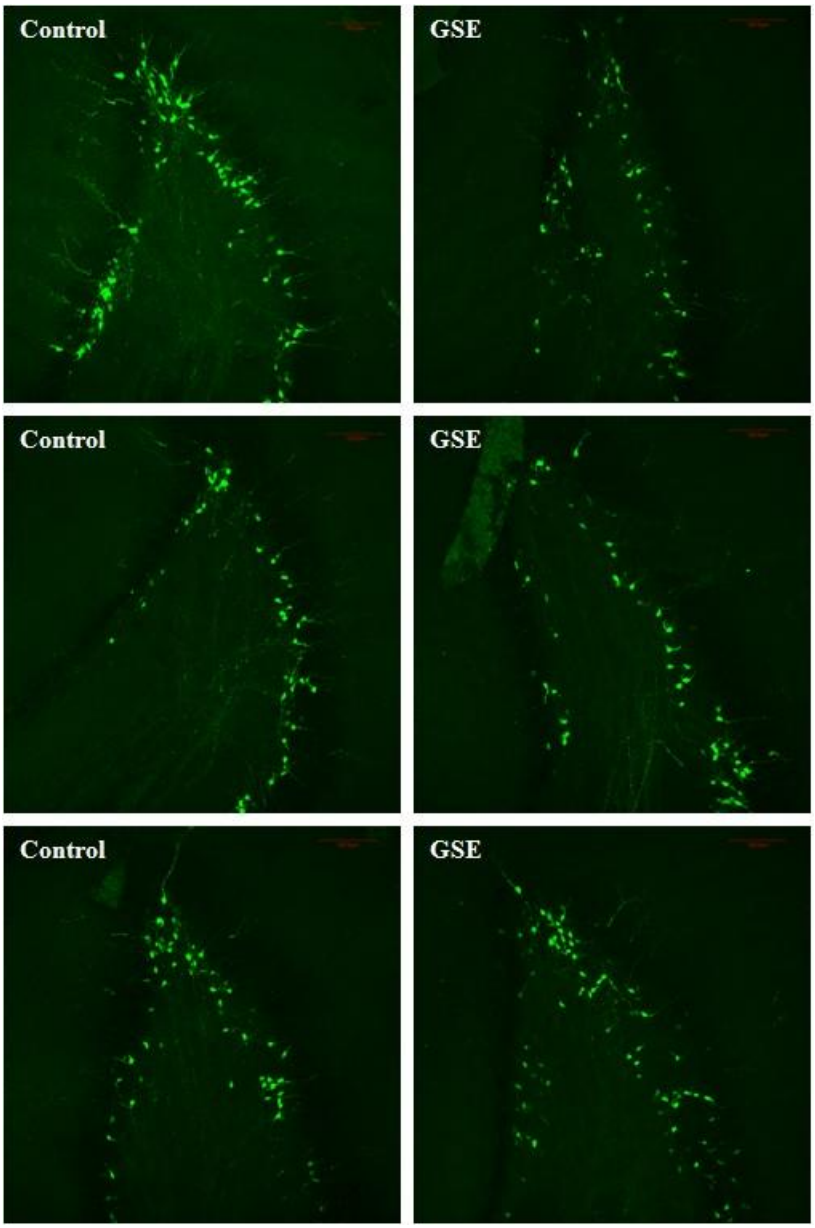



Figure 13. New neurons labeled with GFP in the DG of 5-month-old POMC-EGFP mice given control or GSE (5%) diets. The left panels contain DG sections from 3 different POMC-EGFP mice given the control diet. The right panels contain DG sections from 3 different POMC-EGFP mice given the GSE diet. These micrographs were taken with a confocal microscope of anti-GFP labeled cells.

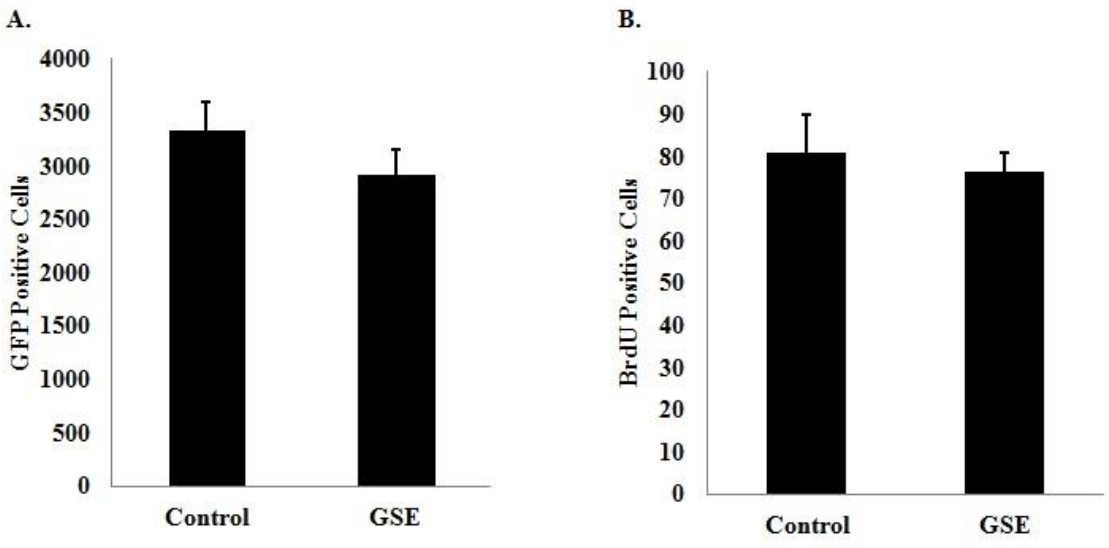



Figure 14. No difference in the number of new neurons or proliferation of neuronal progenitor cells in the DG of 5-month-old POMC-EGFP mice given the GSE (5%) diet vs. POMC-EGFP mice given the control diet. (A) Number of new neurons determined by GFP positive cells in the DG of mice given the control diet (n=3) compared to mice given the GSE diet (n=3, p=0.314). (B) Number of proliferating progenitor cells determined by BrdU positive cells in the DG of mice given the control diet compared to mice given the GSE diet (p=0.689). Data are expressed as the mean ± SEM.

Body weights were measured at the beginning and the end of the study. As with the 10- to 11-month-old mice, the 4-month-old mice given the GSE diet did not gain body weight over the course of the study whereas the 4-month-old mice given the control diet gained 22% of their starting body weight (Table 2). Similar with the older mice both groups had similar body weights at the beginning of the study ($p=0.709$) whereas by the end of the study the control group had higher body weights compared to the GSE group ($p=0.012$). Once again mice from both groups appeared healthy and due to the dispersal of the food it is not known whether the mice given the GSE diet had a lower intake of food.

Table 2.

*Effect of GSE on body weight in 4-month-old POMC-EGFP mice*

| Group (n) | Starting Weight (g) | Final Weight (g) | Difference in Weight (%) |
|---|---|---|---|
| Control Diet (3) | 30.2 ± 0.4 | 36.7 ± 1.5 | 22 |
| GSE Diet (3) | 28.8 ± 3.5 | 25.3 ± 2.2 | -12 |

Difference in weight is the percent body weight gained or lost from starting weight to final weight in each group. Data are expressed as the mean ± SEM.

While the number of new neurons was not affected by GSE, it was important to consider the maturation of these neurons and to determine whether GSE intake affected dendrite morphology. New neurons in the DG that were positive for GFP were examined with regard to the morphology of their dendrites. The software NeuroLucida was used to quantify the total length of dendrites, the number of branch points, and the number of intersections. The total dendrite length in POMC-EGFP mice given the GSE diet was 145 ± 20 μm whereas in POMC-EGFP mice given the control diet was 176 ± 20 μm (Fig. 15). The number of branch points in POMC-EGFP mice given the GSE diet was 2.8 ± 0.6 whereas in POMC-EGFP mice given the control diet was 3.7 ± 0.3 (Fig. 16). The number of intersections in POMC-EGFP mice given the GSE diet was 12.3 ± 1.8 whereas in POMC-EGFP mice given the control diet was 14.9 ± 1.8 (Fig. 17). There was no statistical difference in total dendrite length ($p=0.340$), number of branch points ($p=0.230$), and number of intersections ($p=0.350$). These data suggest that GSE does not affect the maturation of new neurons in the DG of 5-month-old POMC-EGFP mice.

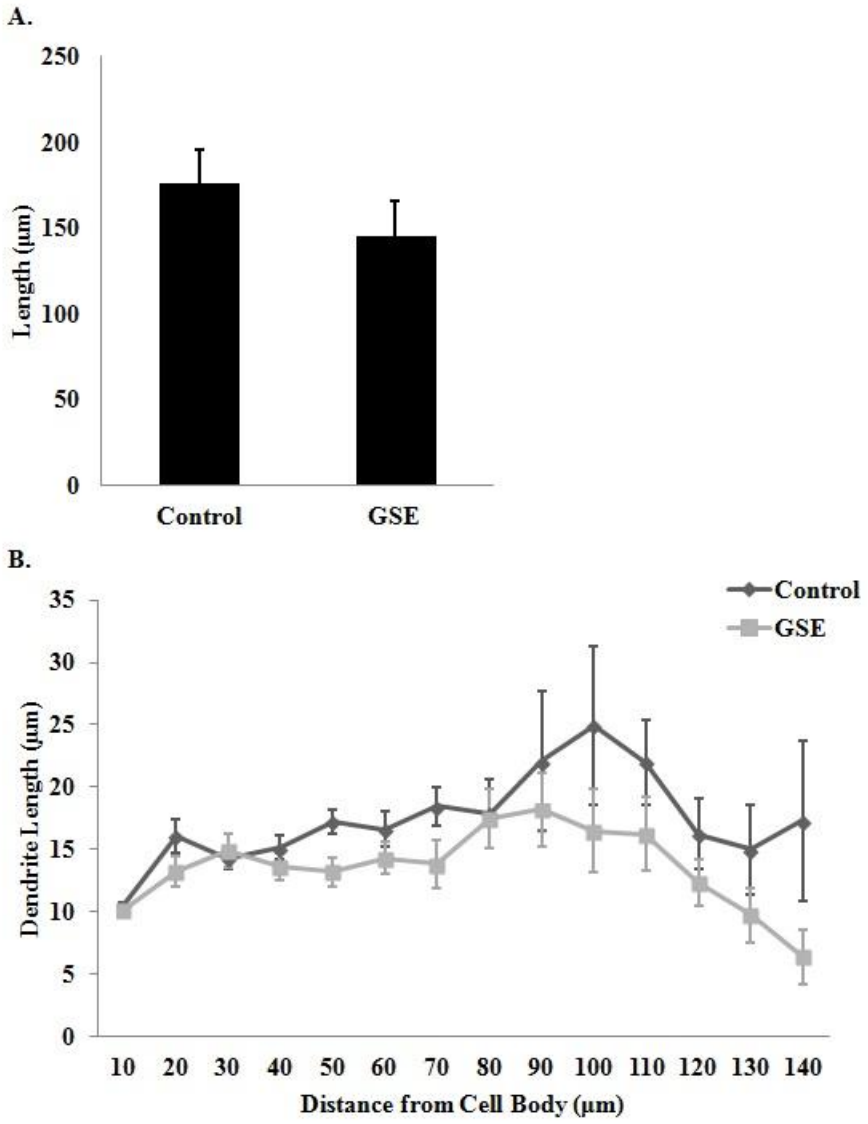



Figure 15. No difference in dendrite length of new neurons in the DG of 5-month-old POMC-EGFP mice given the GSE (5%) diet vs. POMC-EGFP mice given the control diet. (A) Total length of dendrites of new neurons in the DG of mice given the control diet (n=3) compared to mice given the GSE diet (n=3, p=0.340). (B) Length of dendrites at increments of 10 µm away from the cell body in the DG of mice given the control diet compared to mice given the GSE diet. Data are expressed as the mean ± SEM.

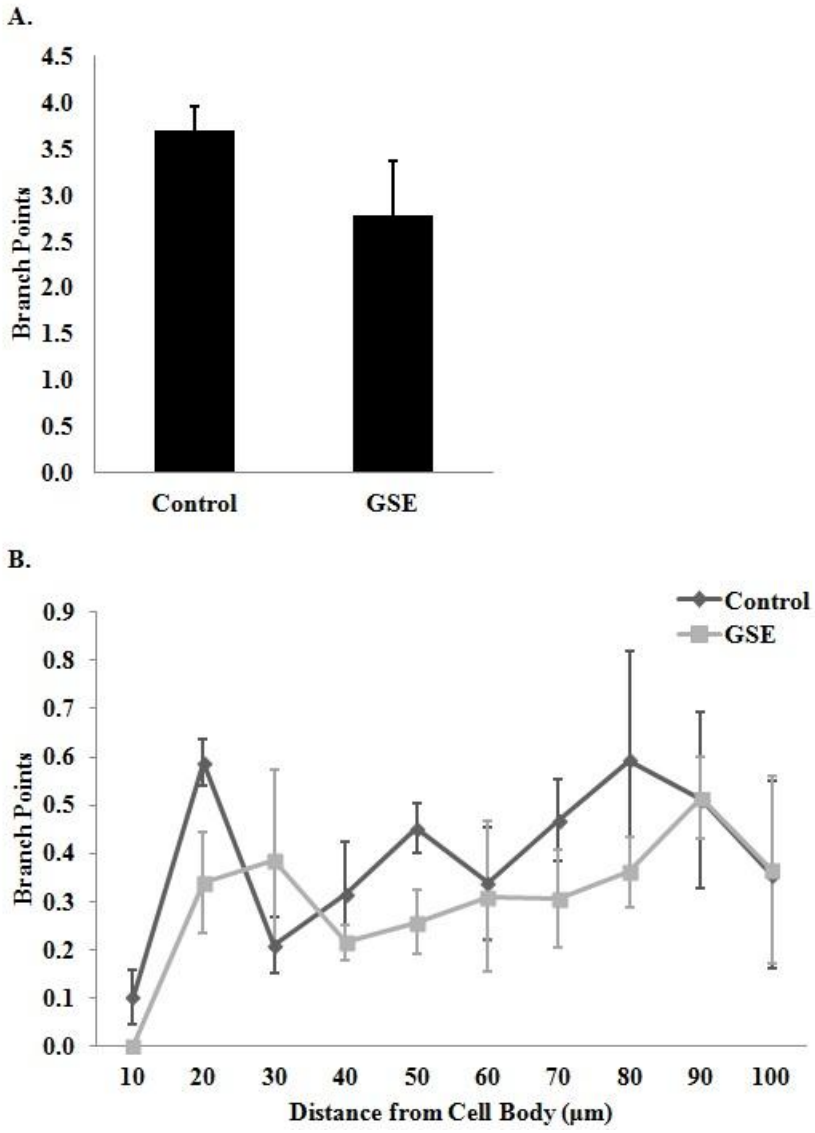



Figure 16. No difference in the number of branch points in dendrites of new neurons in the DG of 5-month-old POMC-EGFP mice given the GSE (5%) diet vs. POMC-EGFP mice given the control diet. (A) Number of branch points in dendrites of new neurons in the DG of mice given the control diet (n=3) compared to mice given the GSE diet (n=3, p=0.230). (B) Number of branch points in dendrites at increments of 10 µm away from the cell body in the DG of mice given the control diet compared to mice given the GSE diet. Data are expressed as the mean ± SEM.

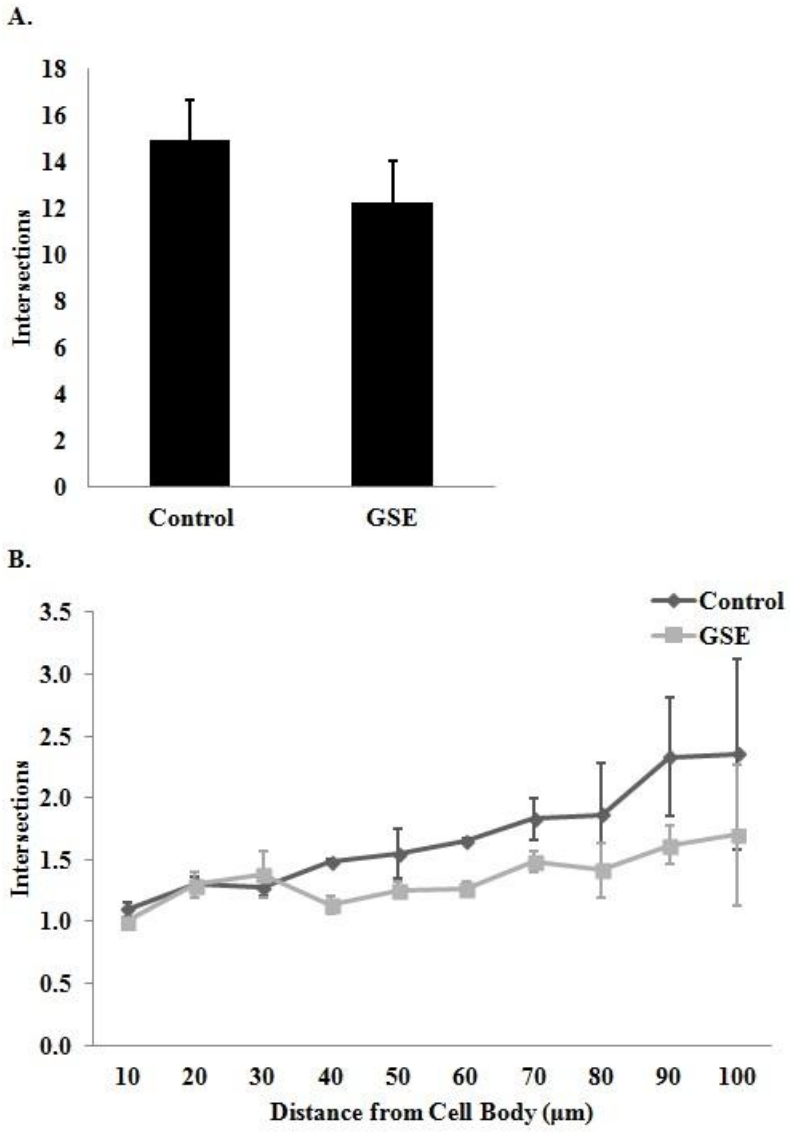



Figure 17. No difference in the number of intersections in dendrites of new neurons in the DG of 5-month-old POMC-EGFP mice given the GSE (5%) diet vs. POMC-EGFP mice given the control diet. (A) Number of intersections of dendrites of new neurons in the DG of mice given the control diet (n=3) compared to mice given the GSE diet (n=3, p=0.350). (B) Number of intersections of dendrites at increments of 10 µm away from the cell body in the DG of mice given the control diet compared to mice given the GSE diet. Data are expressed as the mean ± SEM.

**Changes in Hippocampal Neurogenesis in POMC-EGFP Mice whose Mother was given GSE only while Nursing**

To determine whether neurogenesis is affected in pups whose mother was given GSE only while nursing two pregnant POMC-EGFP mice were maintained on standard rodent chow until they gave birth, at which time they were switched to either control diet or GSE diet. The nursing mothers were maintained on these diets for 26 days at which time the pups were weaned, euthanized, and their brains removed and analyzed for markers of neurogenesis. The number of new neurons (indicated by GFP immunoreactivity) in the DG of 26-day-old mice (n=3) whose mother was given the GSE diet was 11764 ± 553 which was lower compared to 26-day-old mice (n=3) whose mother was given the control diet was 15055 ± 98 ($p=0.004$) (Fig. 18). These data suggest that the exposure of GSE in the diet to a mother nursing attenuates the number of new neurons in the DG of 26-day-old POMC-EGFP mice.

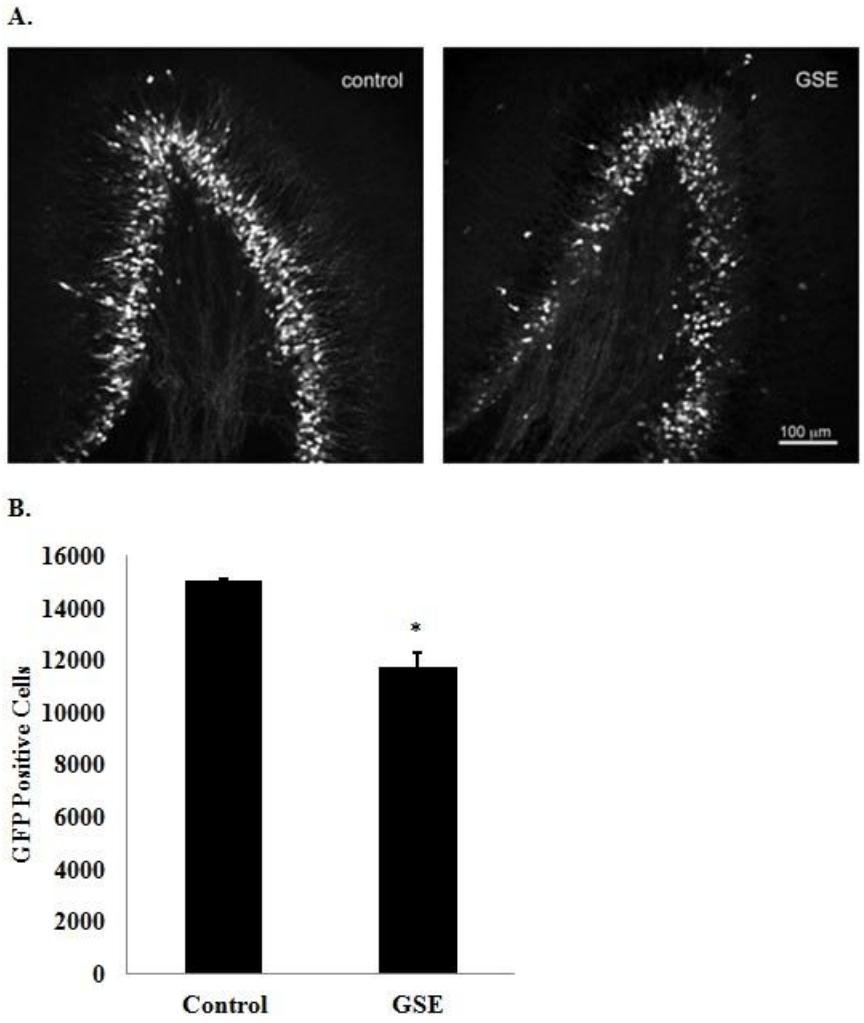



Figure 18. Lowered numbers of new neurons in the DG of 26-day-old POMC-EGFP mice whose mother was given the GSE (5%) diet only while nursing vs. 26-day-old POMC-EGFP mice whose mother was given the control diet. (A) New neurons labeled with GFP in the DG of a 26-day-old mice whose mother was given the control diet (left panel) and a 26-day-old mice whose mother was given the GSE diet (right panel). These micrographs were taken with a confocal microscope of anti-GFP labeled cells in the DG. (B) Number of new neurons determined by GFP positive cells in the DG of 26-day-old mice (n=3) whose mother was given the control diet compared to 26-day-old mice (n=3) whose mother was given the GSE diet ($p=0.004$). Data are expressed as the mean ± SEM. * p value $\leq 0.01$.

The number of proliferating progenitor cells (indicated by Ki-67 immunoreactivity) in the DG of 26-day-old POMC-EGFP mice (n=3) whose mother was given the GSE diet was 6328 ± 653 whereas 26-day-old POMC-EGFP mice (n=3) whose mother was given the control diet was 6668 ± 418 (Fig. 19A). There was no statistical difference between the number of proliferating progenitor cells in the DG of 26-day-old mice whose mother was given the GSE diet compared to the mice whose mother was given the control diet ($p=0.683$). These data suggest that exposure of GSE in the diet to a mother nursing does not affect proliferation of neural progenitor cells in the DG of 26-day-old POMC-EGFP mice.

The number of mature neurons (indicated by NeuN immunoreactivity) in the DG of 26-day-old POMC-EGFP mice (n=3) whose mother was given the GSE diet was 56942 ± 2294 whereas 26-day-old POMC-EGFP mice (n=3) whose mother was given the control diet was 66406 ± 9087 (Fig. 19B). There was no statistical difference between the number of mature neurons in the DG of 26-day-old mice whose mother was given the GSE diet compared to the mice whose mother was given the control diet ($p=0.370$). These data suggest that exposure of GSE in the diet to a mother nursing does not affect the number of mature neurons in the DG of 26-day-old POMC-EGFP mice. Together these data suggest that exposure of GSE in the diet to mothers only while nursing attenuates the number of new neurons but does not affect the proliferation of neural progenitor cells or the number of mature neurons in the DG of their nursing pups.

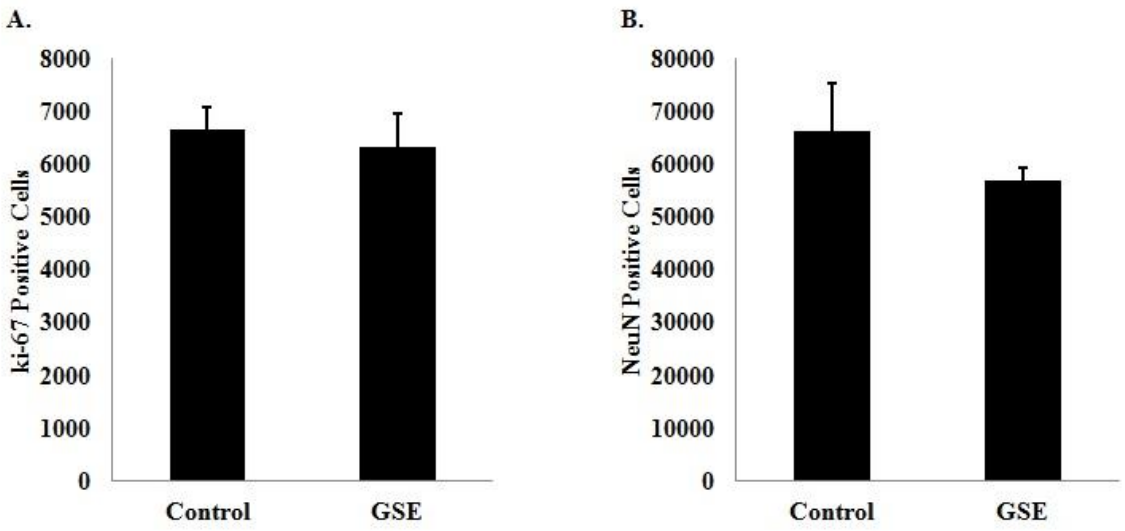



Figure 19. No difference in the proliferation of neural progenitor cells and the number of mature neurons in the DG of 26-day-old POMC-EGFP mice whose mother was given the GSE (5%) diet only while nursing vs. 26-day-old POMC-EGFP mice whose mother was given the control diet. (A) Number of proliferating progenitor cells determined by Ki-67 positive cells in the DG of 26-day-old mice (n=3) whose mother was given the control diet compared to 26-day-old mice (n=3) whose mother was given the GSE diet (p=0.683). (B) Number of mature neurons determined by NeuN positive cells in the DG of 26-day-old mice (n=3) whose mother was given the control diet (n=3) compared to 26-day-old mice (n=3) whose mother was given the GSE diet (p=0.370). Data are expressed as the mean ± SEM.

## DISCUSSION

In the present study, we showed that consumption of GSE does not enhance hippocampal neurogenesis as there was no difference in the number of new neurons or proliferating progenitor cells in the DG of 5-month-old POMC-EGFP mice given GSE compared to control 5-month-old POMC-EGFP mice. Also, we found lowered numbers of new neurons and no difference in proliferation of progenitor cells in the DG of 26-day-old POMC-EGFP mice whose mother was given GSE only while nursing compared to control 26-day-old POMC-EGFP mice. This suggests that dietary intake of GSE does not affect hippocampal neurogenesis in adults but that dietary intake of GSE in nursing mothers causes reduced survival of new neurons in their offspring.

There was no difference in the number of proliferating progenitor cells and new neurons in the DG of 5-month-old POMC-EGFP mice given GSE for 4 weeks compared to control POMC-EGFP mice. This indicates that consumption of GSE does not affect the proliferation of neural stem cells or the survival of new neurons in the DG of adults and that although GSE can have benefits in the brain; these benefits affect pathways that are independent of hippocampal neurogenesis. Whereas dietary interventions may enhance hippocampal neurogenesis in diseased or compromised animals (23, 25, 28, 72), we found no evidence for enhanced neurogenesis in young healthy adult mice. GSE

given to a transgenic mouse model of AD had improved learning and memory when tested in the Morris water maze, a hippocampal dependent task, compared to control AD transgenic mice (25). GSE given to young OVX rats had improved performance in the eight-arm-radial maze, another hippocampal-dependent task, compared to control OVX rats (23). In both of these experiments normal healthy animals were not tested; therefore, it may be that GSE restores hippocampal learning and memory to a normal level and that this may involve enhancing hippocampal neurogenesis. It would be of interest to determine if ablating hippocampal neurogenesis would affect the improvement in learning and memory in the same transgenic mouse model of AD and OVX rats given GSE.

It is to be noted that the POMC-EGFP mice given the control diet had 4 injections of BrdU (50 mg/kg body weight) one more than the POMCE-EGFP mice given the GSE diet. This was due to having injected the control animals with BrdU for the fourth time without having enough BrdU to inject the GSE animals for a fourth time. This may have lead to a higher number of proliferating progenitor cells to be labeled with BrdU in the control animals compared to the GSE animals. Therefore, GSE may have increased the number of proliferating progenitor cells in the DG and the comparison made may not be valid. Also, stereological counts were not performed on BrdU positive cells due to brain sections not staining well for BrdU or sections being cut or broken. This may have lead to a bias count of the number of BrdU positive cells. However, as no differences were detected in GFP and Ki-67 labeling, which are independent of BrdU labeling, this suggest

that the potential confounds arising from the BrdU injections did not mask an effect of GSE on neurogenesis.

Our experiments indicate that GSE in the diet does not affect hippocampal neurogenesis in young adult mice. After the completion of our experiments, Yoo *et al.* (158) reported that 12-month-old mice given GSE had enhanced hippocampal neurogenesis including increased numbers of proliferating progenitor cells and mature neurons along with increased tertiary dendrites in new neurons. Yoo *et al*. used 12-month-old male C57BL/6J mice that were given GSE by gavage daily at a dose of 25, 50, or 100 mg/kg body weight for 28 days. There are several potential explanations for the discrepancies between our data and that of Yoo *et al*. First, the doses Yoo *et al.* gave are equivalent to 1, 2, and 4 mg of GSE per day for a 40 g mouse. Our mice were given a diet supplemented with 5% GSE and as mice consume roughly 4 g of food each day (159), our mice would have ingested roughly 200 mg of GSE per day, much higher than what Yoo *et al*. gave their mice. Second, the mode of administration of GSE to the animals; Yoo *et al.* gavaged their animals with GSE once daily with a known amount of GSE, whereas the mice in our study had access to their food *ad libitum* and therefore the amount they consumed day to day may have varied. Also, it is possible that the animals gavaged with GSE experienced a spike in concentration of GSE and its metabolites that may result in a higher, if transient, local concentration of GSE and its metabolites in tissue to elicit an effect whereas animals given GSE *ad libitum* would not reach a high enough concentration to elicit an effect. Third, the preparation of the GSE; the GSE used by Yoo *et al*. was ground into a fine powder before being extracted whereas the GSE

used in our studies was not ground into powder before extraction (17). The age of the mice could have also caused a difference. In young mice, the proliferation of new neurons is higher than in older mice and this may mask effects of dietary interventions, such as GSE, whereas in older mice these changes would be more prevalent. Although we initially used 12-month-old mice to determine whether GSE enhances neurogenesis we did not detect sufficient number of proliferating progenitor cells or new neurons to perform stereological counts. One pitfall of Yoo *et al*. was they did not perform stereological counts. Instead they took 15 sections and averaged the counts of those sections and the counts from the mice given GSE were shown as a percent of control rather than as raw data. Further studies are needed to determine which of these rationales underlie the differences detected between our study and that of Yoo *et al*. (158). Also, as we and Yoo *et al*. did not correlate our studies with a physiological outcome it would be important to measure LTP in the DG and/or test for hippocampal-dependent learning and memory to correlate any changes in hippocampal neurogenesis with a physiological outcome.

Another parameter to consider when determining whether an intervention like GSE affects neurogenesis is to examine the morphology of the dendrites of new neurons. Differences in the morphology of the dendrites of neurons in the DG are correlated with the level of the maturation of these neurons (160). Mature neurons have long dendrites that go into the outer molecular layer of the DG and have many branches whereas the dendrites in immature neurons are shorter and have fewer branches. Voluntary exercise increased branching of dendrites in new neurons in the DG of rats (160). Pilocarpine-

induced seizures in mice increased the length and branching of dendrites in new neurons and promoted their functional integration into the neuronal circuitry (50). There was no difference in the length or branching of dendrites in new neurons in the DG of 5-month-old POMC-EGFP mice given GSE compared to control POMC-EGFP mice. The morphology of these dendrites was consistent with that of immature neurons, shorter dendrites with few branches. These data suggest that under our experimental conditions, consumption of GSE does not affect the maturation of new neurons.

There were lower numbers of new neurons in the DG of 26-day-old POMC-EGFP mice whose mother was given GSE only while nursing compared to control 26-day-old POMC-EGFP mice. There were no differences in the proliferation of progenitor cells or the number of mature neurons in the DG of 26-day-old POMC-EGFP mice whose mother was given GSE only while nursing compared to control 26-day-old POMC-EGFP mice. Although there was not a statistical difference in the number of mature neurons, there were fewer mature neurons in the DG of 26-day-old POMC-EGFP whose mother was given GSE only while nursing. These data suggest that the composition of milk changes in mothers given GSE while nursing which results in lowered number of new neurons in their offspring. One possible reason for this change is the addition of compounds from GSE, such as catechin and epicatechin, in the milk. The lower number of new neurons in the pups of the mother given GSE only while nursing suggests a decrease in survival of new neurons. The indication that there were fewer total neurons in the DG supports this theory. The lower number of new neurons detected in the pups whose mother was given GSE only while nursing suggests that neurogenesis may be regulated at critical time

periods, such as the developmental period immediately after birth. However, as the mice in this study came from two litters, 3 mice from a mother given GSE and 3 mice from a control mother, follow up experiments are required to confirm and extend these results. Initially we tried to produce additional litters to increase the number of mice in our study but due to the difficulty of breeding, mothers eating their young, small litter sizes, and the timing of when mothers gave birth, we were unable to increase the number of mice in our study.

While the finding of fewer new neurons was unexpected, as we hypothesized there would be an increase, another explanation of why there were lower amounts of new neurons in the pups whose mother was given GSE only while nursing may be due to stress on the mother. This stress may be coming from the switch in the diets at the time of birth. This possible stress may have affected the mothers on the 5% GSE diet as breeding them was difficult and caused many of them to cannibalize their young. In adult monkeys that undergo stress there is a decrease in proliferation of progenitor cells in the DG (59). Pregnant rats that underwent restraint stress for 1 week before delivery gave birth to pups that at 1, 3, 10, and 22 months of age had lower levels of cell proliferation in the DG compared to control rats (161). This correlated with a decrease in learning the position of the platform in the Morris water maze at 4 months of age (161). Prenatal stress in mice lowered the proliferation of progenitor cells and the number of new neurons in the DG of their 5-week-old pups (162). Thus, the lowered numbers of new neurons detected in our study may have been caused by stress, most likely due to the new diet given immediately after giving birth. Early (first 10 days of gestation) and late (11th

day until delivery) restraint stress on rat mothers decreased the number of new neurons in the DG of their pups; however, when resveratrol was given to the stressed mothers during pregnancy this attenuated the decrease in the number of new neurons in the DG of their pups (163). This would suggest that GSE, which contains flavonoids, may also attenuate the reduced number of new neurons in the offspring of stressed mothers. If the mother given GSE only while nursing was stressed, then there are several factors that might explain why the pups had lowered number of new neurons. First, it may be that the flavonoids in GSE do not act similar to resveratrol and cannot attenuate reduced number of new neurons in pups of mothers who are stressed. Second, the concentration of GSE compounds found in the milk was too high and was toxic to the pups. Third, the timing of when the GSE was given, only while nursing, was not optimal as the beneficial effects of resveratrol were seen when it was given throughout pregnancy (163). Follow up studies are required to confirm that GSE given to pregnant and/or nursing mothers leads to lower numbers of new neurons in the DG of their pups. Also, in these studies the diet containing GSE will be given before and during pregnancy as well as when they are nursing to reduce any stress caused by the change in diet.

Although no comparison could be made of GSE effects on neurogenesis in 12-month-old mice, mice given the GSE diet for 1 month did not gain weight over the course of the study whereas mice given the control diet gained 20% of their starting body weight. Similarly, 5-month-old mice given the GSE diet for 1 month did not gain weight over the course of the study whereas mice given the control diet gained 22% of their starting body weight. It is possible that the amount of GSE (5%) given could have been

large enough to be toxic or stressful to the mice leading to impaired weight gain; however, previous studies using this same amount of GSE in rats showed no deleterious health effects (27). Soy fiber has been shown to decrease weight in obese individuals (164), and it may be that GSE works in a similar manner. Thus an unexpected finding of this study may be the indication that dietary intervention with GSE could be used to control or prevent body weight gain without apparent negative effects as the mice in our study appeared healthy. However, the change in weight between GSE and control mice is confounded since stress and caloric restriction both alter adult neurogenesis (56, 59).

In conclusion, dietary GSE does not enhance hippocampal neurogenesis in adult mice and, in fact, may lower it in pups whose mothers are given GSE while nursing. Under our experimental conditions, where 4-month-old POMC-EGFP mice were given a 5% GSE diet for a month, there was no effect of GSE on the proliferation of progenitor cells or the number of new neurons in the DG. These data indicate that enhancement of learning and memory from GSE acts through a pathway that is independent of hippocampal neurogenesis. However, it is important to note that behavior analysis was not carried out with the POMC-EGFP mice in these studies. Unexpectedly, our data indicates that 26-day-old POMC-EGFP mice whose mother was given GSE only while nursing lowered the number of new neurons in the DG indicating a decrease in survival of new neurons. This may have been caused by exposure to one or more of GSE components in the milk or have caused unexpected stress on the mothers. Further experiments are required to confirm whether this decrease in new neurons is caused by changes in the mother's milk or stress on the mother. Also, additional experiments in

older POMC-EGFP mice on a similar GSE dose as Yoo *et al.* (158) and given acutely are required to confirm that GSE enhances hippocampal neurogenesis. If an increase in hippocampal neurogenesis is detected, further experiments including hippocampal-dependent learning and memory tasks and physiological analysis (LTP, which is thought of as the cellular correlate of learning and memory) will be required to determine if the increase in hippocampal neurogenesis correlates with an increase in learning, memory, and/or LTP. It is possible that an effect on hippocampal neurogenesis by GSE may only be detected in sick individuals or the elderly as the benefits of GSE on learning, memory, and LTP were seen in a mouse model of AD and not in normal healthy mice (28). Finally, our data suggests that GSE given in the diet may attenuate weight gain, which has interesting clinical implications since age-related obesity is becoming a major public health issue.

# CHAPTER 3

# ACTIONS OF GRAPE SEED EXTRACT ON LEARNING AND MEMORY IN A RODENT MODEL OF MENOPAUSE

## INTRODUCTION

GSE has been studied for health benefits in many diseases that are associated with aging including cancers (18-22), cardiovascular diseases (23, 24), and neurodegenerative diseases (25, 26). Dietary intake of GSE improved learning and memory in a transgenic mouse model of AD (25) and in young OVX rats shortly after OVX (23). Dietary intake of epicatechin, a monomeric catechin found in GSE, improved memory in wild type mice (30). A metabolite of epicatechin, 3'-*O*-methyl epicatechin glucuronide, enhanced LTP when added exogenously to hippocampal slices from a transgenic mouse model of AD (28). These data suggest that GSE, specifically the catechin monomers, plays a role in learning and memory.

All women go through menopause where the ovaries cease to produce estrogens. Menopause is accompanied with increased risks for osteoporosis (31), coronary heart disease (32, 33), Alzheimer's disease (34), and cognitive decline (83-86). Because of these increased risks it is reasonable to propose that estrogens have a role in protecting against each of these conditions. Indeed, hormone therapy studies with estrogen have

indicated that estrogen is protective against osteoporosis (87, 88), cardiovascular disease (89), and some hormone independent cancers (90, 91), especially when treatment is started during perimenopause (165).

The lowered levels of estrogens caused by ovariectomy impairs learning and memory in hippocampal-based behavioral tasks in rodents (98, 102-104), but the administration of 17β-estradiol to OVX rats restores hippocampal-based learning and memory (104-106). While estrogen therapy may restore cognitive function the use of estrogen therapy raises concerns, especially with women who are dealing with or are at risk for hormone-dependent cancer. It has become important to identify alternatives to estrogen that can act in reducing the risk for osteoporosis, cardiovascular disease, neurodegenerative diseases, and cognitive decline without the hazards of estrogens. Botanical dietary supplements enriched in the polyphenols, including GSE, could be viable alternatives. Peng *et al*. showed that in rats OVX at 4 weeks of age and given GSE for 10 weeks had increased spatial learning and memory compared to OVX rats (23). The rats used in Peng *et al*. were OVX at a young age (4 weeks), before puberty, and we hypothesized that OVX of post-puberty rats, to better mimic menopause, will protect against cognitive impairment induced by OVX similar to the rats OVX before puberty (23). Also, control rats (sham-OVX) will be added and tested alongside the OVX rats to determine whether GSE only attenuates OVX-induced learning and memory deficits or whether it enhances learning and memory in sham-OVX and OVX rats.

This hypothesis was tested using spontaneously hypertensive rats (SHRs) that were sham-OVX and OVX at 6 months of age, after which they were maintained on control or GSE diets. Spatial learning and memory were tested at 12 and 16 months of age using the Morris water maze (166) to determine whether GSE protects against cognitive loss in OVX SHRs.

# MATERIALS AND METHODS

## Control and GSE Supplemented Diets

Powdered GSE was provided by Kikkoman Corporation (Chiba, Japan). GSE was sent to TestDiet (Richmond, IN) where AIN-93M maintenance purified diet and GSE were mixed together to generate diets of AIN-93M supplemented with 0.5% GSE and AIN-93M supplemented with 2.0% GSE. These diets were obtained in pellet form. Rats were caged in pairs and were fed *ad libitum*.

## Animals, GSE Administration, and Sample Collection

Female SHRs were purchased from Charles River (Wilmington, MA) at 12 weeks of age. To accommodate the behavior testing of the large numbers of animals in the study, two sets of 30 animals were purchased 6 months apart. At 6 months of age 12 SHRs were sham-OVX and 18 SHRs were OVX. SHRs were put on normal rodent chow immediately upon arrival from the vendor and switched to AIN-93M diet one week before surgery. For the ovariectomy, the animal was kept on a heating pad at approximately 37$^{o}$C covered with a cloth pad to protect the animal against direct contact with heat. Using an isoflurane vaporizer the flow of oxygen was set to 2.0 and the

vacuum was set at slightly less than 10. Rats were put in a box with isoflurane to knock them out (flow of the isoflurane was 5). Rats were transferred to a nose cone and the flow of isoflurane was turned down to 2 or 3. Respiration of rats was checked and if they were breathing too fast isoflurane was increased and if they were breathing too slow isoflurane was decreased. Anesthetized rats were injected subcutaneously with Rimadyl (Carprofen) at a dose of 5 mg/kg body weight and Buprenex (Buprenorphine) at a dose of 0.01 mg/kg body weight for pain relief. After surgery if rats were noticeably uncomfortable an additional dose of Buprenex was given. Following injections with Rimadyl and Buprenex, the backs of the rats were shaved with clippers and wiped three times with chlorohexidine in a circular fashion. Eye drops were administered to keep the eyes from drying. Clear plastic wrap was placed over the rat once wiped. Autoclaved instruments were opened and sterile gloves put on. The plastic wrap was cut through with scissors. Before making any incisions the rat's foot was pinched to check if the rat would react. If the rat reacted no incisions were made and we waited until no reaction before proceeding with the ovariectomy. An incision in the shaved skin of the rat was made half way between the bump of the back of the rat and the tail. The skin was clamped to one side and an incision was made in the underlying muscle. The ovary was found and clamped with forceps. A suture was tied below the forceps three times and the ovary was cut off. If the rat bleeding did not stop immediately; gauze was applied, the fallopian tube was clamped below the original place, and sutured again. The muscle that had been cut was then sutured. The skin was then moved to other side and incisions, removal of the ovary, and suturing were repeated. The skin was than sutured and wiped with gauze wet with water. Rats were left on the heating pad until they regained

consciousness. Rats were then returned to their cages where food, sprinkled with water, was put on the bedding. A second injection of Rimadyl was given 24 h after surgery for pain. Rats were monitored every day for a week after surgery. If any problems arose UAB veterinarians were called. SHRs that underwent sham-ovariectomies were subjected to the same procedure except for the removal of the ovaries.

After surgeries were completed, 6 sham-OVX SHRs were put on a 2.0% GSE diet, 6 OVX SHRs were put on a 2.0% GSE diet, and 6 OVX SHRs were put on a 0.5% GSE diet, 6 sham-OVX SHRs and 6 OVX SHRs were maintained on the AIN-93M diet. All animals had access to food and water *ad libitum*.

After 12 months on the diets, urine was collected (at 18 months of age) over 48 h using metabolic cages and stored at -80$^{o}$C until analysis. Isoflurane and sodium pentobarbital (50 mg/kg body weight) were used to anesthetize SHRs. Between 1-2 mL of blood was taken from the heart and put on ice after which the organs were flushed with 20 mL of ice cold saline (0.9% NaCl) by intracardiac perfusion for 20 min. The brain, heart, liver, kidney, and uterus were removed from each animal, weighed, snap frozen in liquid nitrogen, and stored at -80$^{o}$C. Prior to euthanasia, approximately 100 μL of bile was collected by cannulation of the bile duct from 4 SHRs from each group. Blood was allowed to coagulate and then centrifuged at 8000 x *g* for 5 min. The serum (supernatant) was removed and stored at -80$^{o}$C until analysis. Each brain was cut sagittally and from one half of the brain the cerebellum, hippocampus, and forebrain were dissected out and snap frozen in liquid nitrogen and stored at -80$^{o}$C. The other half was put in 4% PFA

overnight, then in 30% sucrose the next night, and then stored finally in antifreeze (30% ethylene glycol) at -20$^{o}$C. All experimental procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

## Blood Pressure

Blood pressures measurements were taken twice during the study, once before surgery at 6 months and once just before rats were tested in the Morris water maze at 12 months of age, after they had been on diets for 6 months. Systolic and diastolic pressures were recorded using the MC4000 Multi Channel Blood Pressure Analysis System (Hatteras Instrument, Cary, NC) and associated software. Blood pressure was taken from the tail daily over 3-4 days as follows: SHRs were placed in a box and placed on a heating plate. The heating plate was set to 40.5$^{o}$C. SHRs were left on the heating plate for 20 min before recording blood pressure. A heating lamp was used to warm the tails of the SHRs. Each day 5 preliminary and 10 experimental recordings were recorded. The systolic blood pressure was calculated from the mean of the 10 experimental recordings.

## Morris Water Maze

SHRs were tested in the Morris water maze at 12 months of age. The Morris water maze tests for hippocampal-dependent spatial learning and memory in rodents (166). A circular pool was filled with water and contained a submerged platform in the

southwest (SW) quadrant. On each wall of the room where the pool was there were distinct pictures that the rats could use to orient themselves (Fig. 20). The object of the test is for the rat to find the submerged platform. The rats were trained for 5 days, 4 trials per day starting at 4 different positions (N, E, S, and W). Once the rat found the submerged platform it remained for 10 sec before being removed and put into a cage away from the pool for 20 sec before the next trial. If the rat did not find the submerged platform after 2 min it was shown where the platform was and left on the platform for 10 sec. The time it took to find the platform was recorded and analyzed using EthoVision 3.1 tracking software (Noldus Information Technology, Leesburg, VA). After training on the $5^{th}$ day a 60 sec probe trial was conducted. In the probe trial the platform was removed and the pool was divided into four quadrants. The target quadrant is the quadrant where the platform was located. The object of the probe trial is to determine if the rat remembers the location of the platform by the amount of time spent in the target quadrant. If the rat spends the majority of its time in the target quadrant it indicates that the rat remembered the location of the platform. SHRs were tested in a $2^{nd}$ probe trial 72 h after the initial probe trial to evaluate long-term memory. SHRs were trained for one day with the platform in the northeast (NE) quadrant 4 days after the initial probe trial. As was done previously, training consisted of 4 trials per day at different starting positions. SHRs were tested in a probe trial immediately following training to determine if they could remember the new position of the platform in the pool.

SHRs were tested in the Morris water maze again at 16 months of age. SHRs were trained for one day, 4 trials at different starting positions, with the platform in the

SW quadrant. SHRs were tested in a probe trial immediately following training to determine if they could remember the location of the platform.

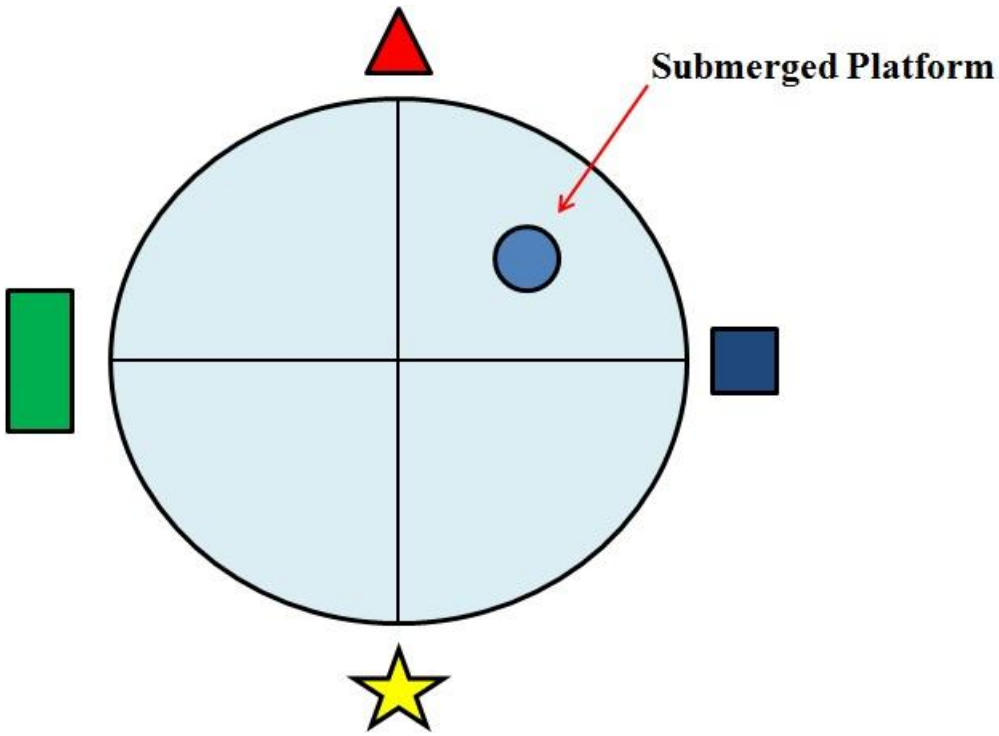



Figure 20. The Morris water maze tests spatial learning and memory in rodents. It consists of a pool of water with a submerged platform and distinct visual cues on each wall. Rats were trained for 5 days to find the submerged platform. After training the platform was removed and rats were tested to determine if they remember the location of the platform, a probe trial. The pool is divided into four quadrants and the quadrant where the platform was located is called the target quadrant. If the rat spends the majority of the time during this test in the target quadrant this indicates it remembers where the platform was located.

## Elevated Plus-Maze

SHRs were tested in the elevated plus-maze at 16 months of age. The elevated plus-maze tests for anxiety in rodents (167). This task was used to determine to what extent OVX increased anxiety in SHRs and whether dietary GSE could attenuate the effects of OVX on anxiety. SHRs were placed on an elevated platform, roughly 4 feet off the ground, with two arms that are closed and two arms that are open (Fig. 21). SHRs were left in the elevated plus-maze for 4 min. The amount of time SHRs spent in the open and closed arms was recorded and analyzed using EthoVision 3.1 tracking software. The percent time spent in the open arms was calculated. An animal that spends most of its time in the open arms indicates it is less anxious than an animal that spends most of its time in the closed arms.

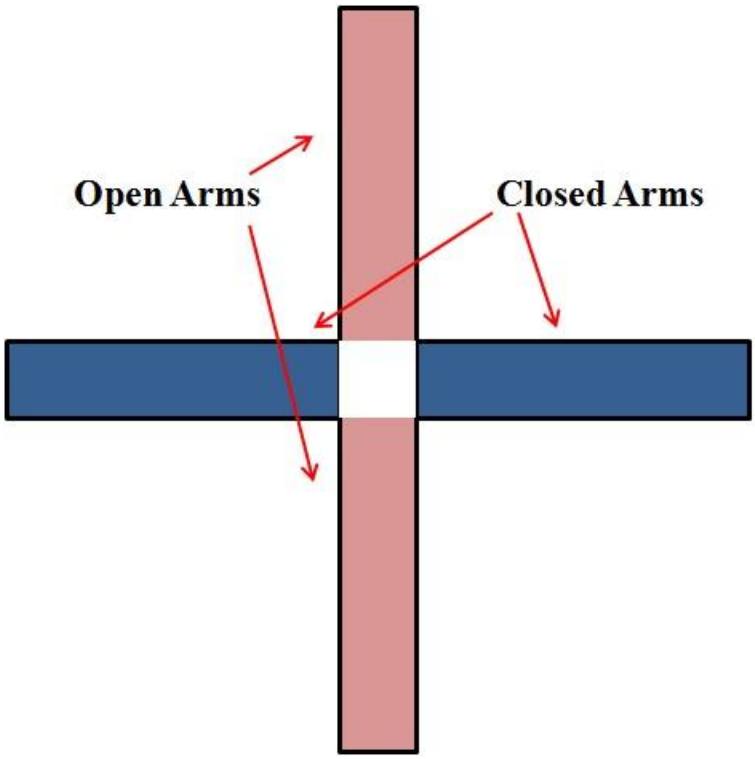



Figure 21. The elevated plus-maze tests for anxiety in rodents. It consists of two closed arms and two open arms elevated off the floor on a pedestal by 4 feet. Closed arms have walls surrounding them whereas open arms are ledges with nothing surrounding them. The test is started by putting a rat in the middle of the maze (the white part); the amount of time a rat spends in the open arms and closed arms was recorded. A rat that spends most of its time in the open arms indicates it is less anxious than a rat that spends most of its time in the closed arms.

## Statistical Analysis

Analysis of variance (ANOVA) with post-hoc tukey test using SYSTAT (Chicago, IL) was used to determine statistical differences between sham-OVX and OVX SHRs groups given control or GSE diets in the Morris water maze. ANOVA with post-hoc tukey test was used to determine statistical differences between sham-OVX and OVX SHRs groups given control or GSE diets in the elevated plus-maze. A p-value of $\leq 0.05$ was considered to be statistically significant. Data are presented as mean ± SEM.

# RESULTS

## Confirmation of Ovariectomy and Effects of GSE in SHRs

OVX rats showed an increase in body weight and a decrease in uterine weight compared to sham-operated rats, confirming ovariectomy. Body weights were measured throughout the study (Fig. 22). All SHRs had similar body weights (210 g) at 6 months of age before they were sham-OVX or OVX and maintained on control or GSE diets. OVX SHRs had significantly higher body weights (277 g) at 9 months of age compared to sham-OVX SHRs (233 g, $p<0.001$). The chronic consumption of GSE did not affect the body weight in OVX and sham-OVX SHRs. The difference in body weight between OVX SHRs and sham-OVX SHRs continued throughout the remainder of the study. Uterine weights were measured at 18 months of age when SHRs were euthanized. OVX SHRs had lower uterine weights (0.19 g OVX SHRs, 0.20 g OVX SHRs + GSE [0.5%], and 0.25 g OVX SHRs + GSE [2.0%]) than sham-OVX SHRs (0.66 g sham-OVX SHRs and 0.62 g sham-OVX SHRs + GSE [2.0%]) and consumption of GSE did not affect the uterine weight in OVX and sham-OVX SHRs (Table 3). Together these data confirm that these rats were ovariectomized and indicate that dietary GSE does not impact body weight gain or uterine weight loss in OVX rats.

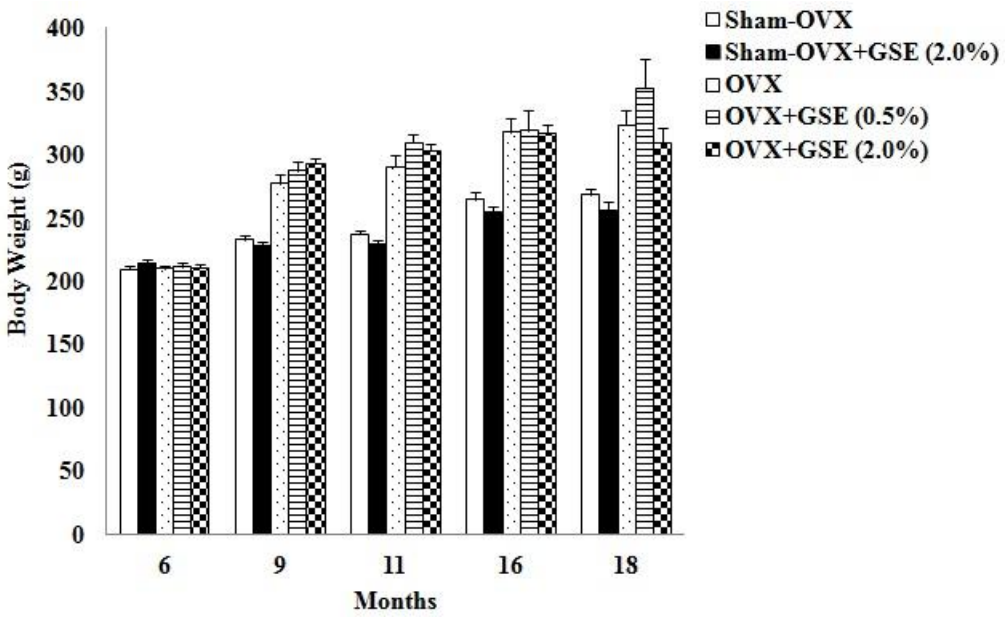



Figure 22. Body weights of sham-OVX and OVX SHRs fed control or GSE diets. SHRs were sham-OVX or OVX at 6 months of age and immediately following were put on control, 0.5% GSE, or 2.0% GSE diets. OVX (n=10), OVX+GSE (0.5%) (n=6), and OVX+GSE (2.0%) (n=11) SHRs had higher body weights than sham-OVX (n=11) and sham-OVX+GSE (2.0%) (n=10) SHRs. OVX caused an increase in body weight whereas GSE did not affect body weight. Data are expressed as the mean ± SEM.

Table 3.

*Effects of Ovariectomy and GSE on uterine weight in 18-month-old SHRs*

| Group (n) | Uterine Weight (g) |
|---|---|
| Sham-OVX (11) | 0.66 ± 0.09 |
| Sham-OVX+GSE (2.0%) (10) | 0.62 ± 0.04 |
| OVX (9) | 0.19 ± 0.05* |
| OVX+GSE (0.5%) (6) | 0.20 ± 0.05* |
| OVX+GSE (2.0%) (11) | 0.25 ± 0.04* |

* P-value < 0.05 as compared to the sham-OVX group. SHRs were sham-OVX or OVX at 6 months of age and maintained on control or GSE diets for 12 months. Data are expressed as the mean ± SEM.

Systolic blood pressure was measured at 6 months of age, before rats were sham-OVX/OVX and placed on diets, and at 12 months of age, before being tested in the Morris water maze (Table 4). Systolic blood pressure increased in all groups when measured at 12 months of age (178-201 mmHg) compared to 6 months of age (169-175 mmHg); however, there was no statistical difference in systolic blood pressure between all groups at 12 months of age ($p=0.155$). These data indicate that under our conditions ovariectomy and dietary GSE does not affect systolic blood pressure in SHRs.

Table 4.

*Effects of Ovariectomy and GSE on systolic blood pressure in 12-month-old SHRs*

| Group (n) | 6 Months Systolic Blood Pressure (mmHg) | 12 Months Systolic Blood Pressure (mmHg) |
|---|---|---|
| Sham-OVX (6) | 170 ± 6 | 201 ± 6 |
| Sham-OVX+GSE (2.0%) (5) | 172 ± 5 | 199 ± 8 |
| OVX (6) | 169 ± 6 | 178 ± 6 |
| OVX+GSE (0.5%) (6) | 175 ± 10 | 189 ± 9 |
| OVX+GSE (2.0%) (6) | 175 ± 3 | 188 ± 6 |

Systolic blood pressure was measured in SHRs at 6 months of age, before being sham-OVX or OVX, and again at 12 months of age, before being tested in the Morris water maze. Data are expressed as the mean ± SEM.

**Effects of Ovariectomy and GSE on Learning and Memory in SHRs**

To confirm that hippocampal-based learning and memory is impaired in OVX SHRs and to determine whether hippocampal-based learning and memory is enhanced in sham-OVX and OVX SHRs given GSE, all SHR groups were tested in the Morris water maze. SHRs were first tested in the Morris water maze at 12 months of age after 6 months on control or GSE diet. SHRs were trained for 5 days; each day it took less time to find the submerged platform (in the SW quadrant) for all SHR groups. Ovariectomy or GSE did not affect SHRs learning the position of the platform. There was no statistical difference in latency to the platform between the SHR groups for each day (day 1 $p=0.587$, day 2 $p=0.971$, day 3 $p=0.759$, day 4 $p=0.893$, day 5 $p=0.692$) (Fig. 23 and Table 5). These data indicate that all SHR groups learned where the submerged platform was and through successive training days took less time to find the submerged platform. At the end of training on day 5 SHRs were immediately tested in a 60 sec probe trial. All SHR groups spent the majority of their time, 23 sec, in the target quadrant (SW) with no statistical difference between SHR groups ($p=0.738$) (Fig. 24). These data indicate that all SHR groups remembered where the platform was located as they spent the majority of the 60 sec in the target quadrant. SHRs were once again tested in a probe trial 72 h after the last day of training. There was no statistical difference in the amount of time, 16-22 sec, spent in the target quadrant between SHR groups ($p=0.301$) (Fig. 25). These data indicate that 72 h after training SHRs had trouble remembering the location of the platform. SHRs underwent further training 96 h after the last day of training with the submerged platform now in the NE quadrant. Immediately following training SHRs were

tested in a 60 sec probe trial. All SHR groups spent the majority of their time, 20-25 sec, in the NE quadrant with no statistical difference between SHR groups ($p=0.513$) (Fig. 26). These data indicate that SHRs were able to learn and remember the new position of the submerged platform even after only one day of training. Together these data indicate that OVX SHRs did not have impaired learning and memory compared to sham-OVX SHRs when tested in the Morris water maze. Also, GSE did not affect learning or memory in sham-OVX or OVX SHRs.

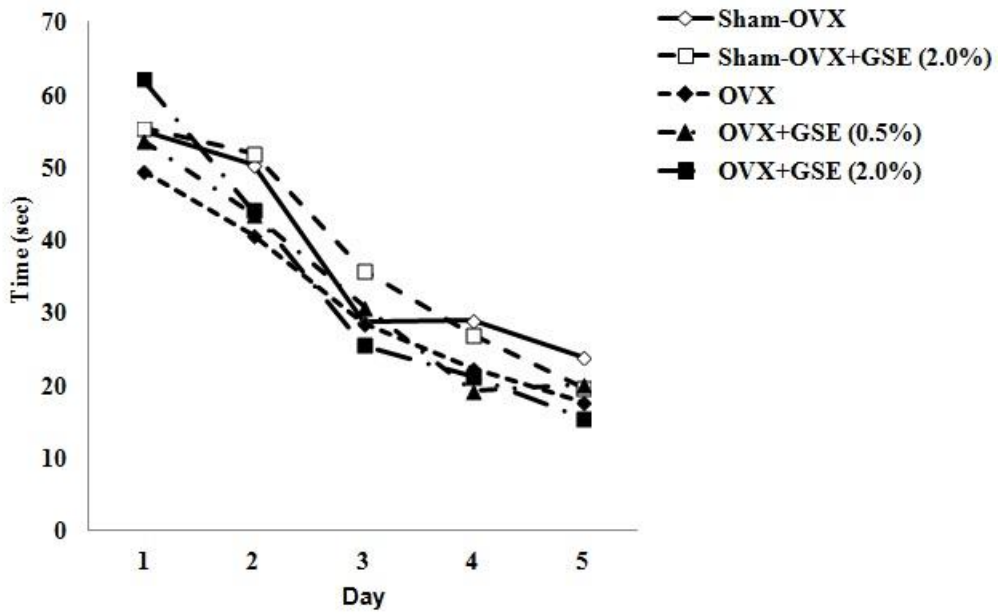



Figure 23. Ovariectomy at 6 months of age in SHRs did not impair learning the location of the submerged platform when tested by the Morris water maze in 12-month-old SHRs. GSE given in the diet for 6 months did not enhance learning the location of the submerged platform when tested by the Morris water maze in sham-OVX and OVX SHRs. All groups found the submerged platform in less time on day 5 compared to day 1 suggesting that all groups had learned the location of the submerged platform. SHRs were trained for 5 days, 4 trials per day, to find the submerged platform in the pool. Latency to platform was recorded. Data are expressed as the mean.

Table 5.

*Effects of Ovariectomy and GSE on learning the Morris water maze in 12-month-old SHRs*

| Group (n) | Day 1 Time (s) | Day 2 Time (s) | Day 3 Time (s) | Day 4 Time (s) | Day 5 Time (s) |
|---|---|---|---|---|---|
| Sham-OVX (11) | 55.1 ± 7.6 | 50.4 ± 7.7 | 28.8 ± 5.2 | 29.0 ± 4.4 | 24.0 ± 6.9 |
| Sham-OVX+GSE (2.0%) (11) | 55.5 ± 5.2 | 52.0 ± 4.9 | 35.9 ± 4.5 | 27.0 ± 6.9 | 19.8 ± 3.5 |
| OVX (10) | 49.5 ± 6.9 | 40.8 ± 4.3 | 28.5 ± 5.0 | 22.5 ± 3.5 | 17.7 ± 3.7 |
| OVX+GSE (0.5%) (12) | 53.7 ± 6.2 | 43.7 ± 6.9 | 30.9 ± 7.2 | 19.3 ± 3.0 | 20.2 ± 3.3 |
| OVX+GSE (2.0%) (12) | 62.2 ± 6.4 | 44.1 ± 7.3 | 25.5 ± 4.0 | 21.3 ± 1.9 | 15.5 ± 1.7 |

Data are expressed as the mean ± SEM.

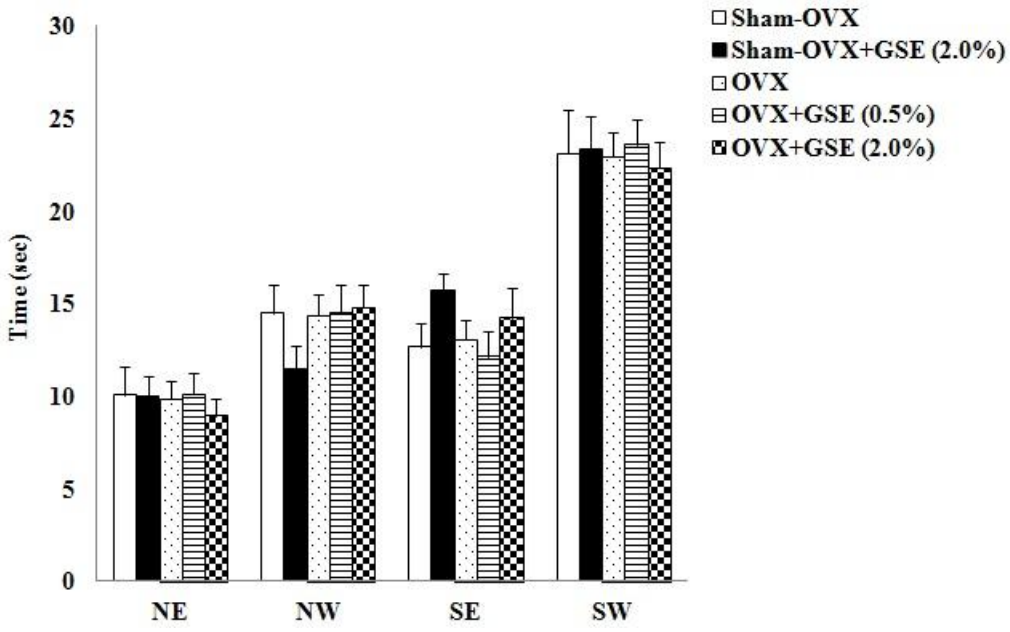



Figure 24. Ovariectomy at 6 months of age in SHRs did not impair memory of the location of the submerged platform in 12-month-old SHRs tested in a probe trial immediately after the last day of training. GSE given in the diet for 6 months did not enhance memory of the location of the submerged platform in sham-OVX and OVX SHRs. All groups spent the majority of their time in the target quadrant suggesting that all groups remembered the location of the submerged platform. SHRs were tested in a 60 sec probe trial where the target quadrant was the SW quadrant. The amount of time spent in each quadrant was recorded. Data are expressed as the mean ± SEM.

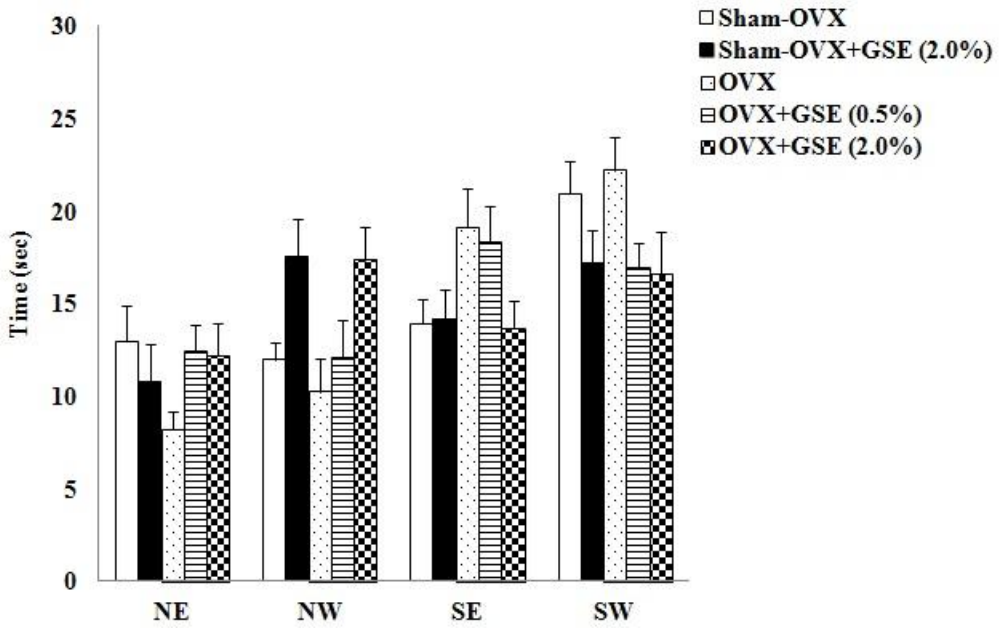



Figure 25. Ovariectomy at 6 months of age in SHRs did not impair memory of the location of the submerged platform in 12-month-old SHRs tested in a probe trial 72 h after the last day of training. GSE given in the diet for 6 months did not enhance memory of the location of the submerged platform in sham-OVX and OVX SHRs. The amount of time spent in each quadrant was similar within groups suggesting that all groups had trouble remembering the location of the submerged platform 72 h after knowing its location. SHRs were tested in a 60 sec probe trial where the target quadrant was the SW quadrant. The amount of time spent in each quadrant was recorded. Data are expressed as the mean ± SEM.

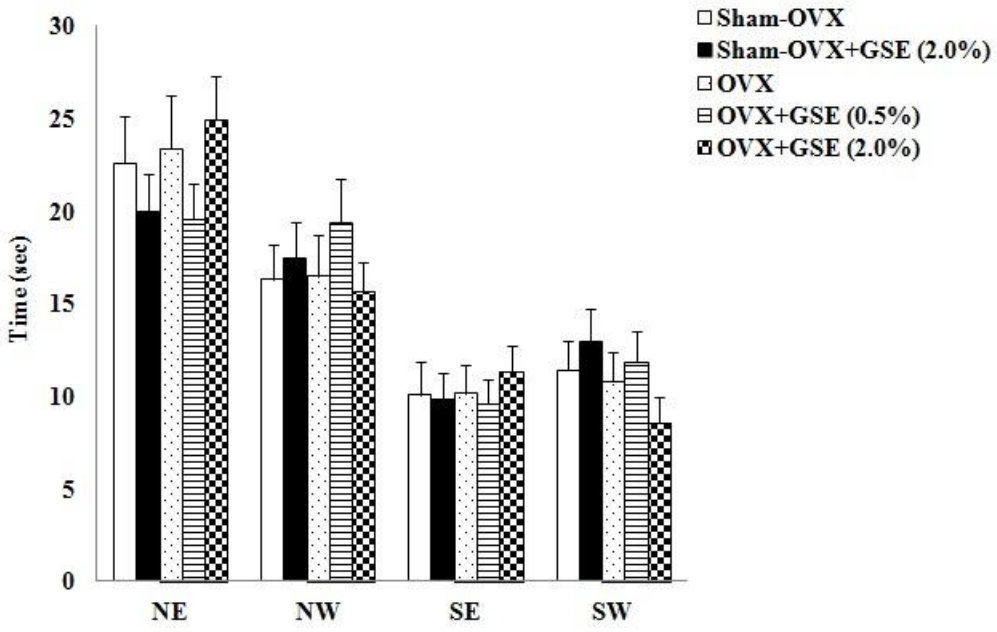



Figure 26. Ovariectomy at 6 months of age in SHRs did not impair memory of the location of the submerged platform in 12-month-old SHRs tested in a reverse probe trial after 1 day of training. GSE given in the diet for 6 months did not enhance memory of the location of the submerged platform in sham-OVX and OVX SHRs. All groups spent the majority of their time in the target quadrant suggesting that all groups learned and remembered the new location of the submerged platform. SHRs were tested in a 60 sec probe trial where the target quadrant was the NE quadrant. The amount of time spent in each quadrant was recorded. Data are expressed as the mean ± SEM.

As no difference was detected in the Morris water maze due to OVX at 12 months of age, it was decided to determine whether testing at a later time point might reveal either the anticipated deficit due to ovariectomy and/or the enhancement due to GSE on hippocampal-dependent learning and memory. The sham-OVX and OVX SHRs were maintained on their respective diets for an additional 4 months and tested in the Morris water maze at 16 months of age. SHRs were trained for one day with the platform in the SW quadrant. No statistical difference was detected during training ($p=0.337$). Immediately following training SHRs were tested in a 60 sec probe trial. There was no statistical difference in the amount of time, 16-18 sec, spent in the target quadrant ($p=0.390$); in fact the amount of time spent in each quadrant was similar across all groups with no preference for any one quadrant (Fig. 27). These data indicate that sham-OVX and OVX SHRs had not retained what they had learned during their first encounter with the Morris water maze at 12 months of age and that more than one day of training at 16 months of age was required for SHRs to re-learn the concept of finding the submerged platform.

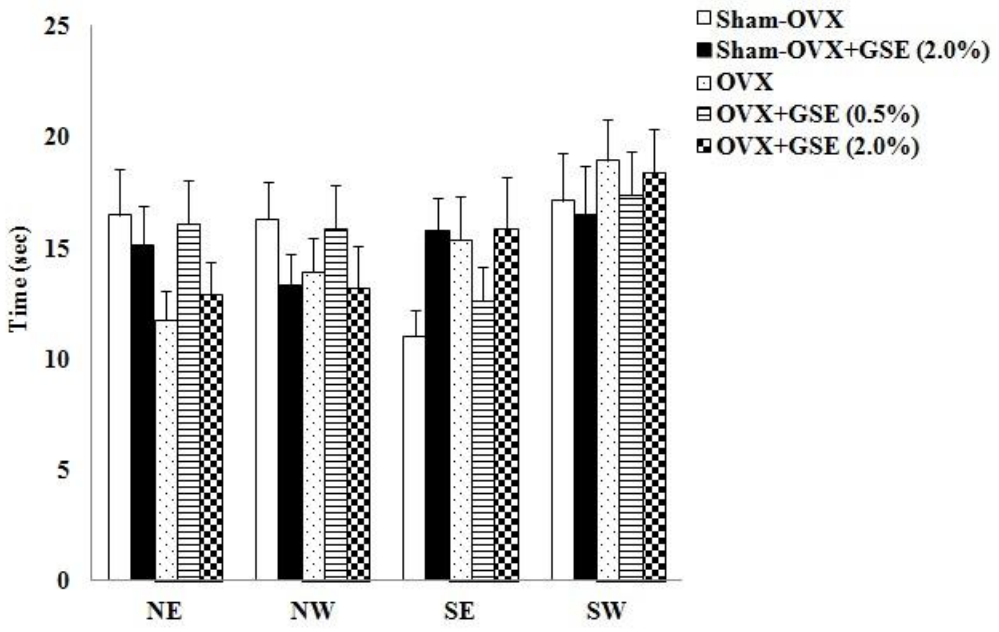



Figure 27. Ovariectomy at 6 months of age in SHRs did not impair memory of the location of the submerged platform in 16-month-old SHRs tested in a probe trial immediately after 1 day of training. GSE given in the diet for 10 months did not enhance memory of the location of the submerged platform in sham-OVX and OVX SHRs. The amount of time spent in each quadrant was similar within groups suggesting that all groups did not learn the location of the submerged after 1 day of training 4 months after initial testing in the Morris water maze. SHRs were tested in a 60 sec probe trial where the target quadrant was the SW quadrant. The amount of time spent in each quadrant was recorded. Data are expressed as the mean ± SEM.

As no learning or memory differences were detected in the Morris water maze at 12 and 16 months of age in sham-OVX and OVX SHRs, it was decided to test whether ovariectomy and/or dietary GSE had affects on anxiety. The anxiety of 16-month-old sham-OVX and OVX SHRs given control or GSE diets was tested using the elevated plus-maze. There was no statistical difference in the amount of time spent in the open arms ($p=0.744$) and the closed arms ($0=0.751$). All SHR groups spent less than 50% of the time in the open arms (Fig. 28). These data indicate that ovariectomy and dietary GSE do not affect anxiety of SHRs.

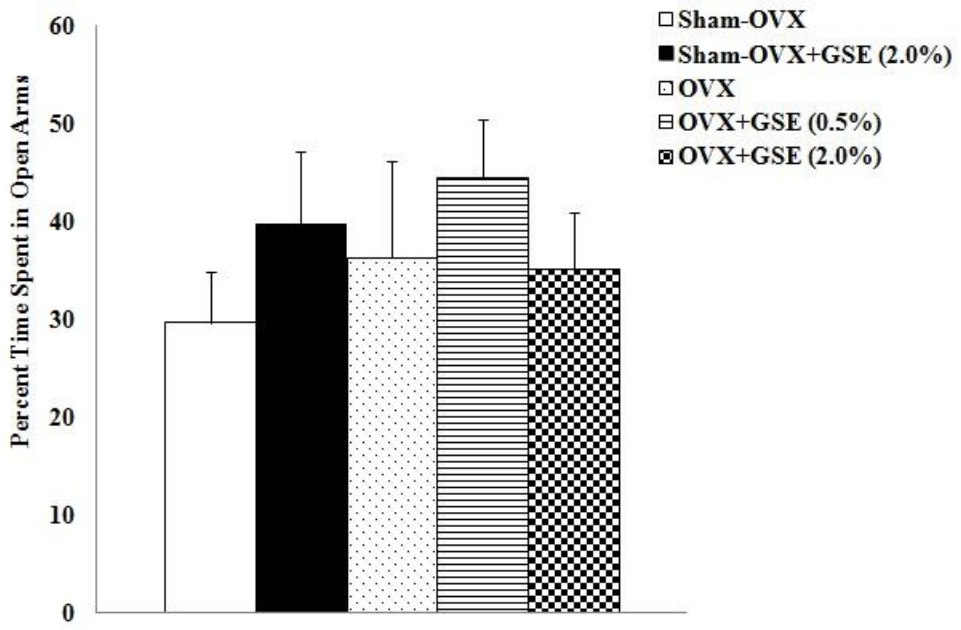



Figure 28. Ovariectomy at 6 months of age in SHRs did not affect anxiety in 16-month-old SHRs tested by the elevated plus-maze. GSE given in the diet for 10 months did not affect anxiety in sham-OVX and OVX SHRs. No difference in the percent of time spent in the open arm indicates that ovariectomy and GSE do not affect anxiety in 16-months-old SHRs. SHRs were tested in the elevated plus-maze for 4 min. The amount of time spent in the open and closed arms was recorded. The percent time spent in the open arm is shown above. Data are expressed as the mean ± SEM.

## DISCUSSION

In the present study, we showed that under the conditions of ovariectomy, which was done at 6 months of age, there was no impairment in learning or memory in SHRs when tested in the Morris water maze at 12 months of age and then again at 16 months of age. The chronic consumption of GSE from 6 months of age and on did not enhance learning or memory in either the sham-OVX or OVX SHRs when tested in the Morris water maze at 12 and 16 months of age. This was contrary to our hypothesis that ovariectomy would lead to impairment in learning and/or memory in rats and that GSE would attenuate this impairment in OVX rats if not also enhance learning and/or memory in sham-OVX rats.

When OVX SHRs were tested in the Morris water maze at 12 months of age it was expected to detect impairment in learning and/or memory compared to sham-OVX SHRs. This was not the case as OVX SHRs learned the location of the submerged platform and remembered its location as well as the sham-OVX SHRs. This was unexpected as others have shown that ovariectomy impaired learning and/or memory in rodents (98, 102-104). Talboom *et al*. showed that Fischer-344 rats OVX at 2 months of age and tested in the Morris water maze at 4 months of age swam further to find the submerged platform compared to sham-OVX rats during 5 days of training (105)

indicating OVX rats had impaired learning. When OVX rats were given 17β-estradiol it improved their learning to that of sham-OVX rats. When tested in a probe trial after training on day 5 there was no difference in the amount of time spent in the target quadrant between sham-OVX and OVX rats. Both sham-OVX and OVX rats spent more time in the target quadrant compared to the opposite quadrant suggesting they remembered equally well the location of the submerged platform. Talboom *et al*. showed that rats OVX at 14 months of age and tested in the Morris water maze at 16 months of age no longer had a statistical difference in the distance swum compared to sham-OVX rats. When these 14-month-old rats were tested in a probe trial there was also no difference in the amount of time spent in the target quadrant between sham-OVX and OVX rats and like the 4-month-old rats spent more time in the target quadrant compared to the opposite quadrant suggesting they had learned and remember the location of the submerged platform. Overall learning and memory was slightly decreased in the 16-month-old rats compared to the 4-month-old rats. Our rats were tested at 12 months of age in the Morris water maze and no difference in learning or memory were detected which mirrors the older rats in these studies done by Talboom *et al*. which suggests that our rats were too old to detect a learning impairment. Talboom *et al*. did show a non-statistical decrease in memory of OVX rats that were OVX at 22 months of age and tested in the Morris water maze at 24 months of age which suggests our rats were too young to detect memory impairment. Overall the 24-month-old rats spent less time in the target quadrant during the probe trial compared to the 4-month-old rats but the 24-month-old rats given 17β-estradiol had a statistically improved memory where their memory was similar to that of the 4-month-old rats. Compared with the findings of Talboom *et al*., it

appears that the age we selected, 6 months to OVX, to induce impaired learning and memory may not have been ideal as it was the rats OVX at 2 and 22 months of age and tested at 4 and 24 months of age respectively showed impaired learning and memory.

We selected to OVX at 6 months as others have done this to test for learning and memory impairment. Sarkaki *et al*. showed that Wistar rats that were OVX at 5 months of age and tested in the Morris water maze 6 weeks later had impaired learning and memory compared to rats with intact ovaries (97). Horvath *et al*. showed that Wistar rats that were OVX at 6 months of age and 3 weeks after OVX given 17β-estradiol for 2 weeks had improved learning of the two-way active shock avoidance task compared to OVX rats (168). Sarkaki *et al*. showed that Wistar rats that were OVX at 5-6 months of age, given a soy isoflavone diet for 4 weeks, and given lesions in the substantia nigra pars compacta by 6-hydroxydopamine (a model of postmenopausal Parkinson's disease) had improved learning and memory in the Morris water maze compared to OVX rats not on the soy isoflavone diet and similar learning and memory to rats with intact ovaries (169). These studies show that others have OVX rats at 6 months of age and detected impairment in learning and memory and enhancement when given 17β-estradiol or a soy isoflavone diet.

We also selected to test 6 months after OVX as Lowry *et al*. did with Long Evans hooded rats that were OVX at 12-13 months of age and given 17β-estradiol, 17β-estradiol plus progesterone, or 17β-estradiol plus medroxyprogesterone acetate for 6 months and then tested in the Morris water maze (170). The OVX rats given 17β-estradiol in Lowry

*et al*. had improved learning on the fourth day of training and they did spend more time in the target quadrant during the probe trial although this was not a statistical significant more than OVX rats. This study shows another group that treated their OVX rats for 6 months before testing for improved learning and memory. Taken together it may be that OVX at a younger or older age, from the 6 months we selected, causes more of a change in learning or memory and that SHRs are resilient to the removal of hormones at 6 months of age in regards to learning and memory performance.

In another study by Monterio *et al*., Wistar rats that were OVX at 80 days of age and tested in the Morris water maze 30 days later did take longer to find the submerged platform but only on the fifth day of training (102). When tested in the probe trial 24 h after the last day of training OVX rats spent less time in the target quadrant compared to sham-OVX rats. Monterio *et al*. showed furthermore that when OVX rats were given soy isoflavones in the diet their impaired learning and memory was attenuated. No improvement in learning and memory in older OVX rats given GSE may be due to those catechins in GSE not being estrogenic like soy isoflavones (171, 172). Therefore, if the improvement of learning and memory in older OVX rats is dependent on estrogen pathways GSE may not be able to improve learning and memory but until this has been tested in older OVX rats with impaired learning and memory it is difficult to conclude that GSE has no benefits in improving learning and memory in an older rodent model of menopause.

After we had completed our experiments Patki *et al*. showed that dietary intake of grape powder, made from whole grape which contains other flavonoids such as resveratrol and quercetin, improved short-term but not long-term memory in OVX Wistar rats when tested in the radial arm water maze (173). There was no behavioral effect of grape powder on sham-OVX rats; therefore, grape powder only attenuated the cognitive impairment induced by OVX. Patki *et al*. also tested for LTP in the DG and found that OVX rats had lower LTP compared to sham-OVX rats and that OVX rats given grape powder had improved LTP compared to OVX rats and the enhanced LTP did not exceed the amplitude of the LTP measured for the sham-OVX rats. Consistent with the behavior study, there was no effect of grape powder on LTP in the DG from sham-OVX rats; thus, grape powder attenuated the OVX induced impairment of the perforant pathway synapses in the DG. Although the study by Patki *et al*. did show differences in short-term memory and LTP as a consequence of OVX and that grape powder enhanced short-term memory and LTP in OVX rats there were several differences between our studies including: differences in what part of the grape was given (grape powder vs. GSE), how we tested for learning and memory (radial arm water maze vs. Morris water maze), the time of treatment (3 weeks vs. 6 months), and age differences (Patki *et al*. did not indicate the age or weight of their rats). The biggest difference between the studies was the use of whole grape powder instead of GSE. The study by Patki *et al*. suggests that grape powder, comprised of more diverse flavonoids than GSE, is also beneficial in preventing cognitive loss due to ovariectomy and could be useful in future studies with SHRs. Although our data described here were inconclusive, it remains a valid hypothesis that GSE can improve learning and memory in a rodent model of menopause since Peng *et al*.

showed that GSE given to young OVX rats improved their learning and memory (23). Also, lowered estrogen is a risk factor for AD (35-38) and GSE has been shown to have benefit in a transgenic mouse model of AD (25, 28).

Marcondes *et al*. showed that Wistar rats in the diestrus phase (low levels of 17β-estradiol) had higher anxiety than when they were in the proestrus phase (high levels of 17β-estradiol) when tested in the elevated plus-maze (174). When these rats were given 17β-estradiol during the diestrus phase their anxiety was reduced to that of when they were in the proestrus phase. These data suggest that levels of 17β-estradiol modulate anxiety in rats. Marcondes *et al*. also showed that OVX rats were slightly more anxious than proestrus rats and less anxious than diestrus rats but these differences were not statistical. In this regard, the study by Marcondes *et al*. corresponds with our study where we did not detect a difference in anxiety between sham-OVX and OVX rats when tested in the elevated plus-maze. However, it should be kept in mind that we did not specify what phase in the estrous cycle our sham-OVX rats were in when tested in the Morris water maze and the elevated plus-maze.

Previous animal studies showed benefits of GSE in learning and memory using a transgenic mouse model of AD (25, 28), in a rat model of cerebral hypoperfusion/ischemia (72), and in young OVX hypertensive rats (23). Xu and Zhang showed that ICR mice OVX at 8 weeks of age had impaired learning in the Morris water maze that was restored when OVX mice were given estradiol benzoate for 4-5 weeks (175). Whereas GSE and estrogen may restore learning and/or memory in diseased or

compromised animals, we found no impairment in learning or memory in our OVX rats relative to sham-OVX rats and therefore found no enhancement in learning or memory when GSE was given to either sham-OVX or OVX rats. No differences were detected in learning, memory, anxiety, or blood pressure in our OVX rats which raises the possibility that either through differences in experimentation or because of the exact ages at which we implemented OVX and tested for behavioral differences suggest that OVX in these SHRs did not sufficiently compromise them in terms of learning and memory.

We addressed whether surgical ovariectomy in our study was successful by measuring differences in body and uterine weights. OVX SHRs had higher body weights compared with sham-OVX SHRs, consistent with the weight gain previously shown to be a consequence of ovariectomy (99, 176). OVX SHRs had lower uterine weights compared with sham-OVX SHRs, consistent with the lower uterine weight previously shown to be a consequence of ovariectomy (176). Thus we confirmed ovariectomy, in spite of the lack of measured difference in cognitive function induced by ovariectomy. We noted that the administration of GSE did not affect body or uterine weights in sham-OVX or OVX SHRs which is consistent with Goodin *et al*. who found no difference in uterine weight in mice treated with EGCG, epicatechin gallate, and epigallocatechin (177). This is of interest as this indicates that GSE does not have estrogenic actions on reproductive tissues, which confirms the safety of GSE as an estrogen alternative.

In conclusion, we determined that SHRs that underwent ovariectomy at 6 months of age did not have impaired learning or memory when tested in the Morris water maze at

12 months of age compared with SHRs that underwent sham-OVX. Our findings also indicate that GSE does not enhance learning or memory in sham-OVX or in OVX SHRs. Several rationales were discussed as to why the OVX in our study did not induce cognitive impairment. These studies underscore the importance of a pre-experiment to determine whether the experimental model, OVX of SHRs at the selected age, is going to be an appropriate model for testing a dietary intervention such as GSE on OVX-induced cognitive impairment. The results also underscore the importance of determining a robust baseline for the sham-OVX learning and memory data, against which an incremental change in learning and memory might be detected. Finally, the results underscore the importance of doing a positive control, in this case the addition of estrogen, such as 17β-estradiol, to each group. In our study we do not know whether the sham-OVX group itself was cognitively impaired in some way. If we had an OVX group to which we had administered 17β-estradiol, presumably that would have attenuated OVX-induced cognitive impairment, which would have given a measure of robustness to our study that was lacking.

# CHAPTER 4

# CATECHIN METABOLISM IN A RAT MODEL OF MENOPAUSE

## INTRODUCTION

GSE is a dietary supplement that has demonstrated health benefits in mammalian models of diseases including cancers (18-22), cardiovascular diseases (23, 24), and neurodegenerative diseases (25, 26). GSE comprises of catechin and epicatechin monomers and oligomers. Although these oligomers make up roughly 90% of GSE they are poorly absorbed in the small intestine (152, 153). Catechin and epicatechin comprise only 4-8% of GSE by weight (17), nonetheless they, and/or their metabolites, are thought to be the bioactive components of GSE (28-30). Catechin and epicatechin are absorbed in the small intestine and are metabolized in the small intestine and the liver to methylated, glucuronidated, and sulfonated forms (139, 143, 145, 146). The methylated forms of catechin and epicatechin can also be glucuronidated or sulfonated. Catechin, epicatechin, and their metabolites have been detected in the blood, urine, bile, and brain after ingestion of catechin, epicatechin, or GSE (28, 144-150).

Circulating steroidal hormones play an important role in regulating enzymes that metabolize endogenous and exogenous compounds. As catechin and epicatechin are

known substrates for phase II metabolism by COMT, UGTs, and SULTs (138-143) it is important to understand whether their metabolism changes with changes in hormone levels such as at menopause when the ovaries cease to produce hormones. Progesterone induces both COMT (127, 128) and UGT1A1 (132); whereas 17β-estradiol down-regulates COMT (130). Tamoxifen, an estrogen antagonist, increases the activity and expression of COMT (131). 17β-Estradiol reduces mRNA levels of UGT1A1 (133) and reduces the activity of UGT1A1 and UGT1A9 (134). 17β-Estradiol along with dihydrotestosterone reduces the activity of the UGTs that are involved in the glucuronidation of dihydrotestosterone and androsterone (135). Thus hormones produced in the ovaries modulate either expression and/or activity of enzymes involved in catechin metabolism.

Although beneficial actions of GSE polyphenols have been reported in healthy rat brain (27), in a transgenic mouse models of AD (25), and in a young rat model of menopause (23), it is unknown whether the metabolism of these polyphenols are affected due to low levels of circulating estrogen. We hypothesized that lower levels of circulating estrogens caused by menopause/ovariectomy increase methylation and/or glucuronidation of catechins. Experiments were carried out to determine whether ovariectomy increased the metabolism of catechin and epicatechin. This was determined by giving OVX SHRs, a rodent model of menopause, GSE and measuring catechin, epicatechin, and their metabolites in the urine and the serum.

## MATERIALS AND METHODS

### Standards and Diet

(+)-Catechin, (-)-epicatechin, and apigenin were purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ), 3'-*O*-methyl epicatechin from Nacalai (San Diego, CA), *Helix pomatia* β-glucuronidase, 4-methylumbelliferyl sulfate, and phenolphthalein β-D-glucuronide from Sigma-Aldrich (St. Louis, MO). Powdered GSE was provided by Kikkoman Corporation (Chiba, Japan). AIN-93M diet was purchased from TestDiet (Richmond, IN). Sham-OVX and OVX rats were housed with three rats per cage and were fed *ad libitum* AIN-93M.

### Animals, GSE Administration, and Sample Collection

Female SHRs were purchased from Charles River (Wilmington, MA) at 12 weeks of age. At 13 weeks of age SHRs were taken off the normal rodent chow and put onto AIN-93M diet. They were maintained on this latter diet for the remainder of the study. At 14 weeks of age half (n=12) of the SHRs underwent bilateral OVX and the other half (n=12) sham-OVX; each group was allowed to post-operatively recover for 3 weeks.

At 17 weeks of age SHRs were split into 4 dietary groups: sham-OVX+vehicle (n=6), sham-OVX+GSE (n=6), OVX+vehicle (n=6), and OVX+GSE (n=6). SHRs were gavaged once daily with 0.5 mL of either vehicle (water) or GSE dissolved in water (300 mg/kg body weight) for 6 days. Urines samples were collected over the 24 h after gavage on day 4 and stored at -80$^{o}$C until analysis. SHRs were fasted for 16 hours before being euthanized on day 6, 1 h after rats were gavaged with vehicle or GSE. SHRs were anesthetized and decapitated. Blood was collected from the trunk and immediately put on ice and allowed to coagulate. Serum was collected after centrifugation at 8000 x *g* for 5 minutes and stored at -80$^{o}$C until analysis. The uterus was removed, weighed, snap frozen in liquid nitrogen, and stored at -80$^{o}$C. All experimental procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

**Extraction of Monomeric and Oligomeric Catechins from GSE**

In preparation for analysis of by mass spectrometry, powdered GSE (0.2 g) was mixed with 5 mL of 80% aqueous methanol for 2 h. The mixture was then centrifuged at 200 x *g* for 15 min. The supernatant was removed and centrifuged at 16,000 x *g* for 15 min. A portion of the supernatant (10 μL) was removed and diluted 100 fold in 80% aqueous methanol and used for analysis of catechin, epicatechin, proanthocyanidin dimers, and proanthocyanidin trimers.

## Extraction of Catechins from Urine

In preparation for analysis by mass spectrometry, urine samples (100 μL) were incubated for 1 h at 37°C with or without 300 U of *H. pomatia* β-glucuronidase/sulfatase, after mixing with 300 mM ammonium acetate, pH 5 (50 μL), acetic acid (5 μL), internal standards (6 μL; 0.6 nmole each of 4-methylumbelliferyl sulfate, phenolphthalein β-D-glucuronide, and apigenin) and water to a total volume of 200 μL. After incubation, samples were treated with ice-cold methanol: 0.5% acetic acid (400 μL) and the precipitated material were removed by centrifugation at 16,000 x *g* for 15 min at 4°C. The supernatant was used for analysis of catechin, epicatechin, and their metabolites.

## Extraction of Catechins from Serum

In preparation for analysis by mass spectrometry, serum samples (200 μL) were incubated for 1 h at 37°C with or without 300 U of *H. pomatia* β-glucuronidase/sulfatase, after mixing with 300 mM ammonium acetate, pH 5 (50 μL), acetic acid (5 μL), and internal standards (5 μL; 0.5 nmole each of 4-methylumbelliferyl sulfate, phenolphthalein β-D-glucuronide, and apigenin). After incubation, samples were treated with ice-cold methanol: 0.1% formic acid (800 μL) and the precipitated material were removed by centrifugation at 16,000 x *g* for 15 min at 4°C. The supernatant was dried and reconstituted in 100 μL of 80% aqueous methanol and used for analysis of catechin, epicatechin, and their metabolites.

**Analysis of Catechin, Epicatechin, and their Metabolites by Liquid Chromatography-Tandem Mass Spectrometry**

LC-MS/MS was carried out on GSE, urine, and serum extracts to analyze catechin, epicatechin, their metabolites, proanthocyanidin dimers, and proanthocyanidin trimers as described by Prasain *et al*. (148) using a model SIL-HT refrigerated Shimadzu autosampler, a Prominence HPLC (Shimadzu Scientific Instruments, Columbia, MD) and an API 4000 triple quadrupole mass spectrometer (AB Sciex, Concord, Ontario, Canada). Aliquots (10 μL) of GSE, urine, and serum extracts were injected onto a Phenomenex (Torrance, CA) Synergy Hydro-RP 80R $C_{18}$ column (250 x 2.0 mm i.d. 4 μm particle size) and peaks were eluted by reverse-phase LC using a 15 min gradient at a flow rate of 0.2 mL/min. Mobile phase A consisted of 0.1% formic acid and mobile phase B consisted of 0.1% formic acid in methanol. The column was pre-equilibrated with 90% A and 10% B. The gradient applied was from 0 to 7 min, 10% to 60% B; from 7 to 8 min, 60% to 100% B; from 8 to 9 min, 100% B; from 9 to 10 min, 100% to 10% B; and re-equilibration from 10 to 15 min in 10% B. Analyst 1.4.2 software (AB Sciex) was used for collection and analysis of data.

MS/MS was carried out on catechin and epicatechin standards and corresponding peaks in GSE, urine, and serum extracts. MS/MS spectra were recorded in positive ion mode for catechin and epicatechin (*m/z* 291) and their *O*-methyl ethers (*m/z* 305). MS/MS spectra for the glucuronides and sulfates of these flavanols were recorded in negative ion mode at *m/z* 369 (catechin/epicatechin sulfates), *m/z* 383 (methyl

catechin/epicatechin sulfates), *m/z* 465 (catechin/epicatechin glucuronides), and *m/z* 479 (methyl catechin/epicatechin glucuronides). MS/MS spectra were recorded in negative ion mode at *m/z* 577 (proanthocyanidin dimers) and *m/z* 577 (proanthocyanidin trimers). Using these product ion spectra, MRM was used to detect and quantitate catechin, epicatechin, their metabolites, proanthocyanidin dimers, and proanthocyanidin trimers.

MRM analysis in positive ion mode was used to quantify catechin and epicatechin using the mass transition *m/z* 291/139, and methyl catechin and methyl epicatechin using the mass transition *m/z* 305/139. MRM analysis in negative ion mode was used to detect the glucuronides of catechin and epicatechin using the mass transition *m/z* 465/289 and of methyl catechin and epicatechin using the mass transition *m/z* 479/303. The sulfates of catechin and epicatechin using the mass transition *m/z* 369/289 and of methyl catechin and epicatechin using the mass transition *m/z* 383/303 were also assessed using MRM in negative ion mode. MRM analysis in negative ion mode was used to detect proanthocyanidin dimers and proanthocyanidin trimers using the mass transitions *m/z* 577/289 and *m/z* 865/577, respectively.

Catechin, epicatechin, and 3'-*O*-methyl epicatechin standards were spiked into control urine and serum samples (from sham-OVX SHRs on control diet or gavaged with vehicle), before extraction to generate standard curves. The standard curve for 3'-*O*-methyl epicatechin was used for quantification of 3'-*O*-methyl catechin. The peak that eluted 1 min before 3'-*O*-methyl epicatechin was designated as 3'-*O*-methyl catechin. The standard curves in urine and 80% methanol for catechin and epicatechin used

concentrations of 5, 1, 0.5, 0.1, and 0.05 μg/ml and for 3'-*O*-methyl epicatechin concentrations of 1, 0.5, 0.1, and 0.05 μg/ml. The standard curves in serum for catechin and epicatechin used concentrations of 1, 0.5, 0.1, 0.05, 0.01, and 0.005 μg/ml and for 3'-*O*-methyl epicatechin concentrations of 1, 0.5, 0.1, 0.05, 0.01 and 0.005 μg/ml. Correlation coefficients for each of the standard curves for catechin, epicatechin, and 3'-*O*-methyl epicatechin were > 0.99.

## Urine Creatinine Analysis

Creatinine concentrations were measured for each urine sample using an isotope dilution procedure with $^{2}H_{3}$-creatinine by LC-MS (178). Urinary outputs of catechin, epicatechin, and their metabolites were corrected for creatinine output and were expressed as μg/mg creatinine.

## Statistical Analysis

A two-sided equal variance Student's t-test was used to determine statistical differences in the amount or concentration of catechin, epicatechin, and their metabolites in urine and serum of 18-week-old and 18-month-old sham-OVX and OVX SHRs given control or GSE diet. A p-value of $\leq 0.05$ was considered to be statistically significant. Data are presented as mean ± SEM.

# RESULTS

## Confirmation of Catechin Monomers and Oligomers in GSE

Catechin and epicatechin are epimers with the same molecular weight of 290 Da; as such, when analyzed by MS in positive ion mode, they have the same *m/z* of 291. Because of this MS is unable to distinguish between the two and LC is necessary to separate catechin and epicatechin before entering the mass spectrometer. Initially catechin and epicatechin were run separately to determine their retention times ($t_R$). Catechin elutes first, roughly 1 min before epicatechin. LC-MS/MS was performed on catechin (5 μg/mL) and epicatechin (5 μg/mL) standards with catechin eluting at $t_R$ 9.97 min and epicatechin eluting at $t_R$ 10.93 min (Fig. 29A). The product ions, *m/z* 123, 139, 147, and 165, from the two peaks were consistent with catechin (Fig. 29B) and epicatechin (Fig. 29C) product ions. Using the precursor ion *m/z* 291 and the most abundant product ion *m/z* 139, the mass transition of *m/z* 291/139 was created for the selective detection of catechin and epicatechin by MRM.

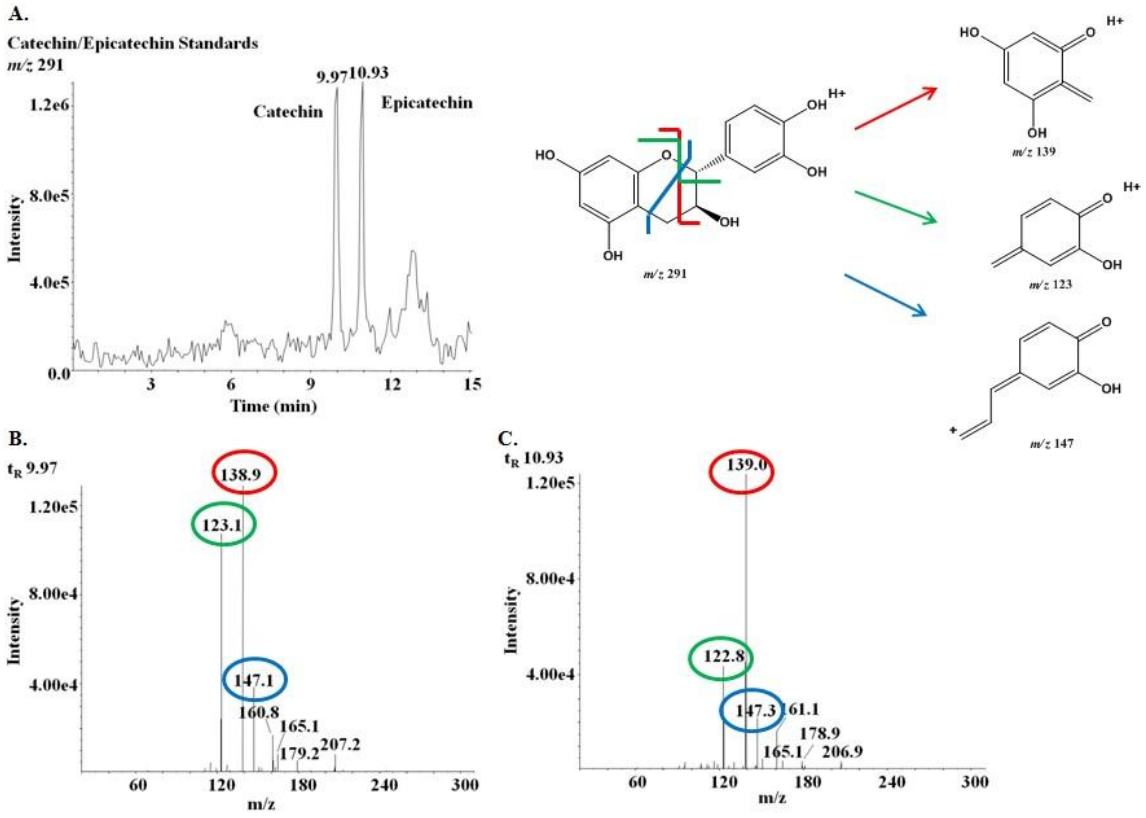



Figure 29. LC-MS/MS of catechin and epicatechin standards in positive ion mode. Extracted ion chromatogram (*m/z* 291) of (A) catechin ($t_R$ 9.97 min, 5 µg/mL) and epicatechin ($t_R$ 10.93 min, 5 µg/mL) standards. Product ion mass spectra of the peaks at (B) $t_R$ 9.97 min (catechin) and (C) $t_R$ 10.93 min (epicatechin). The most abundant product ions are *m/z* 139, *m/z* 123, and *m/z* 147.

To confirm the presence of catechin monomers and oligomers in the GSE that was given to the sham-OVX and OVX SHRs the composition of GSE was analyzed by LC-MRM MS. Catechin (10 μg/mL) and epicatechin (10 μg/mL) standards eluted at $t_R$ 9.39 min and $t_R$ 10.39 min, respectively, with mass transition *m/z* 291/139 (Fig. 30A). GSE had two peaks elute at $t_R$ 9.37 min and $t_R$ 10.38 min with mass transition *m/z* 291/139 (Fig. 30B) confirming the presence of catechin and epicatechin in GSE. GSE had multiple peaks elute between $t_R$ 8-11 min with mass transitions *m/z* 577/289 (Fig. 30C) and *m/z* 865/577 (Fig 30D) which correspond to catechin dimers and trimers, respectively, confirming their presence in GSE. Multiple peaks were detected using the mass transitions for catechin dimers and trimers which represent the composition of catechin and epicatechin in the dimers and trimers. Each batch of GSE from Kikkoman Cooperation contains slightly different composition of catechin and epicatechin and it was necessary to quantify the amount of catechin and epicatechin in the GSE given to the rats in these studies. Quantification of 1 g of GSE had 16 mg of catechin and 20 mg of epicatechin (Fig. 30E), indicating that catechin and epicatechin comprised 3.6% of the GSE.

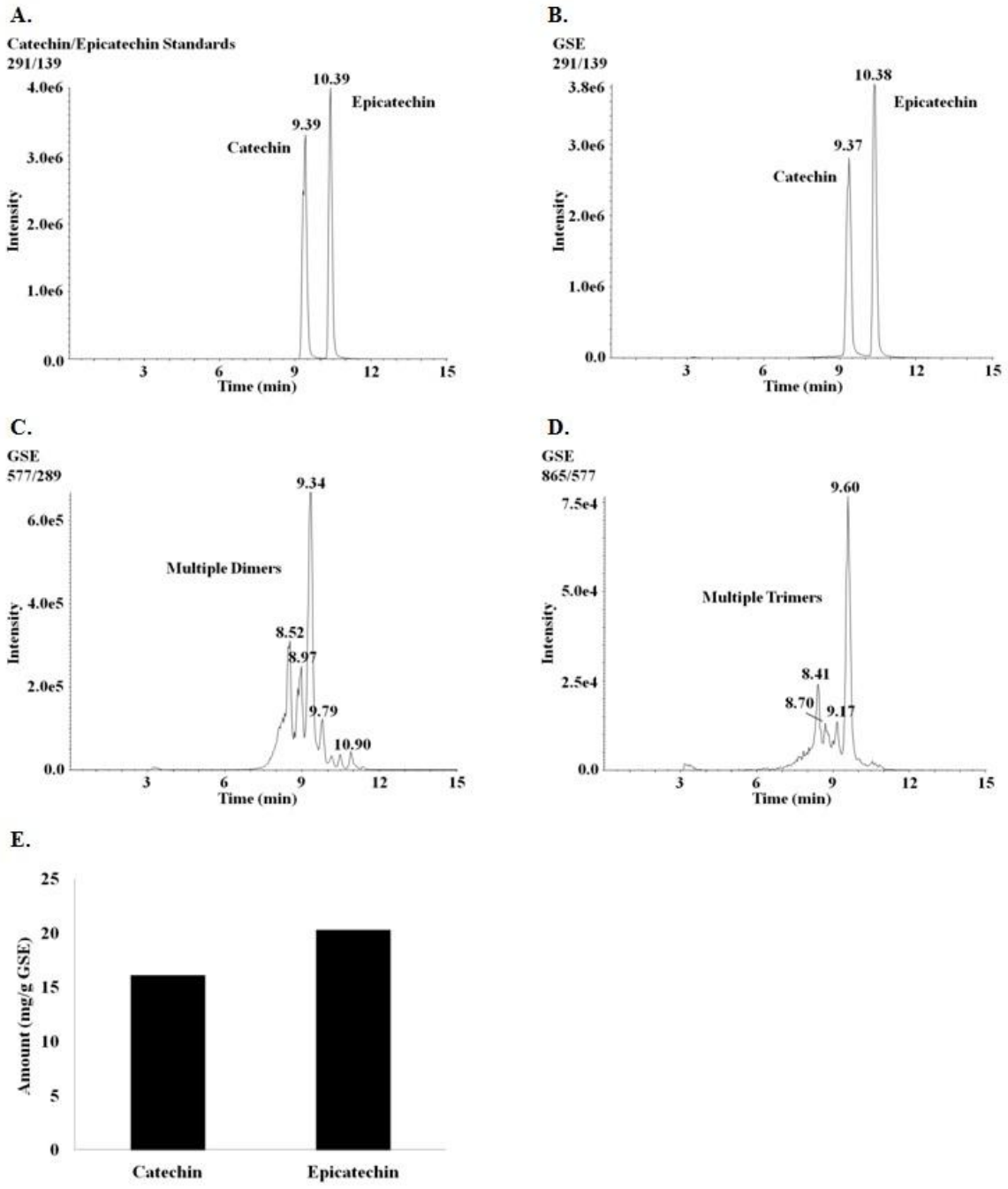



Figure 30. Confirmation of catechin monomers and oligomers in the GSE used in these studies. MRM ion chromatogram of (A) catechin ($t_R$ 9.39 min, 10 µg/mL) and epicatechin ($t_R$ 10.39 min, 10 µg/mL) standards using the mass transition *m/z* 291/139 in positive ion mode. MRM ion chromatogram of GSE using the mass transitions of (B) *m/z* 291/139 (catechin and epicatechin) in positive ion mode. MRM ion chromatograms of GSE using the mass transitions (C) *m/z* 577/289 (catechin dimers) and (D) *m/z* 865/577 (catechin trimers) in negative ion mode. (E) Quantification of catechin and epicatechin in 1 g of GSE.

## Confirmation of Catechin, Epicatechin, and their Metabolites in Urine of Rats given GSE

Urine samples were collected from sham-OVX and OVX SHRs at 18 months of age. SHRs had been sham-OVX or OVX at 6 months of age and immediately following surgery were put on a 2.0% GSE diet for 12 months. Urine samples were analyzed by LC-MS/MS to confirm the presence of catechin, epicatechin, methyl catechin/epicatechin, catechin/epicatechin glucuronide, methyl catechin/epicatechin glucuronide, catechin/epicatechin sulfate, and methyl catechin/epicatechin sulfate. The presence of catechin and epicatechin was confirmed by LC-MS/MS of *m/z* 291 in positive ion mode. The product ions *m/z* 123, 139, 147, and 165 were consistent with those detected with catechin and epicatechin standards (Fig. 31). The presence of methyl catechin/epicatechin (289 [catechin/epicatechin] + 14 [methyl]) was confirmed by LC-MS/MS of *m/z* 303 in negative ion mode. The precursor ion *m/z* 303 and the product ion *m/z* 137 were consistent with methyl catechin/epicatechin (Fig. 32). The peaks at $t_R$ 11.00, $t_R$ 11.59, and $t_R$ 11.85 all contained similar precursor and product ions. The presence of catechin/epicatechin glucuronide (289 [catechin/epicatechin] + 176 [glucuronic acid]) was confirmed by LC-MS/MS of *m/z* 465 in negative ion mode. The precursor ion *m/z* 465 and the product ions *m/z* 289, 137, and 145 were consistent with catechin/epicatechin glucuronide (Fig. 33). The presence of methyl catechin/epicatechin glucuronide (289 [catechin/epicatechin] + 14 [methyl] + 176 [glucuronic acid]) was confirmed by LC-MS/MS of *m/z* 479 in negative ion mode. The precursor ion *m/z* 479 and the product ions *m/z* 303 and 137 were consistent with methyl catechin/epicatechin

glucuronide (Fig. 34). The presence of catechin/epicatechin sulfate (289 [catechin/epicatechin] + 80 [sulfonic acid]) was confirmed by LC-MS/MS of *m/z* 369 in negative ion mode. The precursor ion *m/z* 369 and the product ions of *m/z* 289 and 137 were consistent with catechin/epicatechin sulfate (Fig. 35). The presence of methyl catechin/epicatechin sulfate (289 [catechin/epicatechin] + 14 [methyl] + 80 [sulfonic acid]) was confirmed by LC-MS/MS of *m/z* 383 in negative ion mode. The precursor ion *m/z* 383 and the product ions of *m/z* 303 and 137 were consistent with methyl catechin/epicatechin sulfate (Fig. 36). These data indicate that urine samples from sham-OVX and OVX SHRs given a 2.0% GSE diet contained catechin, epicatechin, methyl catechin/epicatechin, catechin/epicatechin glucuronide, methyl catechin/epicatechin glucuronide, catechin/epicatechin sulfate, and methyl catechin/epicatechin sulfate.

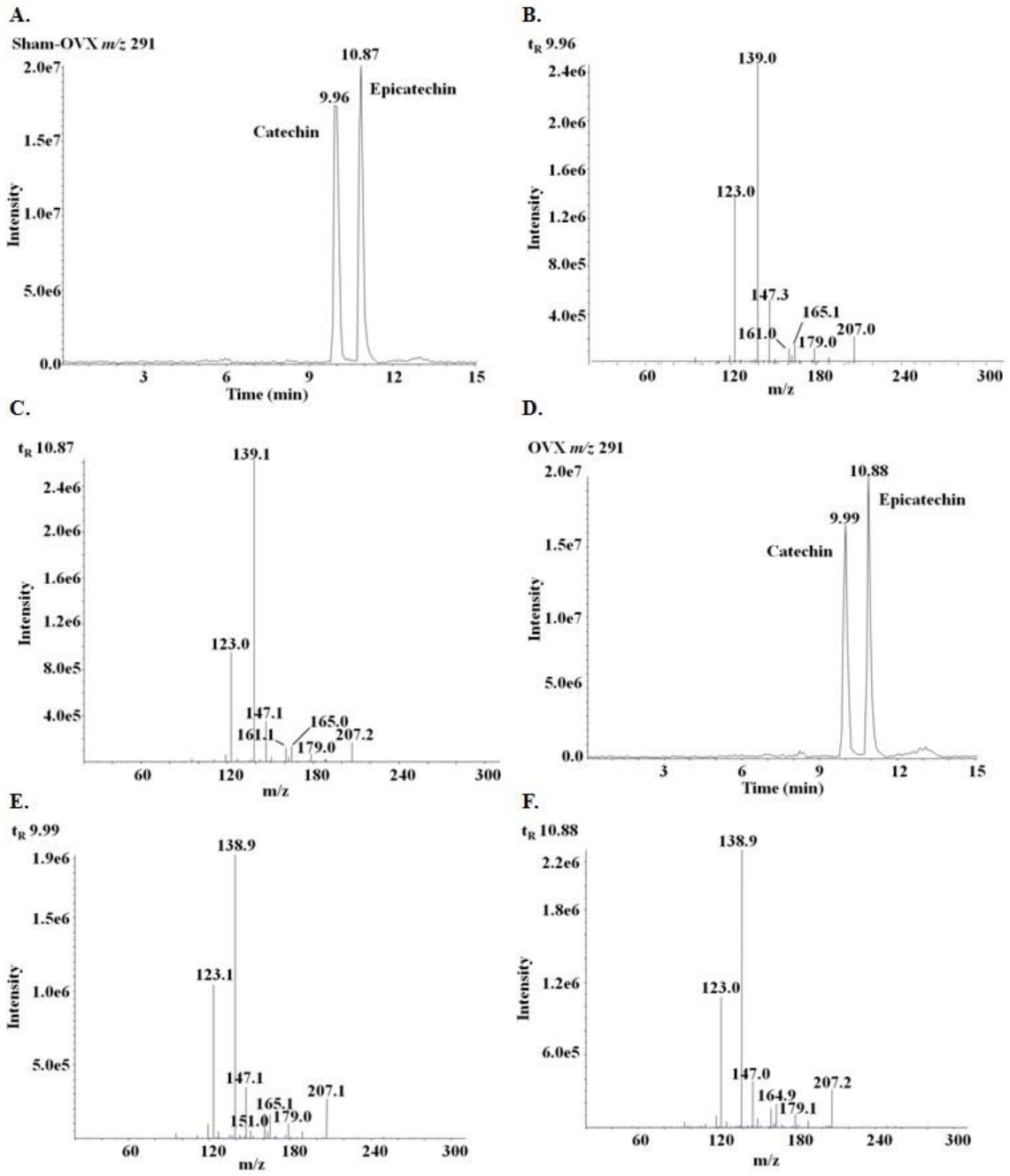



Figure 31. Catechin and epicatechin are present in the urine of 18-month-old sham-OVX and OVX SHRs given a 2.0% GSE diet. Extracted ion chromatogram (*m/z* 291) of a urine sample from a (A) sham-OVX SHR in positive ion mode. Product ion mass spectra of the peaks at (B) $t_R$ 9.96 min (catechin) and (C) $t_R$ 10.87 min (epicatechin). Extracted ion chromatogram (*m/z* 291) of a urine sample from an (D) OVX SHR in positive ion mode. Product ion mass spectra of the peaks at (E) $t_R$ 9.99 min (catechin) and (F) $t_R$ 10.88 min (epicatechin).

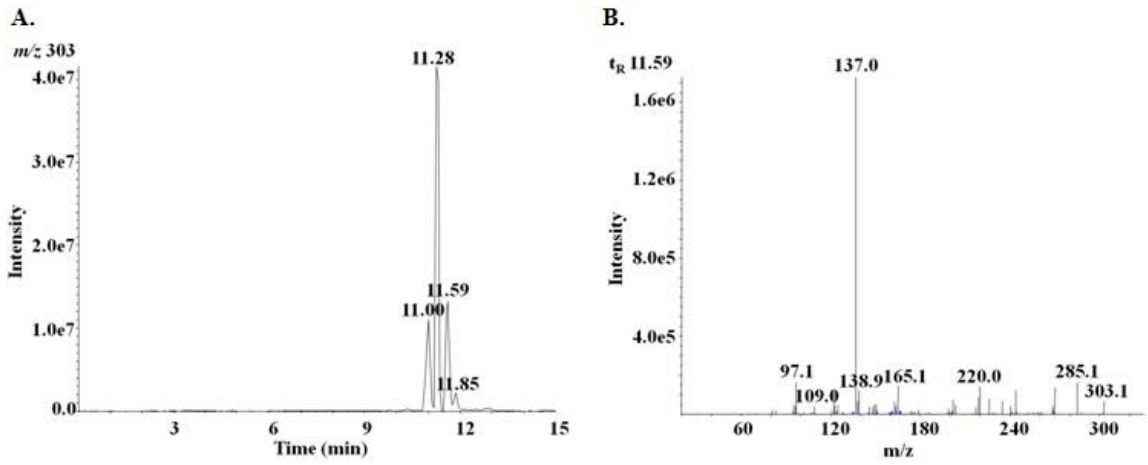



Figure 32. Methylated catechin and epicatechin are present in the urine of 18-month-old sham-OVX and OVX SHRs given a 2.0% GSE diet. Extracted ion chromatogram (*m/z* 303) of a urine sample from a (A) sham-OVX SHR in negative ion mode. Product ion mass spectra of the peak at (B) $t_R$ 11.59 min. Peaks at $t_R$ 11.00 min and $t_R$ 11.85 min had similar product ion mass spectra as the peak at $t_R$ 11.59 min.

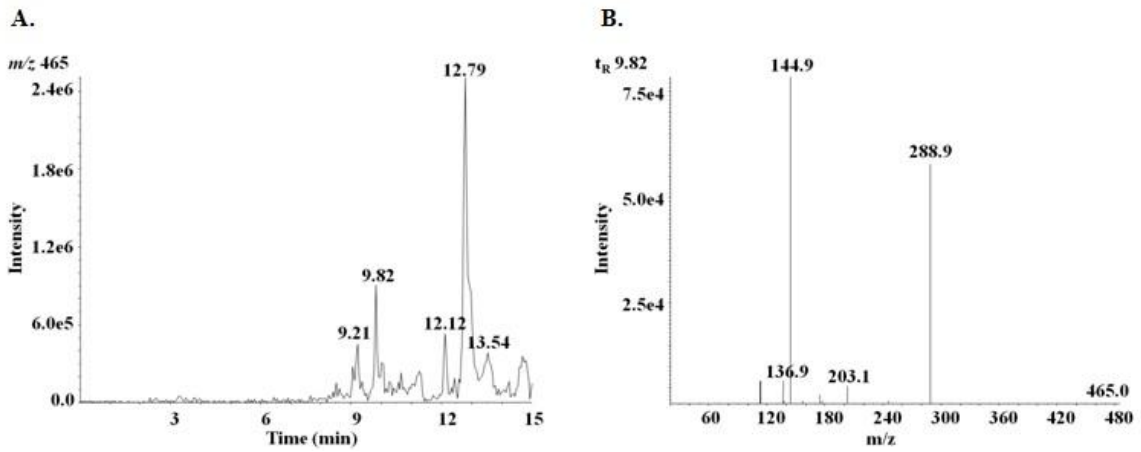



Figure 33. Glucuronidated catechin and epicatechin are present in the urine of 18-month-old sham-OVX and OVX SHRs given a 2.0% GSE diet. Extracted ion chromatogram (*m/z* 465) of a urine sample from a (A) sham-OVX SHR in negative ion mode. Product ion mass spectra of the peak at (B) $t_R$ 9.82 min.

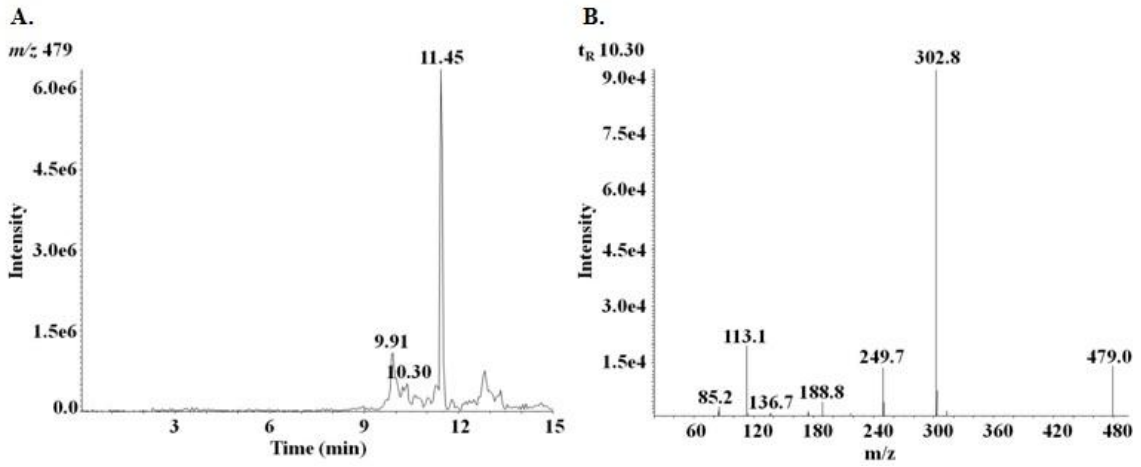



Figure 34. Methylated and glucuronidated catechin and epicatechin are present in the urine of 18-month-old sham-OVX and OVX SHRs given a 2.0% GSE diet. Extracted ion chromatogram (*m/z* 479) of a urine sample from a (A) sham-OVX SHR in negative ion mode. Product ion mass spectra of the peak at (B) $t_R$ 10.30 min.

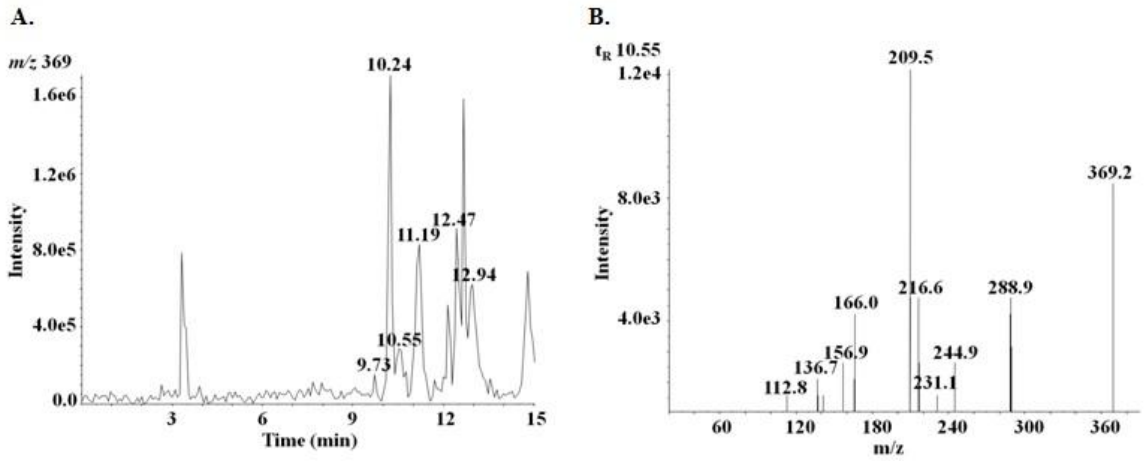



Figure 35. Sulfonated catechin and epicatechin are present in the urine of 18-month-old sham-OVX and OVX SHRs given a 2.0% GSE diet. Extracted ion chromatogram (*m/z* 369) of a urine sample from a (A) sham-OVX SHR in negative ion mode. Product ion mass spectra of the peak at (B) $t_R$ 10.55 min.

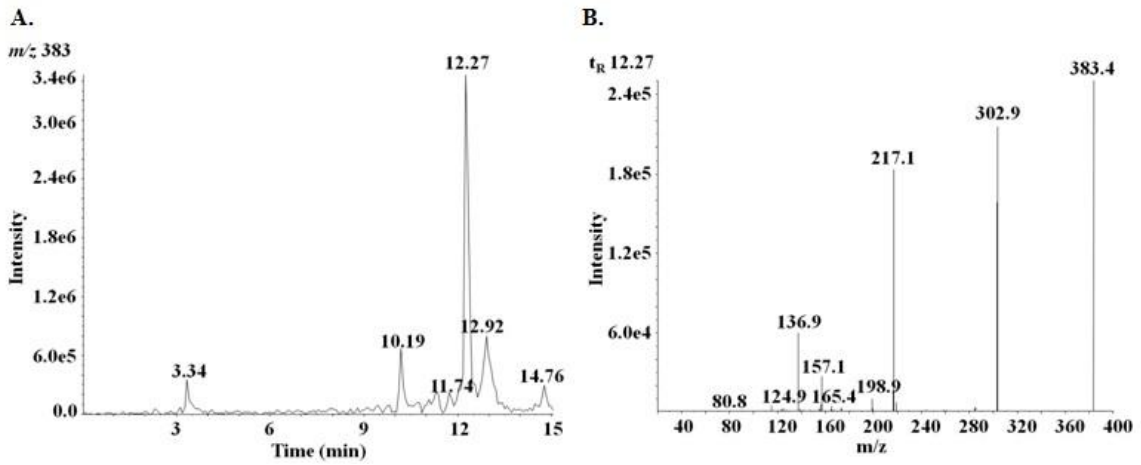



Figure 36. Methylated and sulfonated catechin and epicatechin are present in the urine of 18-month-old sham-OVX and OVX SHRs given a 2.0% GSE diet. Extracted ion chromatogram (*m/z* 383) of a urine sample from a (A) sham-OVX SHR in negative ion mode. Product ion mass spectra of the peak at (B) $t_R$ 12.27 min.

**Effects of Ovariectomy on Urinary Catechin, Epicatechin, and their Metabolites in 18-month-old SHRs**

Urine samples from 18-month-old sham-OVX (n=10) and OVX (n=11) SHRs given a 2.0% GSE diet were analyzed by LC-MRM MS for catechin, epicatechin, and their methylated metabolites. The amount of free catechin and epicatechin in the urine was determined by LC-MRM MS. Free catechin/epicatechin represents catechin/epicatechin that is not methylated and/or conjugated with glucuronic acid or sulfonic acid (Fig. 37). Urine samples from OVX+GSE SHRs contained 276 ± 87 μg of free catechin and 851 ± 300 μg of free epicatechin, whereas urine samples from sham-OVX+GSE SHRs contained 289 ± 108 μg of free catechin (p=0.929 compared to OVX+GSE SHRs) and 848 ± 395 μg of free epicatechin (p=0.995 compared to OVX+GSE SHRs) (Fig. 38A). Incubation with β-glucuronidase/sulfatase before extraction was done to determine the amount of total catechin and epicatechin, the amount of catechin and epicatechin plus their glucuronidated and sulfonated forms, in the urine. It was thought that β-glucuronidase/sulfatase (*H. pomatia*) would remove glucuronides and sulfates from catechin and epicatechin but as is shown later in this dissertation the preparation of β-glucuronidase/sulfatase used in these studies only removed the glucuronides and not the sulfates; therefore, total catechin represents catechin and catechin glucuronides (Fig. 37). Urine samples from OVX+GSE SHRs contained 418 ± 110 μg of total catechin and 1262 ± 382 μg of total epicatechin, whereas urine samples from sham-OVX+GSE SHRs contained 604 ± 185 μg of total catechin

(p=0.388 compared to OVX+GSE SHRs) and 1648 ± 540 μg of total epicatechin (p=0.561 compared to OVX+GSE SHRs) (Fig. 38C).

As catechin and epicatechin are known to be methylated, the amount of free methyl catechin and methyl epicatechin in the urine was also determined by LC-MRM MS. Free 3'-*O*-methyl catechin/epicatechin represents 3'-*O*-methyl catechin/epicatechin that is not conjugated with glucuronic acid or sulfonic acid (Fig. 37). Urine samples from OVX+GSE SHRs contained 290 ± 90 μg of free 3'-*O*-methyl catechin and 256 ± 82 μg of free 3'-*O*-methyl epicatechin, whereas urine samples from sham-OVX+GSE SHRs contained 226 ± 81 μg of free 3'-*O*-methyl catechin (p=0.605 compared to OVX+GSE SHRs) and 237 ± 90 μg of free 3'-*O*-methyl epicatechin (p=0.878 compared to OVX+GSE SHRs) (Fig. 38B). Incubation with β-glucuronidase/sulfatase before extraction was done to determine the amount of total 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin, the amount of 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin plus their glucuronidated forms, in the urine (Fig. 37). Urine samples from OVX+GSE SHRs contained 381 ± 94 μg of total 3'-*O*-methyl catechin and 368 ± 96 μg of total 3'-*O*-methyl epicatechin, whereas urine samples from sham-OVX+GSE SHRs contained 438 ± 134 μg of total 3'-*O*-methyl catechin (p=0.732 compared to OVX+GSE SHRs) and 463 ± 144 μg of total 3'-*O*-methyl epicatechin (p=0.582 compared to OVX+GSE SHRs) (Fig. 38D). OVX+GSE SHRs excreted roughly 20 mL of urine over 48 h whereas sham-OVX+GSE SHRs excreted roughly 26 mL of urine over 24 h (p=0.091). These data suggest that there is no difference in the amounts of free or total catechin, epicatechin, 3'-*O*-methyl catechin, 3'-*O*-methyl epicatechin, and their glucuronidated forms between

sham-OVX and OVX SHRs given GSE. However, the intake of food for these animals was not recorded; therefore, the intake of GSE was unknown. To understand whether ovariectomy leads to changes in catechin metabolism experiments where sham-OVX and OVX SHRs were given a known amount of GSE each day were performed.

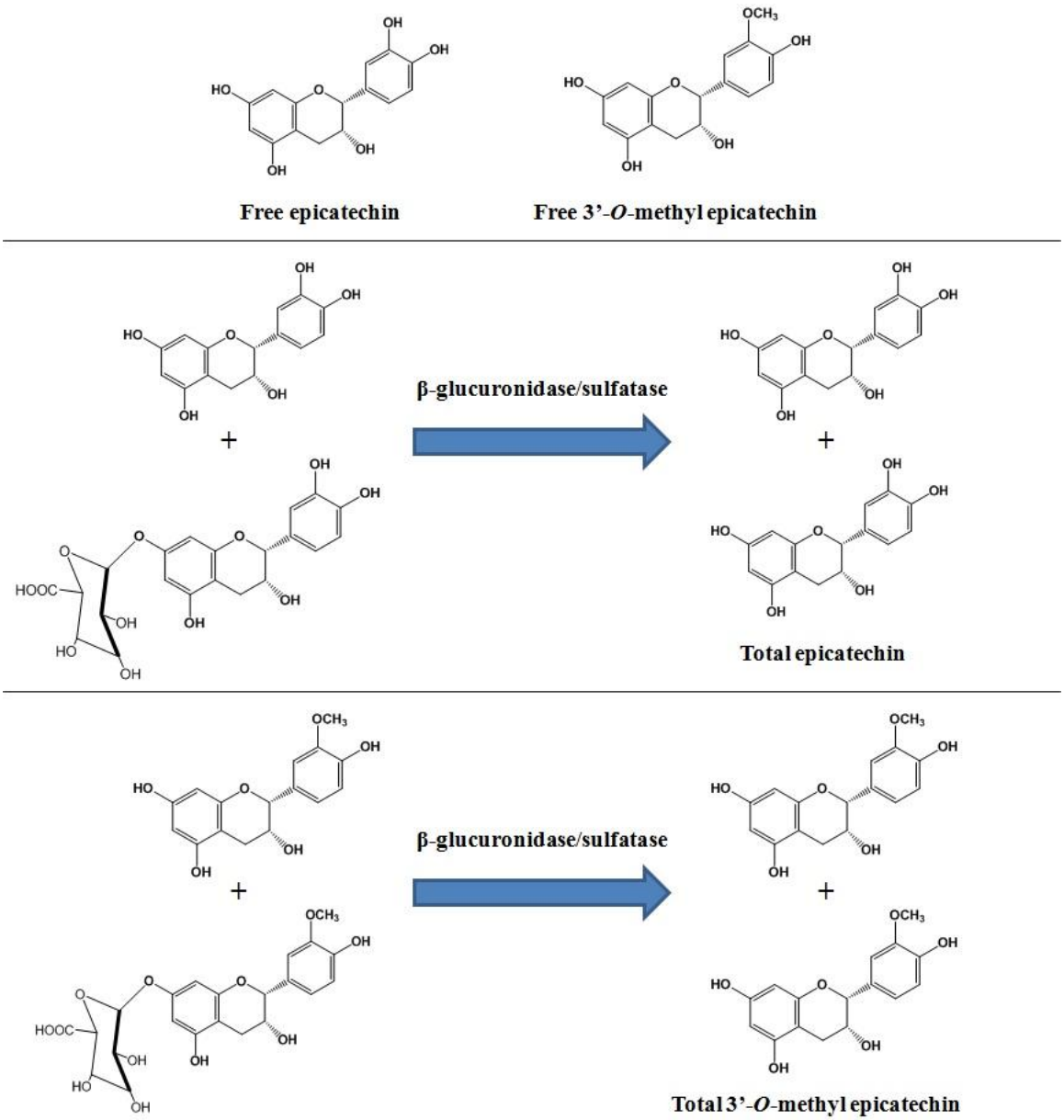



Figure 37. Determining the amount of free and total catechin/epicatechin. Free catechin/epicatechin represents catechin/epicatechin that is not methylated and/or conjugated with glucuronic acid or sulfonic acid. Free 3'-*O*-methyl catechin/epicatechin represents 3'-*O*-methyl catechin/epicatechin that is not conjugated with glucuronic acid or sulfonic acid. Total catechin/epicatechin represents catechin, epicatechin, and their glucuronidated forms. Total 3'-*O*-methyl catechin/epicatechin represents 3'-*O*-methyl catechin, 3'-*O*-methyl epicatechin, and their glucuronidated forms.

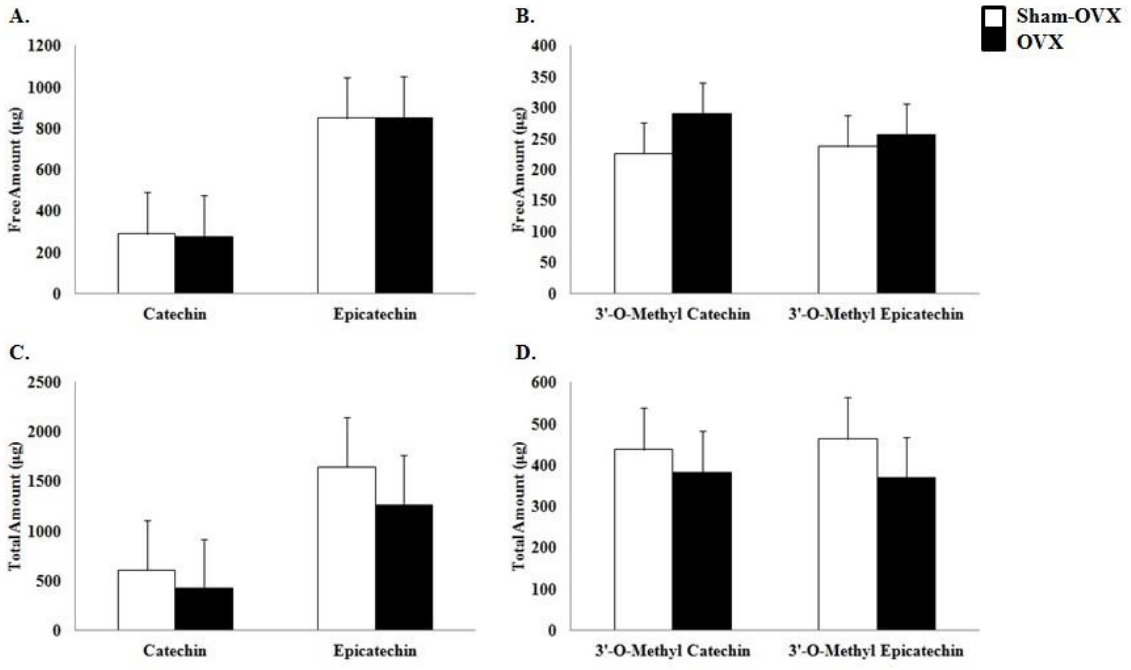



Figure 38. No difference in levels of GSE-derived urinary catechin, epicatechin, 3'-*O*-methyl catechin, 3'-*O*-methyl epicatechin, and their glucuronidated forms in OVX SHRs vs. sham-OVX SHRs. SHRs were sham-OVX or OVX at 6 months of age and given a 2.0% GSE diet for 12 months. The amount of (A) free and (C) total catechin and epicatechin in the urine of 18-month-old sham-OVX (n=10) and OVX (n=11) SHRs given a 2.0% GSE diet. The amount of (B) free and (D) total 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin in the urine of 18-month-old sham-OVX and OVX SHRs given a 2.0% GSE diet. Free catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin represents catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin that are not conjugated with glucuronic acid or sulfonic acid. Total catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin represents catechin, epicatechin, 3'-*O*-methyl catechin, 3'-*O*-methyl epicatechin, and their glucuronidated forms. White bars represent sham-OVX SHRs and black bars represent OVX SHRs. Data are expressed as the mean ± SEM.

## Confirmation of Ovariectomy and Effects of GSE in SHRs

Similar to the 18-month-old OVX SHRs, the 18-week-old OVX SHRs showed an increase in body weight and a decrease in uterine weight compared to sham-operated SHRs, confirming ovariectomy. Body weights were measured at 17 weeks of age, 3 weeks after surgery and before rats were gavaged with either GSE or vehicle. OVX SHRs (237 g OVX SHRs + vehicle and 241 g OVX SHRs + GSE) had significantly higher body weights compared to sham-OVX SHRs (203 g OVX SHRs + vehicle and 201 g OVX SHRs + GSE) (Table 6). OVX SHRs continued to have higher body weights at 18 weeks of age compared to sham-OVX SHRs. All SHRs whether given GSE or vehicle had lower body weights at 18 weeks of age compared to 17 weeks of age (Table 6). Uterine weights were measured at 18 weeks of age when SHRs were euthanized. OVX SHRs had lower uterine weights (0.10 g OVX SHRs + vehicle and 0.10 g OVX SHRs + GSE) than sham-OVX SHRs (0.56 g sham-OVX SHRs + vehicle and 0.53 g sham-OVX SHRs + GSE) and GSE did not affect uterine weight in OVX and sham-OVX SHRs (Table 6). Together these data confirm that these rats were ovariectomized and indicate that GSE may attenuate body weight gain but does not affect uterine weight loss in OVX rats.

*Table 6.*

*Effects of Ovariectomy and GSE on body and uterine weight in SHRs*

| Group (n) | Week 17 Body Weight (g) | Week 18 Body Weight (g) | Week 18 Uterine Weight (g) |
|---|---|---|---|
| Sham-OVX+vehicle (5) | 203 ± 1 | 194 ± 2 | 0.56 ± 0.06 |
| Sham-OVX+GSE (4) | 201 ± 2 | 181 ± 6* | 0.53 ± 0.10 |
| OVX+vehicle (4) | 237 ± 2* | 231 ± 1* | 0.10 ± 0.00* |
| OVX+GSE (3) | 241 ± 3* | 217 ± 1* | 0.10 ± 0.00* |

* P-value < 0.05 as compared to the sham-OVX group. SHRs were sham-OVX or OVX at 14 weeks of age. GSE (300 mg/kg) or vehicle was given once daily for six days starting at 17 weeks of age. Data are expressed as the mean ± SEM.

**Effects of Ovariectomy on the Glucuronidation and Sulfation of Catechin, Epicatechin, and their Methylated Metabolites in 18-week-old SHRs**

To determine differences in urinary glucuronidated and sulfonated forms of catechin, epicatechin, 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin between 18-week-old sham-OVX (n=4) and OVX (n=4) SHRs given GSE urine samples were analyzed by LC-MRM MS. Urine samples incubated without β-glucuronidase/sulfatase (monitored with the mass transition *m/z* 465/289) had two dominant peaks elute at $t_R$ 8.23 min and $t_R$ 8.93 min and another smaller peak at $t_R$ 9.85 min (Fig. 39A), confirming the presence of catechin and epicatechin glucuronides in the urine. Urine samples incubated without β-glucuronidase/sulfatase (monitored with the mass transition *m/z* 479/303) had two dominant peaks elute at $t_R$ 9.38 min and $t_R$ 9.96 min and another smaller peak at $t_R$ 8.73 min (Fig. 39C), confirming the presence of 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin glucuronides in the urine. Multiple peaks were detected in the urine using the mass transitions for catechin/epicatechin glucuronide and methyl catechin/epicatechin glucuronide which could represent the position of where the glucuronic acid binds either at carbon 3', 5, or 7 for catechin/epicatechin glucuronide or carbon 5 or 7 carbon for the methyl catechin/epicatechin glucuronide. Each of these peaks disappeared when the urines were incubated with β-glucuronidase/sulfatase (Fig. 39B & 39D). All urine samples had phenolphthalein β-D-glucuronide added to confirm hydrolysis of glucuronides by β-glucuronidase/sulfatase. Using the mass transition *m/z* 317/93, urine samples incubated without β-glucuronidase/sulfatase contained low amounts of phenolphthalein (Fig. 39E) whereas urine samples incubated with β-

glucuronidase/sulfatase contained high amounts of phenolphthalein (Fig. 39F) at $t_R$ 12.61 min confirming the hydrolysis of phenolphthalein β-D-glucuronide.

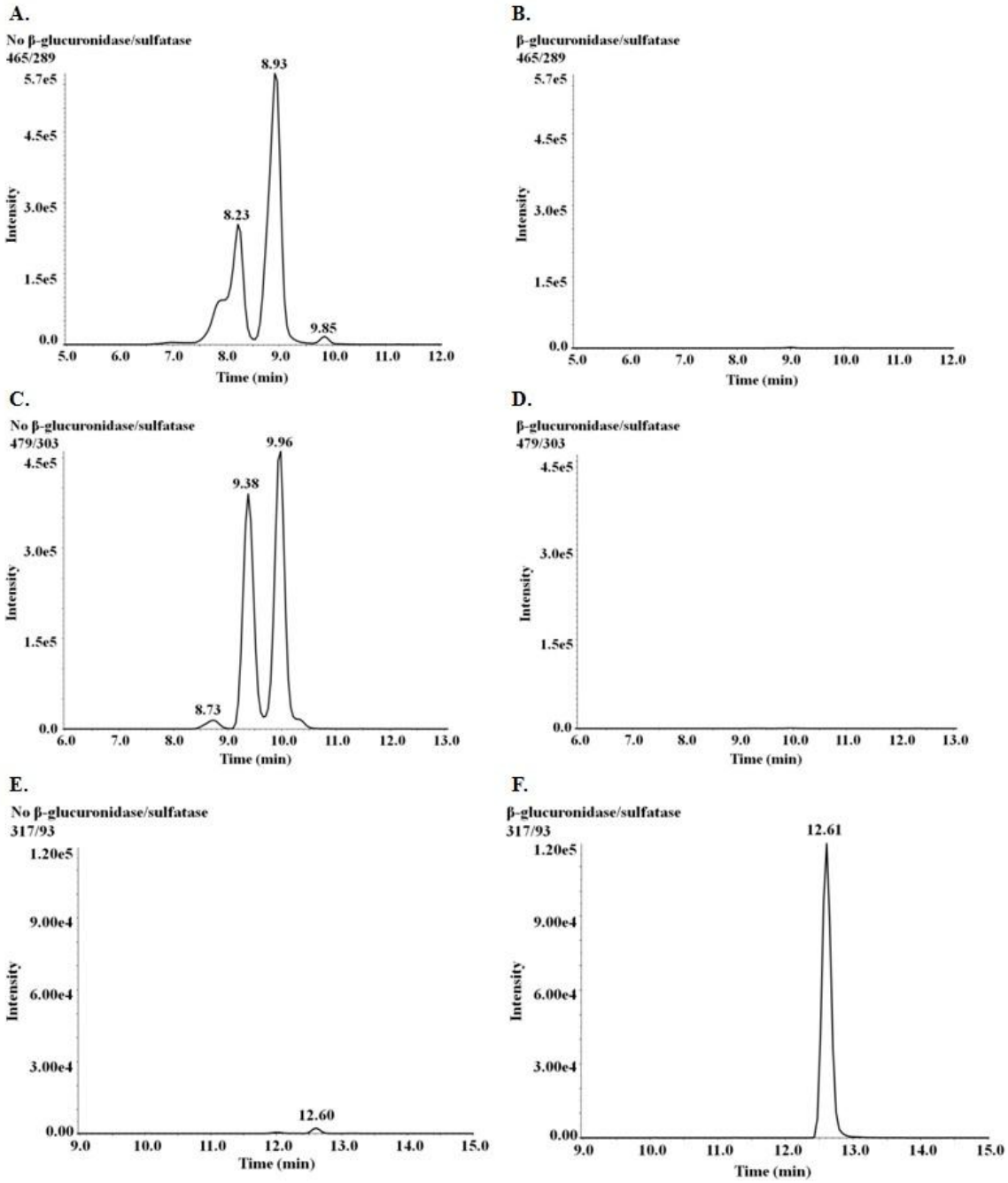



Figure 39. β-Glucuronidase/sulfatase (*H. pomatia*) hydrolyzes glucuronides of catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin. MRM ion chromatograms of catechin and epicatechin glucuronides in urine of an 18-week-old OVX SHR incubated (A) without and (B) with β-glucuronidase/sulfatase using the mass transition *m/z* 465/289 in negative ion mode. MRM ion chromatograms of 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin glucuronides in urine of an 18-week-old OVX SHR incubated (C) without and (D) with β-glucuronidase/sulfatase using the mass transition *m/z* 479/303 in negative ion mode. MRM ion chromatograms of phenolphthalein in urine from the same OVX SHR incubated (E) without and (F) with β-

glucuronidase/sulfatase using the mass transition *m/z* 317/93 in negative ion mode, showing the appearance of the phenolphthalein released from phenolphthalein β-D-glucuronide.

Using the mass transition *m/z* 369/289 for catechin and epicatechin sulfates, urine samples incubated without β-glucuronidase/sulfatase had low intensity peaks at $t_R$ 9.51 min, 10.15 min, 10.85 min, and 12.05 min (Fig. 40A). Using the mass transition *m/z* 383/303 for 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin sulfates, urine samples incubated without β-glucuronidase/sulfatase had dominant peaks elute at $t_R$ of 10.82 min and 11.28 min (Fig. 40C). Multiple peaks were detected in the urine using the mass transition for catechin/epicatechin sulfate which could represent the position of where the sulfonic acid binds either at carbon 3', 5, or 7 for catechin/epicatechin sulfate. However, unlike for the β-glucuronides, the amounts of catechin and epicatechin sulfates (Fig. 40B) and 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin sulfates (Fig. 40D) were unaffected by incubation with β-glucuronidase/sulfatase. There was a slight increase in the peaks at $t_R$ 9.51 min and 10.15 min for catechin/epicatechin sulfate suggesting a small amount of sulfonated and glucuronidated catechin/epicatechin. All urine samples had 4-methylumbelliferyl sulfate added prior to extraction to monitor sulfatase activity in the β-glucuronidase/sulfatase preparation. Using the mass transition *m/z* 175/119 for 4-methylumbelliferone, LC-MRM MS determined that the urine extracts that were incubated without β-glucuronidase/sulfatase contained only trace amounts of 4-methylumbelliferone (Fig. 40E), whereas samples incubated with the enzyme contained the expected amount of 4-methylumbelliferone (Fig. 40F) at $t_R$ 12.34. These data confirm that *H. pomatia* β-glucuronidase/sulfatase did not hydrolyze the sulfate esters of catechin and epicatechin and their methylated metabolites even though it successfully carried out the hydrolysis of 4-methylumbelliferyl sulfate. Therefore, the increase in the amounts of catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin

following incubation with β-glucuronidase/sulfatase represents β-glucuronides of these flavanols and not the sulfates.

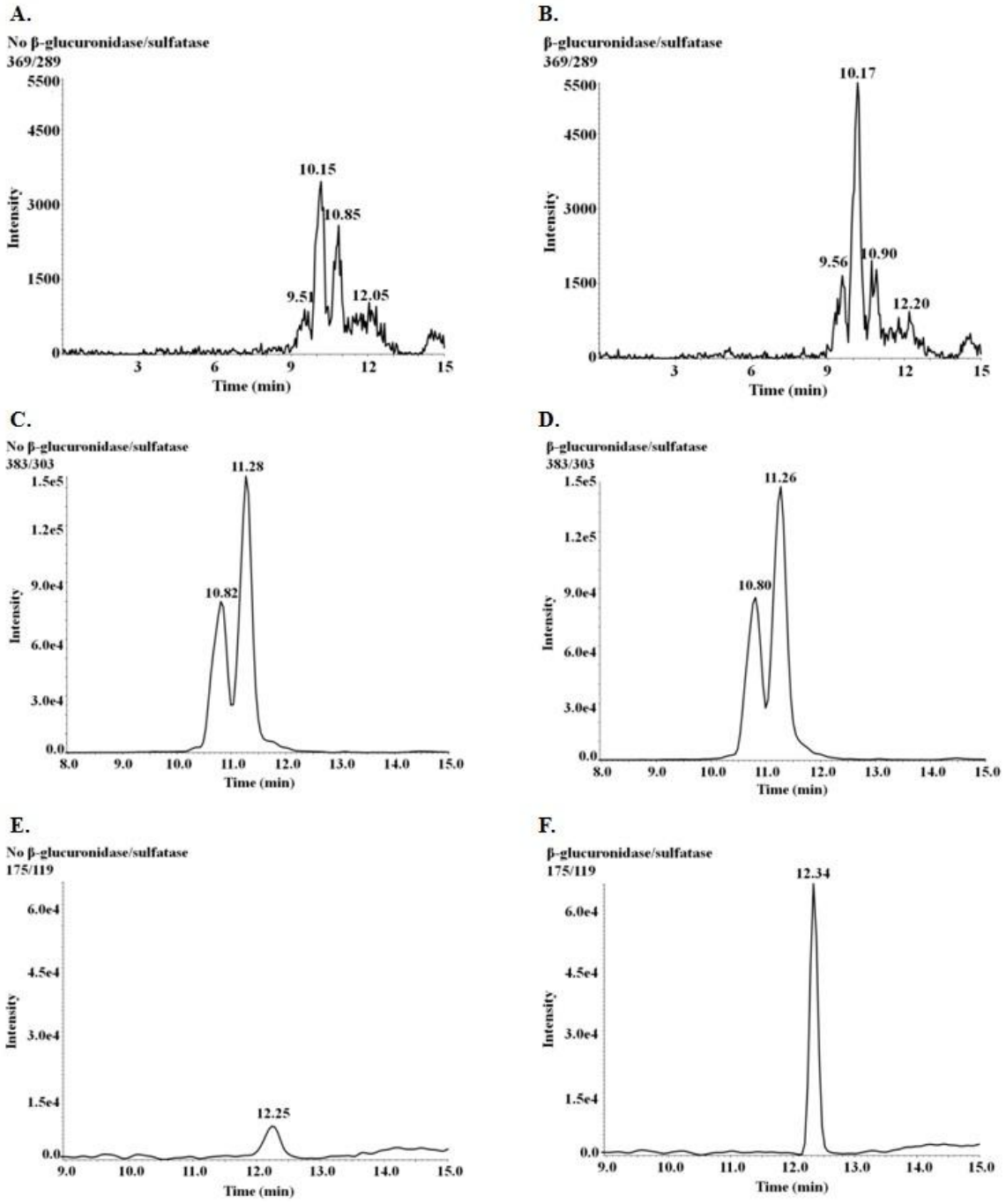



Figure 40. β-Glucuronidase/sulfatase (*H. pomatia*) does not hydrolyze sulfate esters of catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin, although it hydrolyzes 4-methylumbelliferyl sulfate. MRM ion chromatograms of catechin and epicatechin sulfates in urine of an 18-week-old OVX SHR incubated (A) without and (B) with β-glucuronidase/sulfatase using the mass transition *m/z* 369/289 in negative ion mode. MRM ion chromatograms of 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin sulfates in urine of an 18-week-old OVX SHR incubated (C) without and (D) with the β-glucuronidase/sulfatase using the mass transition *m/z* 383/303 in negative ion mode.

Incubation (A&C) without and (B&D) with this β-glucuronidase/sulfatase preparation showed no or very little differences in peak areas. Conversely, MRM ion chromatograms of 4-methylumbelliferone in urine from the same OVX SHR incubated (E) without and (F) with β-glucuronidase/sulfatase using the mass transition *m/z* 175/119 in negative ion mode show that this preparation of β-glucuronidase/sulfatase has sulfatase activity, albeit with poor specificity for the flavanol sulfate esters.

**Effect of Ovariectomy on Urinary Catechin, Epicatechin, and their Metabolites in 18-week-old SHRs**

To determine differences in amounts of urinary catechin and epicatechin urine samples from 18-week-old sham-OVX (n=4) and OVX (n=4) SHRs given GSE were analyzed by LC-MRM MS. In these experiments catechin and epicatechin spiked in control urine samples, from a SHR given vehicle, eluted at $t_R$ 8.98 min and $t_R$ 9.99 min, respectively, using the mass transition *m/z* 291/139. Urine samples from both sham-OVX+GSE (Fig. 41A) and OVX+GSE (Fig. 41B) SHRs had two peaks elute at $t_R$ of 8.97 min and $t_R$ 9.99 min with the mass transition *m/z* 291/139 confirming that urine from sham-OVX and OVX SHRs contained catechin and epicatechin. As was measured with the 18-month-old SHRs, free catechin/epicatechin represents catechin/epicatechin that is not methylated and/or conjugated with glucuronic acid or sulfonic acid and total catechin/epicatechin is determined by incubating with β-glucuronidase/sulfatase to remove glucuronides. Urinary catechins were normalized to the urinary creatinine concentrations (Table 7). Urine samples from OVX+GSE SHRs contained 18.6 ± 1.0 µg/mg creatinine of free catechin and 12.8 ± 5.7 µg/mg creatinine of free epicatechin, whereas urine samples from sham-OVX+GSE SHRs contained 61.0 ± 21.8 µg/mg creatinine of free catechin (p=0.100 compared to OVX+GSE SHRs) and 142.3 ± 57.5 µg/mg creatinine of free epicatechin (p=0.066 compared to OVX+GSE SHRs) (Fig. 41C). In urines incubated with β-glucuronidase/sulfatase before extraction, OVX+GSE SHRs contained 220.5 ± 34.6 µg/mg creatinine of total catechin and 226.8 ± 43.6 µg/mg creatinine of total epicatechin, whereas urine samples from sham-OVX+GSE SHRs

contained 291.9 ± 42.2 µg/mg creatinine of total catechin ($p=0.238$ compared to OVX+GSE SHRs) and 330.7 ± 59.7 µg/mg creatinine of total epicatechin ($p=0.209$ compared to OVX+GSE SHRs) (Fig. 41D). Urine samples from sham-OVX+vehicle and OVX+vehicle SHRs did not contain catechin or epicatechin. These data indicate that OVX causes an increase in glucuronidated catechin and epicatechin in the urine of SHRs.

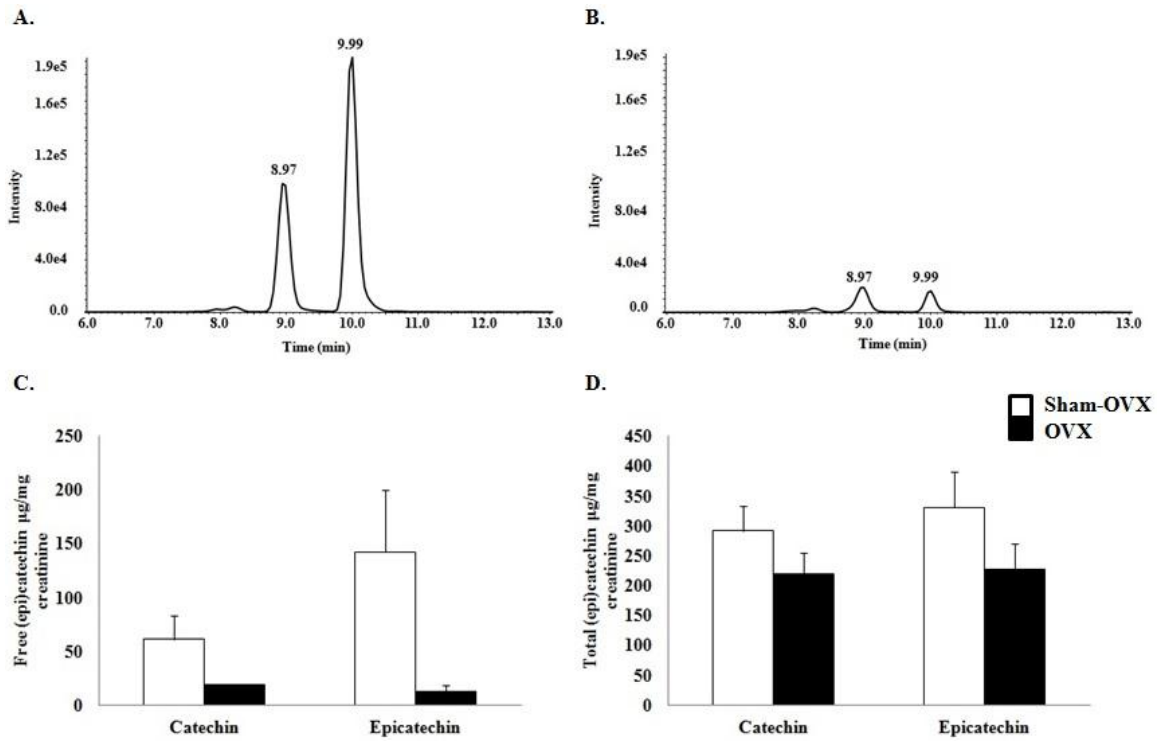



Figure 41. Lower levels of GSE-derived urinary catechin and epicatechin were detected in OVX SHRs vs. sham-OVX SHRs. SHRs were sham-OVX or OVX at 14 weeks of age. GSE (300 mg/kg) was given once daily for six days starting at 17 weeks of age. MRM ion chromatograms of urines from (A) an 18-week-old sham-OVX SHR and (B) an 18-week-old OVX SHR given GSE using the mass transition *m/z* 291/139 in positive ion mode. Quantification of (C) free and (D) total catechin and epicatechin in urines from 18-week-old sham-OVX (n=4) and OVX (n=4) SHRs given GSE. Lower levels of free catechin (p=0.100) and epicatechin (p=0.066) were detected in the urine of OVX SHRs vs. sham-OVX SHRs. Similar levels of total catechin (p=0.238) and epicatechin (p=0.209) were detected in the urine of OVX SHRs vs. sham-OVX SHRs. Free catechin and epicatechin represents catechin and epicatechin that are not conjugated with glucuronic acid or sulfonic acid. Total catechin and epicatechin represents catechin, epicatechin, and their glucuronidated forms. White bars represent sham-OVX SHRs and black bars represent OVX SHRs. Data are expressed as the mean ± SEM.

*Table 7.*

*Creatinine concentrations in urines of 18-week-old sham-OVX and OVX SHRs*

| Rat | Group | Concentration (μM) | mg/dL (x0.0113) | Urine Volume (mL) |
|---|---|---|---|---|
| 4 | sham-OVX+GSE | 3465 | 39.15 | 10 |
| 8 | sham-OVX+GSE | 4131 | 46.68 | 13 |
| 12 | sham-OVX+GSE | 3550 | 40.12 | 15 |
| 24 | sham-OVX+GSE | 2152 | 24.32 | 23 |
| 7 | OVX+GSE | 7470 | 84.41 | 6 |
| 11 | OVX+GSE | 4467 | 50.48 | 14 |
| 15 | OVX+GSE | 3854 | 43.55 | 7 |
| 19 | OVX+GSE | 2506 | 28.32 | 26 |

Urines were collected for 24 h after GSE (300 mg/kg) was given on the fourth day.

To determine differences in amounts of urinary 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin urine samples from 18-week-old sham-OVX (Fig. 42A) and OVX (Fig. 42B) SHRs given GSE were analyzed by LC-MRM MS. 3'-*O*-methyl epicatechin spiked in control urine samples eluted at $t_R$ 10.86 min with the mass transition *m/z* 305/139. The peak at $t_R$ 10.09 min was designated as 3'-*O*-methyl catechin. Urinary catechins were normalized to the urinary creatinine concentrations. Urine samples from OVX+GSE SHRs contained 11.4 ± 3.7 μg/mg creatinine of free 3'-*O*-methyl catechin and 20.0 ± 4.5 μg/mg creatinine of free 3'-*O*-methyl epicatechin, whereas urine samples from sham-OVX+GSE SHRs contained 46.2 ± 18.5 μg/mg creatinine of free 3'-*O*-methyl catechin ($p=0.115$ compared to OVX+GSE SHRs) and 111.5 ± 35.1 μg of free 3'-*O*-methyl epicatechin ($p=0.041$ compared to OVX+GSE SHRs) (Fig. 42C). In urines incubated with β-glucuronidase/sulfatase before extraction, OVX+GSE SHRs contained 163.6 ± 11.2 μg/mg creatinine of total 3'-*O*-methyl catechin and 185.3 ± 17.7 μg/mg creatinine of total 3'-*O*-methyl epicatechin, whereas urine samples from sham-OVX+GSE SHRs contained 174.6 ± 9.8 μg/mg creatinine of total 3'-*O*-methyl catechin ($p=0.489$ compared to OVX+GSE SHRs) and 200.9 ± 13.4 μg/mg creatinine of total 3'-*O*-methyl epicatechin ($p=0.509$ compared to OVX+GSE SHRs) (Fig. 42D). Multiple peaks were detected in the urine using the mass transition for 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin which could represent 4'-*O*-methyl catechin and/or 4'-*O*-methyl epicatechin. Urine samples from sham-OVX+vehicle and OVX+vehicle SHRs did not contain 3'-*O*-methyl catechin or 3'-*O*-methyl epicatechin. These data indicate that OVX does not affect the amount of methylated catechin and epicatechin but does increase the

amount of glucuronidated 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin in the urine of SHRs.

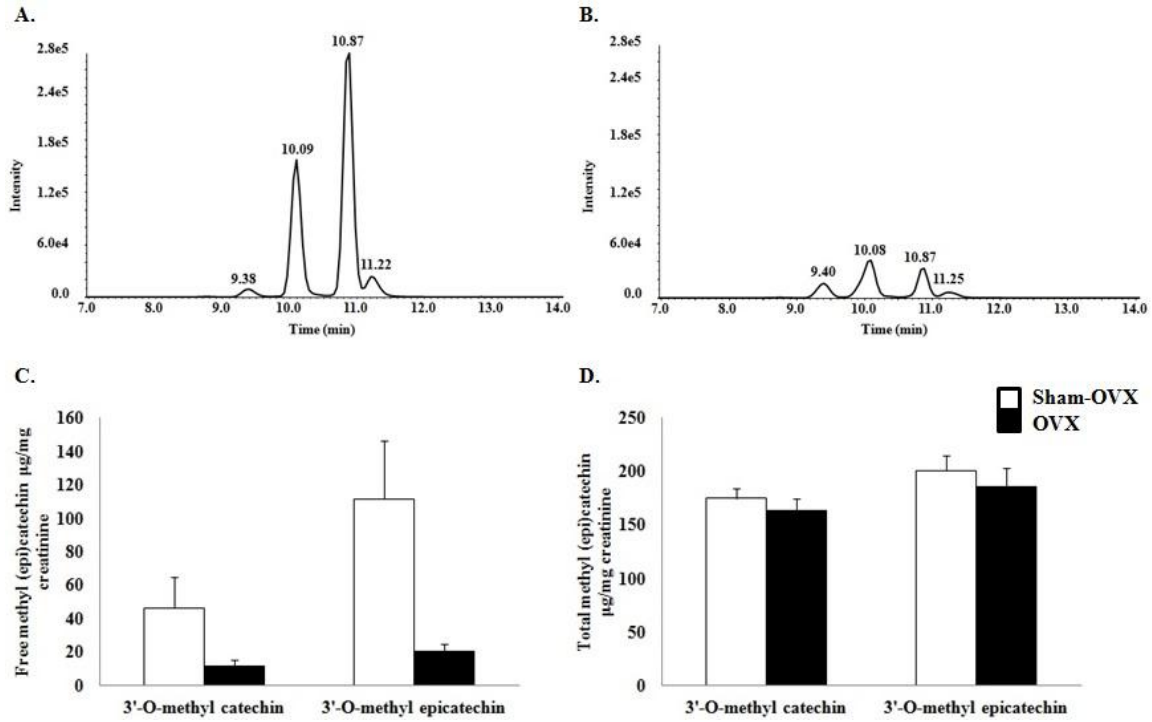



Figure 42. Lower levels of GSE-derived urinary 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin were detected in OVX SHRs vs. sham-OVX SHRs. SHRs were sham-OVX or OVX at 14 weeks of age. GSE (300 mg/kg) was given once daily for six days starting at 17 weeks of age. MRM ion chromatograms of urines from (A) an 18-week-old sham-OVX SHR and (B) an 18-week-old OVX SHR given GSE using the mass transition *m/z* 305/139 in positive ion mode. Quantification of (C) free and (D) total 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin in urines from sham-OVX (n=4) and OVX (n=4) SHRs given GSE. Lower levels of free 3'-*O*-methyl catechin (p=0.115) and 3'-*O*-methyl epicatechin (p=0.041) were detected in the urine of OVX SHRs vs. sham-OVX SHRs. Similar levels of total 3'-*O*-methyl catechin (p=0.489) and 3'-*O*-methyl epicatechin (p=0.509) were detected in the urine of OVX SHRs vs. sham-OVX SHRs. Free 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin represents 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin that are not conjugated with glucuronic acid or sulfonic acid. Total 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin represents 3'-*O*-methyl catechin, 3'-*O*-methyl epicatechin, and their glucuronidated forms. White bars represent sham-OVX SHRs and black bars represent OVX SHRs. Data are expressed as the mean ± SEM.

The amount of free catechin was expressed as a percentage of all flavanol forms in urine samples. All flavanol forms include catechin, epicatechin, 3'-*O*-methyl catechin, 3'-*O*-methyl epicatechin, and their glucuronidated forms from Figures 41D and 42D for sham-OVX and OVX SHRs. This was also done to determine the percentage of free epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin. The percentage of free catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin in urine samples from OVX+GSE SHRs were 2.4%, 1.6%, 1.6%, and 2.6%, respectively, and the percentage of free catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin in urine samples from sham-OVX+GSE SHRs were 6.2%, 14.7%, 4.9%, and 11.9%, respectively (Fig. 43). Based on these calculations, monomeric catechins conjugated with glucuronic acid were 62.3% of total monomeric catechins in urines from sham-OVX SHRs; this percentage increased to 91.8% of monomeric catechins in urines from OVX SHRs. It is to be noted that these totals do not take into account catechin and epicatechin sulfates.

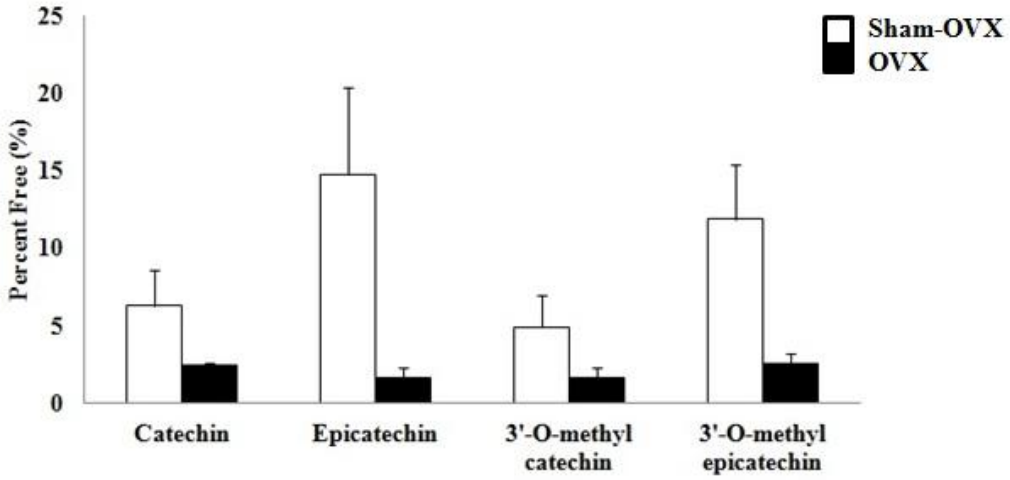
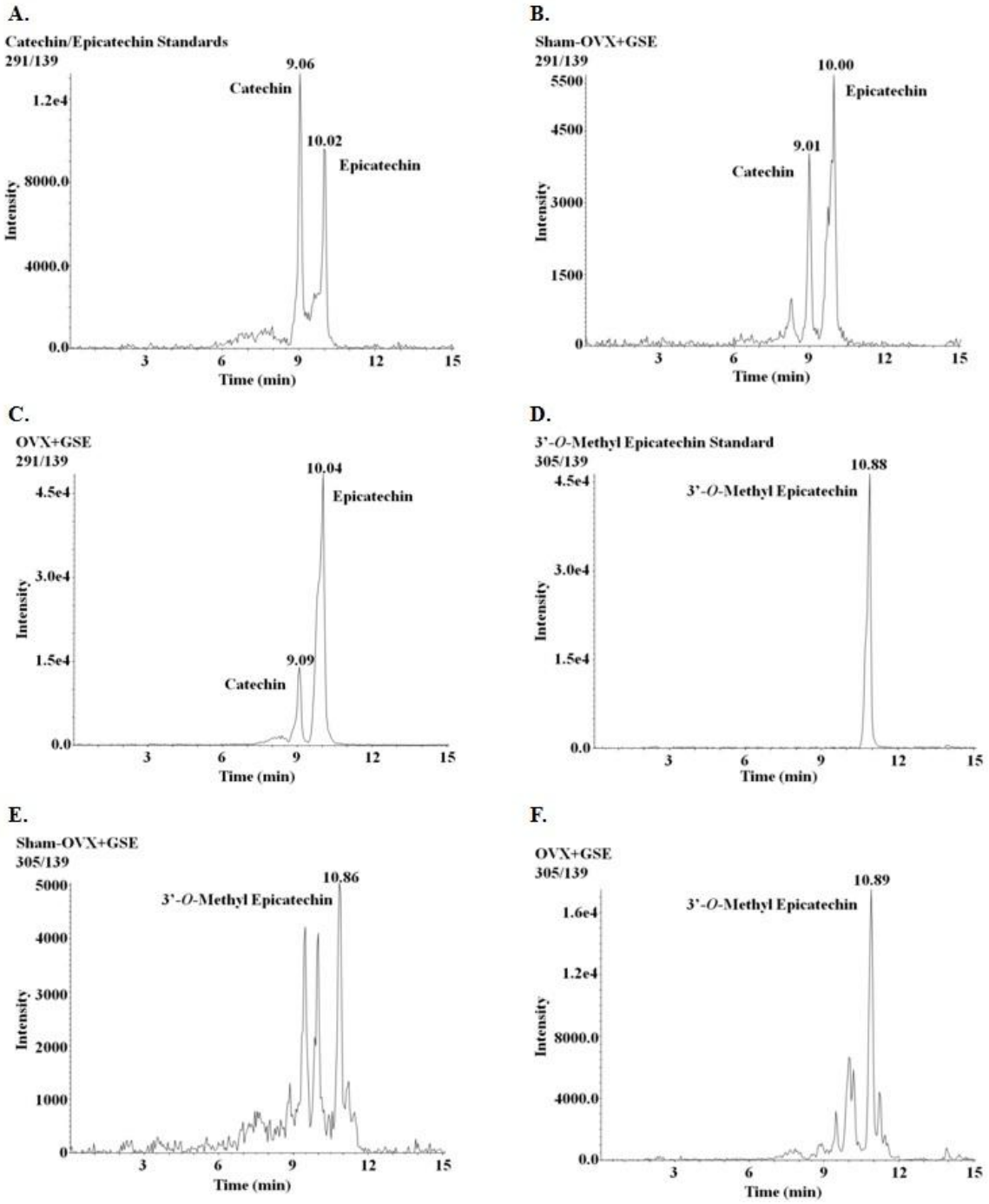



Figure 43. The percentage of free catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin in urines of 18-week-old sham-OVX and OVX SHRs given GSE. These data indicate that 62.3% of total catechin monomers are glucuronidated in the urine of sham-OVX SHRs and 91.8% of total catechin monomers are glucuronidated in the urine of OVX SHRs. Free catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin represents catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin that are not conjugated with glucuronic acid or sulfonic acid. Total catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin represents catechin, epicatechin, 3'-*O*-methyl catechin, 3'-*O*-methyl epicatechin, and their glucuronidated forms. White bars represent sham-OVX SHRs and black bars represent OVX SHRs. Data are expressed as the mean ± SEM.

**Effects of Ovariectomy on Serum Catechin, Epicatechin, and their Metabolites in 18-week-old SHRs**

To determine whether ovariectomy also changed the metabolite profile in the serum, the concentrations of catechin, epicatechin, and 3'-*O*-methyl epicatechin were measured from serum collected 1 h after sham-OVX (n=4) and OVX (n=3) SHRs were gavaged with GSE on the sixth day and analyzed by LC-MRM MS. In these experiments catechin and epicatechin spiked in control serum samples, from a SHR given vehicle, eluted at $t_R$ 9.06 min and $t_R$ 10.02 min, respectively, using the mass transition *m/z* 291/139 (Fig. 44A). Serum samples from sham-OVX+GSE SHRs (Fig. 44B) had two peaks elute at $t_R$ 9.01 min and $t_R$ 10.00 min and OVX+GSE SHRs (Fig. 44C) had two peaks elute at $t_R$ 9.09 min and $t_R$ 10.04 min with the mass transition *m/z* 291/139 confirming that serum from both sham-OVX and OVX SHRs contained catechin and epicatechin. In these experiments 3'-*O*-methyl epicatechin was also spiked in control serum samples and eluted at $t_R$ 10.88 min using the mass transition *m/z* 305/139 (Fig. 44D). Serum samples from sham-OVX+GSE SHRs (Fig. 44E) had a peak elute at $t_R$ 10.86 min and OVX+GSE SHRs (Fig. 44F) had a peak elute at $t_R$ 10.89 min with the mass transition *m/z* 305/139 confirming that serum from both sham-OVX and OVX SHRs contained 3'-*O*-methyl epicatechin. Multiple peaks were detected in the serum using the mass transition for 3'-*O*-methyl epicatechin which could represent 3'-*O*-methyl catechin, 4'-*O*-methyl catechin, and/or 4'-*O*-methyl epicatechin. Serum samples from OVX+GSE SHRs contained 0.26 ± 0.12 μg/mL of free catechin, 0.72 ± 0.35 μg/mL of free epicatechin, and 0.07 ± 0.04 μg/mL of free 3'-*O*-methyl epicatechin, whereas serum

samples from sham-OVX+GSE SHRs contained 0.10 ± 0.06 μg/mL of free catechin (p=0.264 compared to OVX+GSE SHRs), 0.20 ± 0.10 μg/mL of free epicatechin (p=0.165 compared to OVX+GSE SHRs), and 0.02 ± 0.01 μg/mL of free 3'-*O*-methyl epicatechin (p=0.219 compared to OVX+GSE SHRs) (Fig. 44G). The concentration of total catechin, total epicatechin, and total 3'-*O*-methyl epicatechin in serum was determined by analysis of serum samples that had been incubated with β-glucuronidase/sulfatase before extraction. Serum samples from OVX+GSE SHRs contained 1.93 ± 1.72 μg/mL of total catechin, 7.48 ± 6.25 μg/mL of total epicatechin, and 0.53 ± 0.29 μg/mL of total 3'-*O*-methyl epicatechin, whereas serum samples from sham-OVX+GSE SHRs contained 1.68 ± 0.53 μg/mL of total catechin (p=0.881 compared to OVX+GSE SHRs), 5.99 ± 1.49 μg/mL of total epicatechin (p=0.798 compared to OVX+GSE SHRs), and 0.54 ± 0.11 μg/mL of total 3'-*O*-methyl epicatechin (p=0.971 compared to OVX+GSE SHRs) (Fig. 44H). Free catechin, epicatechin, and 3'-*O*-methyl epicatechin represents catechin, epicatechin, and 3'-*O*-methyl epicatechin that are not conjugated with glucuronic acid or sulfonic acid. Total catechin, epicatechin, and 3'-*O*-methyl epicatechin represents catechin, epicatechin, 3'-*O*-methyl epicatechin, and their glucuronidated forms. These data indicate that OVX does not affect serum concentrations of catechin, epicatechin, 3'-*O*-methyl epicatechin, or their glucuronidated forms in SHRs 1 h after the dose of GSE on the sixth day. Therefore, the increase in glucuronidated catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin detected in the urine may be due to selective excretion of glucuronidated catechins that is induced by OVX in SHRs.


A.
Catechin/Epicatechin Standards
291/139
9.06
Catechin
10.02
Epicatechin
Intensity
1.2e4
8000.0
4000.0
0.0
3
6
9
12
15
Time (min)
B.
Sham-OVX+GSE
291/139
10.00
Epicatechin
9.01
Catechin
Intensity
5500
4500
3000
1500
0
Time (min)
C.
OVX+GSE
291/139
10.04
Epicatechin
9.09
Catechin
Intensity
4.5e4
3.0e4
1.5e4
0.0
Time (min)
D.
3'-O-Methyl Epicatechin Standard
305/139
10.88
3'-O-Methyl Epicatechin
Intensity
4.5e4
3.0e4
1.5e4
0.0
Time (min)
E.
Sham-OVX+GSE
305/139
10.86
3'-O-Methyl Epicatechin
Intensity
5000
4000
3000
2000
1000
0
Time (min)
F.
OVX+GSE
305/139
10.89
3'-O-Methyl Epicatechin
Intensity
1.6e4
1.2e4
8000.0
4000.0
0.0
Time (min)

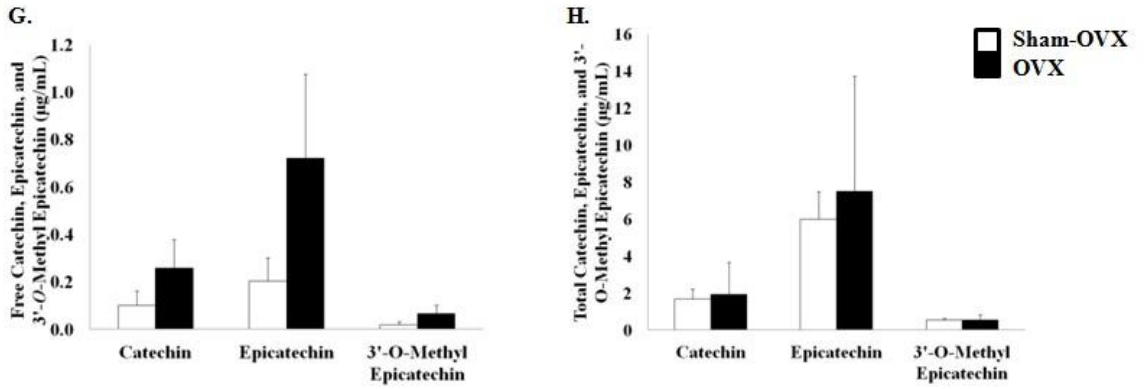



Figure 44. No difference in serum concentrations of catechin, epicatechin, and 3'-*O*-methyl epicatechin in OVX SHRs vs. sham-OVX SHRs. SHRs were sham-OVX or OVX at 14 weeks of age. GSE (300 mg/kg) was given once daily for six days starting at 17 weeks of age. Serum concentrations were measured 1 h after sham-OVX and OVX SHRs were given GSE on the sixth day. MRM ion chromatogram of (A) catechin ($t_R$ 9.06 min, 0.1 µg/mL) and epicatechin ($t_R$ 10.02 min, 0.1 µg/mL) from a serum sample spiked with catechin and epicatechin standards using the mass transition *m/z* 291/139 in positive ion mode. MRM ion chromatograms of serum samples from (B) an 18-week-old sham-OVX and (C) an 18-week-old OVX SHRs given GSE using the mass transition *m/z* 291/139 in positive ion mode. MRM ion chromatogram of (D) 3'-*O*-methyl epicatechin ($t_R$ 10.88 min, 0.1 µg/mL) from a serum sample spiked with 3'-*O*-methyl epicatechin standard using the mass transition *m/z* 305/139 in positive ion mode. MRM ion chromatograms of serum samples from (E) an 18-week-old sham-OVX and (F) an 18-week-old OVX SHRs given GSE using the mass transition *m/z* 305/139 in positive ion mode. Quantification of (G) free and (H) total catechin, epicatechin, and 3'-*O*-methyl epicatechin in serum samples from sham-OVX (n=4) and OVX (n=3) SHRs given GSE. Similar concentrations of free catechin (p=0.264), epicatechin (p=0.165), and 3'-*O*-methyl epicatechin (p=0.219) were detected in the serum of OVX SHRs vs. sham-OVX SHRs. Similar concentrations of total catechin (p=0.881), epicatechin (p=0.798), and 3'-*O*-methyl epicatechin (p=0.971) were detected in the serum of OVX SHRs vs. sham-OVX SHRs. Free catechin, epicatechin, and 3'-*O*-methyl epicatechin represents catechin, epicatechin, and 3'-*O*-methyl epicatechin that are not conjugated with glucuronic acid or sulfonic acid. Total catechin, epicatechin, and 3'-*O*-methyl epicatechin represents catechin, epicatechin, 3'-*O*-methyl epicatechin, and their glucuronidated forms. White bars represent sham-OVX SHRs and black bars represent OVX SHRs. Data are expressed as the mean ± SEM.

## DISCUSSION

In the present study, we showed that ovariectomy altered the metabolism and/or excretion of GSE catechins; specifically, lower levels of catechin, epicatechin, and their methylated metabolites, and higher levels of the glucuronides of these flavanols were quantified in the urines of OVX SHRs vs. sham-OVX SHRs. No differences in the levels of methylated catechin/epicatechin were detected in the urine and no differences in the concentrations of catechin, epicatechin, 3'-*O*-methyl epicatechin, or their glucuronidated forms were detected in the serum between OVX and sham-OVX SHRs.

As hormones, such as estrogens, are known to affect the metabolism of xenobiotics it was determined whether the loss of ovarian hormones, due to ovariectomy, caused a change in GSE metabolism; specifically the flavanols catechin and epicatechin. Quantification of flavanols in urine samples from OVX SHRs gavaged with GSE showed 3-5 fold lower amounts of catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin excreted over 24 h compared to sham-OVX SHRs. These data represent a trend with p-values from 0.04 to 0.11. Failure to achieve full significance may be due to a larger variance in the urine output of catechin, epicatechin, and their methylated metabolites in sham-OVX SHRs than in the OVX SHRs which may be a result of changes in the concentration of 17β-estradiol during the estrous cycle in rats (174).

There are reports of variation of 17β-estradiol concentration during the estrous cycle altering responsiveness to physiological and external stimuli such as nitric oxide synthase (179) and phosphorylation of AKT (protein kinase B) (180). Furthermore, Kulkarni *et al*. has recently shown that the oral bioavailability of another bioflavonoid – genistein – depends on the stage of the estrous cycle of the rat, where higher oral bioavailability of genistein occurs during low levels of estrogen and lower oral bioavailability occurs during high levels of estrogen (181). Lower levels of glucuronidated and sulfonated genistein were detected in the presence of high levels of estrogen compared to higher levels of glucuronidated and sulfonated genistein were detected in the presence of low levels of estrogen (181). This supports the idea that estrogens down regulate phase II enzymes involved in the metabolism of flavonoids and is consistent with the results presented here where an increase of glucuronidated catechin, epicatechin, and their methylated metabolites were detected in the urine of OVX SHRs.

Monomeric catechins are methylated by the phase II enzyme COMT. In this study, there was no difference in the total amount of 3'-*O*-methyl catechin and 3'-*O*-methyl epicatechin excreted in the urine between sham-OVX and OVX SHRs. One reason we may not have detected any changes in methylated catechins in the urine is that 17β-estradiol or the lack of 17β-estradiol (by OVX) effect on COMT is tissue-dependent, as was shown by Schendzielorz et al. (131). Therefore, when we detected no difference in the urine between OVX and sham-OVX rats this may be a global result where the decrease in COMT expression in tissues such as the prefrontal cortex and the kidney is offset by the increase in COMT expression in other tissues. To further understand the

effects of 17β-estradiol on the methylation of catechin future studies will need to be tissue-specific.

LC-MRM MS of the urine samples incubated without β-glucuronidase/sulfatase detected glucuronidated and sulfonated forms of catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin in urines from both OVX and sham-OVX SHRs gavaged with GSE. Some of these conjugated forms of catechin had multiple peaks which may represent isomers of catechin and epicatechin glucuronide/sulfate conjugation on hydroxyl groups at carbon 3', carbon 5, or carbon 7 (139) and methylation on hydroxyl groups at carbon 3' or carbon 4' (143, 144). Whereas urine samples that were incubated with β-glucuronidase/sulfatase no longer contained the glucuronidated forms of catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin, the peak areas of the sulfonated forms of catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin were unchanged. LC-MRM MS indicated that while both phenolphthalein and 4-methylumbelliferone were released from the internal standards phenolphthalein β-D-glucuronide and 4-methylumbelliferyl sulfate when incubated with *H. pomatia* β-glucuronidase/sulfatase, the sulfatase activity of this enzyme preparation did not hydrolyze the sulfate esters of catechins and methyl catechins. These data confirm the findings of Saha *et al*. who have recently shown that flavanols, specifically epicatechin and methyl epicatechin, are poor substrates for many sulfatase-containing commercial preparations including *H. pomatia* β-glucuronidase/sulfatase (182). Accordingly, the increase in levels of the amounts of urinary catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin following incubation with β-glucuronidase/sulfatase

represents increased glucuronides of these catechins, consistent with our hypothesis that OVX would increase glucuronidation of catechins, presumably due to dysregulation of UGTs resulting from the loss of estrogens. These data are consistent with work (133-135) showing that estrogen attenuates UGT expression and activity.

One interesting observation of the data was that the intensity and area of the peaks from methylated catechin/epicatechin sulfate were much greater than the peaks from catechin/epicatechin sulfate. One reason for this may be that methyl catechin and methyl epicatechin are better substrates for SULTs than catechin and epicatechin. Futures studies to address this are required.

In these studies changes in catechin glucuronides were detected in the urine of OVX SHRs. It should be noted that epicatechin was not glucuronidated by human liver and small intestinal microsomes but was glucuronidated by rat liver microsomes (138). Epicatechin was efficiently sulfonated by human liver cytosol whereas the sulfation was less in rat liver cytosol (138). This would suggest that in humans catechin and epicatechin are probably highly sulfonated. In fact, Ottaviani *et al*. showed that in humans given epicatechin, half of its metabolites were glucuronides and the other half sulfates (139). As sulfates make up a large portion of catechin and epicatechin metabolites in humans, follow-up studies using a sulfatase that has efficacy in removing the sulfate from catechins or using sulfate standards are required and may reveal an increase in sulfation due to OVX similar to genistein (181). To avoid metabolism differences between rats and humans future studies may include performing these

experiments in pre- and postmenopausal women, where urine and blood can be collected after ingestion of GSE, catechin, or epicatechin and tested for differences in glucuronidated and sulfonated catechins.

As glucuronidation and sulfation are generally considered to be a mechanism whereby the body excretes xenobiotics, if the results presented here are confirmed in OVX rats or postmenopausal women it would suggest that postmenopausal women may not achieve equivalent physiological levels as premenopausal women of catechin, epicatechin, and their methylated metabolites from intake of the same dose of a dietary supplement like GSE. It has recently been shown that epicatechin and 3'-*O*-methyl epicatechin are glucuronidated *in vitro* most effectively by UGT1A9 (183), which is mainly expressed in the liver (184). Epicatechin has also been shown to be sulfonated by SULT1A1 and 1A3 *in vitro* (138). Follow up studies will determine whether changes in 17β-estradiol affect the expression and/or activity of UGT1A9 in liver microsomes and SULT1A1 and 1A3 in the cytosolic fraction. It should be kept in mind that 3'-*O*-methyl epicatechin glucuronide (300 nM) added to hippocampal slices enhanced long term potentiation (28); however, it is not known whether the bioactive form in the neurons was the glucuronide or whether it had been de-conjugated. It is also to be noted that the increase in LTP only occurred in brain sections from a transgenic mouse model of AD and not in healthy wild-type mice brain sections Others have detected low levels of epicatechin glucuronide and methyl epicatechin glucuronide (147) and catechin and epicatechin (148) in the brain of rats. Abd El Mohsen *et al*. detected low levels (200 nM) of epicatechin glucuronide and methyl epicatechin glucuronide in brain extracts of rats

given 100 mg/kg of epicatechin per day (147). This is of interest as it suggests that catechins and their methylated and glucuronidated metabolites can pass the blood brain barrier. An *in vitro* study showed that catechin and epicatechin pass through cerebral endothelial cells and are actually metabolized to their glucuronide forms by these cells (185).

LC-MRM MS of the serum samples incubated without β-glucuronidase/sulfatase detected glucuronidated and sulfonated forms of catechin, epicatechin, and 3'-*O*-methyl epicatechin in sera from both OVX and sham-OVX SHRs gavaged with GSE. Serum was collected 1 h after SHRs were gavaged with GSE on the sixth day. There was no difference in serum concentrations of catechin, epicatechin, 3'-*O*-methyl epicatechin, or their glucuronidated forms between sham-OVX and OVX SHRs. Of the catechin, epicatechin, and 3'-*O*-methyl epicatechin that were detected in the serum, roughly 90% were conjugated with glucuronic acid. These data indicate that ovariectomy or lower circulating levels of estrogen do not affect the glucuronidation of catechin and epicatechin. It may be that ovariectomy increases the excretion of glucuronidated catechins in the kidneys. As serum was only collected at one time point, 1 h after rats were gavaged with GSE, whereas the urine was collected over 24 h, this difference between serum and urine may be due to when the samples were collected. Further pharmacokinetic studies are required to determine the affects of ovariectomy on serum concentrations of catechin, epicatechin, and their metabolites and whether glucuronidation is increased in OVX rats. Another reason we may not have detected a difference is due to the variance within the groups. At the beginning of the study there

were 6 rats per group but for unknown reasons 5 of the animals died leaving only 4 sham-OVX SHRs and 3 OVX SHRs to collect serum from. An increase in the number of animals per group would decrease the variance allowing us to determine if there were any real differences caused by ovariectomy. Estrogen does affect the kidney as the loss of estrogen increases the excretion of sodium, chloride, and catecholamines in OVX rats and the addition of 17β-estradiol decreases their excretion (186, 187). These studies also suggest that the loss of estrogen leads to an increase in water intake and urinary output with no effect on glomerular filtration (186, 187). In the studies presented here the urinary output over 24 h was random between the two groups, although we did not measure the intake of water over the course of the study. Future studies will measure the intake of water and the output of urine.

The mean body weight of sham-OVX SHRs given GSE was 205 g and the mean body weight of OVX SHRs given GSE was 250 g. SHRs were gavaged with 300 mg/kg of GSE resulting in sham-OVX SHRs receiving 61 mg of GSE per day and OVX SHRs receiving 75 mg of GSE per day. The analysis of the GSE given to the rats determined that catechin comprised 1.6% of GSE and that epicatechin comprised 2.0% of GSE. On average sham-OVX SHRs received 0.98 mg of catechin and 1.22 mg of epicatechin whereas OVX SHRs received 1.2 mg of catechin and 1.5 mg of epicatechin each day. GSE also contains oligomers of catechin and epicatechin that theatrically could be broken down to catechin and epicatechin monomers but Tsang *et al*. showed that these oligomers do not contribute to an increase in catechin or epicatechin levels (146) and therefore the ingested amount is the total amount. In our experiments sham-OVX SHRs excreted

0.246 mg of catechin and 0.277 mg of epicatechin in the urine and OVX SHRs excreted 0.228 mg of catechin and 0.246 mg of epicatechin in the urine. This results in sham-OVX SHRs excreting 25% of ingested catechin and 23% of ingested epicatechin in the urine over 24 h and OVX SHRs excreting 19% of ingested catechin and 16% of ingested epicatechin in the urine over 24 h.

The body can also excrete xenobiotics in the bile and it has been shown that catechin and epicatechin metabolites, including methylated, glucuronidated, and sulfonated forms (144, 149, 150). If there were changes in glucuronidation due to ovariectomy it would be expected to have an increase of these glucuornides in the bile. One reason that supports the idea of ovariectomy causing an increase in glucuronidation of catechins is that catechin glucuronides are entering the enterohepatic circulation and therefore are staying in the body for a longer period of time. Preliminary results detected methyl catechin/epicatechin, catechin/epicatechin glucuronide, methyl catechin/epicatechin glucuronide, and methyl catechin/epicatechin sulfate but not catechin and epicatechin in the bile of the 18-month-old sham-OVX and OVX SHRs. The percentages of catechin and epicatechin excreted in the urine are still only a portion of what was ingested. Ward *et al*. showed that catechins are metabolized to phenolic acids such as 3-hydroxyphenylpropionic acid (151). Future studies would address detecting and quantifying these phenolic acids and their metabolites as well as catechin, epicatechin, and their metabolites.

We addressed whether surgical ovariectomy in our study was successful by measuring differences in body and uterine weights. At the beginning of the study, 3 weeks after ovariectomy, OVX SHRs had higher body weights compared with sham-OVX SHRs, consistent with the weight gain previously shown to be a consequence of ovariectomy (99, 176) and similar to what was detected in the rats that were OVX at 6 months of age. As expected, OVX SHRs had lower uterine weights compared with sham-OVX SHRs, consistent with the lower uterine weights previously shown to be a consequence of ovariectomy (176) and similar to what was detected in the 18-month-old OVX rats. The difference in body weights between OVX and sham-OVX SHRs continued throughout the study. It was noted that SHRs gavaged with GSE had lower body weights compared to SHRs gavaged with vehicle. The SHRs given vehicle lost 3-4% of their starting body weight whereas the SHRs given GSE lost 10% of their starting body weight. Given the short duration of the study (6 days), the loss of body weight could have reflected initial adjustment to being gavaged, particularly with the GSE. The administration of GSE did not affect the uterine weight in sham-OVX or OVX SHRs which once again indicates that GSE does not have estrogenic actions on reproductive tissues.

In conclusion, our findings indicate that ovariectomy lowered urinary levels of GSE-derived catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin, with concomitant higher levels of the glucuronides of these flavanols in OVX SHRs. This is the first report of differences in the urine of glucuronidated catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin in a rodent model of menopause

following oral intake of GSE. As no difference of catechin, epicatechin, or their metabolites were detected in the serum 1 h after receiving GSE, future studies are required to determine whether ovariectomy increases the metabolism and/or the excretion of catechin and epicatechin. As dietary supplements containing flavonoids are used for the treatment and prevention of diseases the clinical ramifications of this data are that postmenopausal women might require a higher dose of catechin containing dietary supplements to achieve the same physiological levels as premenopausal women.

# CHAPTER 5

# DISCUSSION

This dissertation described studies that addressed the following hypotheses: 1) that GSE enhances adult hippocampal neurogenesis; 2) that GSE enhances learning and/or memory in an older rodent model of menopause; and 3) that the metabolism of the GSE monomeric flavanols, catechin and epicatechin, is different in OVX rats due to lowered estrogens.

The first aim of this dissertation was to assess whether enhancement of adult hippocampal neurogenesis is one mechanism that could contribute to GSE-induced learning and memory enhancement. We showed that consumption of GSE did not affect hippocampal neurogenesis as there was no difference in the number of new neurons or proliferating progenitor cells in the DG of 5-month-old POMC-EGFP mice given GSE compared to control 5-month-old POMC-EGFP mice. Also, we found lowered numbers of new neurons and no difference in proliferation of progenitor cells in the DG of 26-day-old POMC-EGFP mice whose mother was given GSE only while nursing compared to control 26-day-old POMC-EGFP mice. This suggests that dietary intake of GSE does not affect hippocampal neurogenesis in adults but that dietary intake of GSE in nursing mothers causes reduced survival of new neurons in their offspring. These results lead to

the conclusion that dietary GSE does not enhance hippocampal neurogenesis in adult mice and, in fact, may lower it in pups whose mothers are given GSE while nursing.

Our study was designed to determine if GSE enhances hippocampal neurogenesis. Our studies did not detect an effect in adult mice. To verify our results we identified several areas that require further research. First, establish that POMC-EGFP mice given GSE have improved learning and/or memory when tested in a hippocampal-dependent task, such as the Morris water maze. Increased hippocampal neurogenesis has been correlated with increased learning and memory in the Morris water maze (39, 54, 55). Once it is established that GSE enhances learning and/or memory by a hippocampal-dependent task, we can determine whether GSE improves learning and/or memory through or partially through hippocampal neurogenesis. If mice given GSE have enhanced learning and/or memory and enhanced hippocampal neurogenesis it would suggest that GSE-induced learning and memory enhancement is due, at least partially, to enhanced hippocampal neurogenesis. If mice given GSE have enhanced learning and/or memory and no difference in hippocampal neurogenesis it would suggest that GSE-induced learning and memory enhancement is through a pathway independent of hippocampal neurogenesis. Second, assess the effects of GSE on LTP in the hippocampus; this experiment could be performed before or alongside the experiment to determine whether GSE enhances hippocampal neurogenesis. LTP would be measured in the DG and CA1, and as increased LTP in the DG, not the CA1, is associated with enhanced hippocampal neurogenesis (39), if LTP increases in the DG in mice given GSE it would suggest that GSE has a role in enhancing hippocampal neurogenesis.

In regards to determining whether GSE enhances or suppresses hippocampal neurogenesis in pups whose mothers are given GSE while nursing, further studies are required to help explain our results. First, the number of litters where mothers are given GSE needs to be increased. Second, collect milk and perform compositional analysis using mass spectrometry to determine whether components of GSE are in the milk and what those components are. Although others have shown that genistein and daidzein, which are structurally similar to catechin and epicatechin, were found in the stomach milk of 7-day-old rats (156, 157), it is necessary to determine that catechin, epicatechin, and/or their metabolites can pass through the mother's milk. Third, to address possible stress that arises due to a new diet at the time of birth, mothers would be given the GSE diet at the time of breeding.

Future directions in this area would be to establish that GSE enhances learning and/or memory in a hippocampal-dependent task in POMC-EGFP mice. As was done before, 4-month-old POMC-EGFP mice would be given a GSE diet or a control diet for 4 weeks before being tested in a hippocampal-dependent task, such as the Morris water maze. Stereological counts would be performed on proliferating progenitor cells, new neurons, and mature neurons in the DG. It may be necessary to give GSE by gavage once daily to achieve a difference as this was one of the differences between our study and that of Yoo *et al.* (158). To help determine whether there are changes in parameters that relate to neurogenesis, it would be of interest to perform LTP in mice that are given GSE, as it is not known whether GSE enhances LTP in healthy rodents; whereas it has been shown that in hippocampal slices (CA1) from a transgenic mouse model of AD

treated with a metabolite of epicatechin (3'-*O*-methyl epicatechin glucuronide) had increased LTP compared to vehicle treated hippocampal slices (28). Future experiments to confirm that the number of new neurons is lowered in pups whose mothers are given GSE would be repeated to include multiple litters per group and to give GSE at the time of breeding through weaning. If differences are detected, the milk from the mothers and in the pups stomachs (156, 188) would be examined to determine what components of GSE are being passed to the pups through the mother's milk.

Whether or not the intake of dietary GSE affects hippocampal neurogenesis, there is evidence that GSE and other catechin containing dietary supplements have beneficial health effects in the brain. GSE given to healthy rats had brain protein changes consistent with neuroprotection (27). GSE attenuated the loss of cognitive function and reduced Aβ plaques in the brains of a transgenic mouse model of AD (25, 26). GSE suppressed hypoxic-ischemic brain injury in rats (189). Cocoa, which contains epicatechin, has been shown to induce vasodilation by nitric oxide; this is significant because increased cerebral blood flow is thought of as improving cerebral function (190, 191). Also, the ingestion of epicatechin has been shown to enhance retention of spatial memory in mice (30).

The second aim of this dissertation was to assess whether GSE enhances learning and/or memory in an older rodent model of menopause, as was detected in a young rodent model of menopause (23). We showed that under our conditions of ovariectomy, which was done at 6 months of age, there was no impairment in learning or memory in

SHRs when tested in the Morris water maze at 12 months of age and then again at 16 months of age. The chronic consumption of GSE from 6 months of age and on did not enhance learning or memory in sham-OVX or OVX SHRs when tested in the Morris water maze at 12 and 16 months of age. This was contrary to our hypothesis that ovariectomy would lead to impairment in learning and/or memory in rats and that GSE would enhance learning and/or memory in OVX rats, if not also in sham-OVX rats. These results lead to the conclusions that under our conditions, where SHRs were OVX at 6 months of age and tested in the Morris water maze at 12 and 16 months of age, OVX SHRs were not a model of learning and memory impairment and therefore it was not possible to determine whether GSE attenuates learning and memory impairment in an older rodent model of menopause.

Our study was designed to determine if GSE enhances learning and/or memory in older OVX rats. As others have shown that ovariectomy impaired learning and memory in rodents (97, 98, 102-104, 168), it was a major concern that our OVX rats showed no impairment in learning or memory between sham-OVX and OVX SHRs when tested in the Morris water maze. This prevented the determination if GSE attenuates impaired hippocampal-dependent learning and/or memory in OVX rats; however, 12-month-old sham-OVX rats are still cycling and as we did not measure parameters to indicate what stage of the estrous cycle the rats were in, this may have caused part of the variance detected in the sham-OVX groups. Rats have a relatively short estrous cycle, 4-5 days (192), and as the Morris water maze takes at least 5 days to perform, it would be difficult to normalize to one stage in the estrous cycle. Future studies would include an OVX rat

group given 17β-estradiol and an OVX rat group given GSE and 17β-estradiol as positive controls. To help decrease variance within groups, rats would be given vehicle or GSE by gavage each day so the exact amount of GSE given to the rats would be known, and to use Sprague Dawley rats instead of SHRs to remove any effects of high blood pressure. Future studies would also test rats in a probe trial 24 h after the last day of training as it has been shown that mice given epicatechin and tested in the Morris water maze had improved memory in the probe trial tested 24 h after the last day of training (30) and that grape powder only enhanced short-term memory (173). Future studies would also collect blood immediately after testing for learning and memory to have an accurate concentration of circulating 17β-estradiol levels that can be correlated to learning and memory.

Further experiments could also include duplicating the work of Peng *et al*. (23) but with older rats and with the inclusion of a sham-OVX rat group, a sham-OVX rat plus GSE group, an OVX rat plus 17β-estradiol group, and an OVX rat plus GSE and 17β-estradiol group. Rats would once again be OVX or sham-OVX at 6 months of age and then put into their different dietary and 17β-estradiol groups for 10 weeks vs. 6 months, followed by testing in the 8-arm-radial maze. Another experiment that could be performed is to test these same groups in the novel object recognition task, another hippocampal-dependent learning and memory task. Vedder *et al*. showed that 17β-estradiol given to OVX rats increased their learning and memory in the novel object recognition task compared to OVX rats (98). If these experiments result in increased learning and/or memory in the OVX rat plus GSE group and the OVX rat plus 17β-

estradiol group with no enhancement in the sham-OVX rat plus GSE group or further enhancement in the OVX rat plus GSE and 17β-estradiol group it would suggest that GSE enhances learning and/or memory through or by affecting an estrogen dependent pathway. If these experiments result in increased learning and/or memory in all rat groups given GSE, it would suggest that GSE enhances learning and/or memory independent of estrogen pathways.

If it is found that GSE enhances learning and/or memory in OVX rats but not in sham-OVX rats, then further studies could be done to determine if GSE works through estrogen pathways. OVX Sprague Dawley rats that were given 17β-estradiol for 2 days had increased LTP, enhanced performance in the novel object recognition task, increased neuron spine density, and increased number of N-methyl-D-aspartate receptor subtype 2B (NR2B) (98, 193). Blocking either estrogen receptors or NR2B inhibited the increased LTP, enhanced performance in the novel object recognition task, increased neuron spine density, and increased number of NR2B suggesting that 17β-estradiol is involved in enhancing learning and memory in OVX rats through estrogen receptors (98, 193). If it is detected that GSE affects one or more of these parameters, it can also be suggested that GSE affects learning and/or memory by impacting an estrogen dependent pathway.

The third aim of this dissertation was to assess whether the metabolism of the GSE monomeric flavanols, catechin and epicatechin, is affected due to ovariectomy. We showed that ovariectomy altered the metabolism and/or excretion of GSE catechins; specifically, lower levels of catechin, epicatechin, and their methylated metabolites, and

higher levels of the glucuronides of these flavanols were quantified in the urines of OVX SHRs vs. sham-OVX SHRs. No differences in the levels of methylated catechin/epicatechin were detected in the urine and no differences in the concentrations of catechin, epicatechin, 3'-*O*-methyl epicatechin, or their glucuronidated forms were detected in the serum between OVX and sham-OVX SHRs. These results lead to the conclusion that ovariectomy causes an increase in glucuronidation of catechins and/or excretion of glucuronidated catechins in the urine.

Our study was designed to determine if ovariectomy, which lowers estrogens, affects the metabolism of catechin and epicatechin. This is the first study to show a change in urinary catechin metabolites in OVX rats given GSE; specifically, increased levels of glucuronidated catechin, epicatechin, and their methylated metabolites. If the metabolism and/or excretion of catechins are increased due to lowered estrogens this suggests that postmenopausal women may not achieve equivalent physiological levels as premenopausal women from intake of the same dose of a catechin containing dietary supplement such as GSE. However, statistical difference with a p value $\leq 0.05$ was only achieved for free 3'-*O*-methyl epicatechin ($p=0.041$). Free catechin ($p=0.100$), epicatechin ($p=0.066$), and 3'-*O*-methyl catechin ($p=0.115$) did approach but did not reach statistical significance. Failure to achieve statistical difference was most likely due to the low number of animals per group and compounded with an increase in variance in the sham-OVX group compared to the OVX groups possibly due to cycling in these rats. Further studies would include an increased number of animals per group, and as positive

controls the addition of an OVX rat plus 17β-estradiol group and an OVX rat plus GSE and 17β-estradiol group.

There was no difference in concentrations of catechin, epicatechin, 3'-*O*-methyl epicatechin, or their glucuronidated forms in the serum suggesting that there may not be a difference in metabolism but a difference in excretion of glucuronidated catechin and epicatechin due to ovariectomy. Large variances were detected within each group suggesting that there were too few animals per group to detect any real differences. Further studies are required to determine whether ovariectomy leads to an increase in glucuronidation and/or excretion of catechins. Future studies would also use the same time frame as before with a possible change to using Wistar or Sprague Dawley rats instead of SHRs and would include monitoring water intake, collecting blood at multiple time points, and collecting bile. First, water intake would be monitored to determine whether there is an increase in catechin and epicatechin excretion in OVX rats. OVX rats have increased excretion of sodium, chloride, and catecholamines and increased water intake and urinary output (186, 187). The urine from our study was normalized to creatinine and as there was not statistical difference in the total amount of catechin, epicatechin, 3'-*O*-methyl catechin, and 3'-*O*-methyl epicatechin after normalization to creatinine it would suggest that the kidney was not affected by ovariectomy in our rats but still needs to be confirmed due to the varying outputs of urine. Second, blood would be collected at multiple time points after GSE administration to determine a range of when catechin metabolites enter the blood after ingestion and whether there are differences between OVX and sham-OVX rats. Third, bile would be collected as it is

another pathway to excrete catechins (144, 145, 149, 150). Preliminary results in the 18-month-old rats detected methylated, glucuronidated, and sulfonated catechin and epicatechin in the bile. If ovariectomy leads to an increase in glucuronidation, it would be expected to detect an increase in catechin and epicatechin glucuronides in the blood and/or bile. If there is no difference in catechin and epicatechin glucuronides in the blood and/or bile but a difference in the urine, this would suggest ovariectomy affects kidney excretion of catechin and epicatechin. Another parameter to change that would help in determining whether ovariectomy affects catechin and epicatechin metabolism is to give pure catechin and epicatechin to the animals. This would remove any effect coming from other flavonoids present in GSE.

Catechin, epicatechin, and their methylated forms are known to be glucuronidated and sulfonated. As standards are not always available, a common practice to determine the amount of glucuronidated and sulfonated catechin, epicatechin, and their methylated forms is to hydrolyze the samples with a commercially available glucuronidase/sulfatase preparation. As was shown in this study and in the study by Saha *et al*. (182), the β-glucuronidase/sulfatase preparation from *Helix pomatia* was insufficient to hydrolyze sulfates from catechin, epicatechin, and their methylated forms. Future studies would include using a sulfatase that is effective in removing the sulfate group from catechin and epicatechin or making sulfate standards to perform direct quantification. Conjugated forms of catechin and epicatechin are not the only forms of metabolites. Ward *et al*. showed that catechins from GSE are metabolized to phenolic acids such as 3-

hydroxyphenylpropionic acid and 4-*O*-methylgallic acid (151). Future studies will include the detection of these metabolites as well.

If future studies confirm that ovariectomy leads to increased glucuronidated or sulfonated catechin and epicatechin, additional studies using LC-MS/MS and activity assays will be performed on liver microsomes and cytosolic fraction to determine whether the expression and/or activity of UGT1A9, SULT1A1, or SULT1A3 are changed due to the higher levels of 17β-estradiol. The rationale to study UGT1A9 expression levels and activity is because of the recent demonstration that UGT1A9 glucuronidates epicatechin and 3'-*O*-methyl epicatechin better than other UGT isoforms *in vitro* (183). The rationale to study SULT1A1 and 1A3 expression levels and activity is because epicatechin has been shown to be sulfonated by SULT1A1 and 1A3 *in vitro* (138).

An experiment that would be helpful in determining metabolite differences due to menopause would be to collect urine and blood samples after ingestion of GSE, catechin, or epicatechin from premenopausal women, postmenopausal women, and postmenopausal women on hormone therapy. Human studies have been performed with flavanols such as epicatechin to determine effects and metabolites (29, 139, 149, 151, 153, 194). Ottaviani *et al*. showed that in humans given epicatechin, half of its metabolites were glucuronides and the other half sulfates (139). This type of experiment would eliminate differences in metabolism due to differences between rat and human (138).

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# APPENDIX

## INSTITUTIONAL ANIMAL AND CARE USE COMMITTEE APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

*Institutional Animal Care and Use Committee (IACUC)*

**MEMORANDUM**

**DATE:** December 27, 2011

**TO:** HELEN KIM, Ph.D.
MCLM-460 0005
FAX: (205) 934-6944

**FROM:** Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

**SUBJECT:** **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

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The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on December 27, 2011.

Title of Application: Cognitive Effects of Grape Seed Extract-Brain Protein Targets
Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)

**Institutional Animal Care and Use Committee**
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