Comparative Immunological Effects of a Natural Estrogen (17β-estradiol) versus a Pharmacologic Synthetic Estrogen (17α-ethinyl estradiol)

Tyson P. Brummer

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Dr. S. Ansar Ahmed, Chair
Dr. R.M. Gogal, Jr.
Dr. S.D. Holladay

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Abstract

Exposure to exogenous estrogens such as synthetic 17α-ethinyl estradiol (EE) occurs via multiple sources (i.e. hormonal contraceptives, environmental contamination, hormone replacement therapy). The natural estrogen, 17β-estradiol (E2), is a well-studied immunomodulatory hormone at both environmental and pharmacologic levels. Conversely, little data exist regarding the immune effects of EE at either environmentally-relevant exposure levels or at pharmacological levels. Further, EE is delivered to patients in a clinical setting via different routes of exposure (e.g. subcutaneous or oral). Many key questions in relation to potential immunological effects of EE are unanswered. Important variables in estrogen-modulation of the immune system include: (i) dose, (ii) age, (iii) gender, and (iv) route of exposure. Thus, pertinent questions emerge. Does exposure to EE at low concentrations for a subacute duration affect the immune or reproductive systems? Are the effects similar in both hormones and between sexes? Are these effects similar in juvenile and aged mice? How do the effects compare across two common routes of exposure (subcutaneous versus oral)? To address these questions, three separate studies were performed. In the first study, we investigated whether very low, but environmentally relevant, doses of EE, E2 (10 ng/kg body weight), or vehicle orally administered every other day for 21 days to young (6 week-old) and aged (>15 month-old) C57BL/6 mice had immunomodulatory effects. As expected, significant gender and age-related differences were noted with regard to thymus weight, thymocyte recovery, spleen weight, and splenocyte recovery. However, low dose treatment of either E2 or EE had no marked effects on the thymus or spleen organ to body weight ratios, cell numbers, or lymphocyte subsets. Low dose oral estrogens did not alter the ability of activated splenocytes to induce interferon-γ or nitric oxide. No effects on male reproductive organ to BW ratios of young or aged mice were found. Similarly, with the exception of E2-stimulating effects on the female reproductive tract of young mice, there were no pronounced effects in females.

In separate studies, intact juvenile female and male C57BL/6 mice were given daily subcutaneous (second study) or oral (third study) doses of either EE or E2 (0.04, 0.4, or 4.0 μg per 25 g BW) for 21 days. In the subcutaneous exposure study, both EE and E2 morphologically altered uterine and seminal vesicle weights. However, EE had a more pronounced effect compared to E2, especially in males, even at the lowest dose administered. Additionally, like E2, EE induced thymic atrophy in both sexes. In female mice, thymic atrophy and thymic cellularity were significantly decreased by subcutaneous EE and E2 at doses of 0.4 and 4.0 μg/25 g body weights. EE elicited significantly more pronounced thymic atrophy-inducing effects compared to E2 at the 4.0 μg/25g dose. In males, thymocyte cellularity was decreased by both subcutaneous EE and E2 only at the highest dose tested (4.0 μg/25 g body weight), whereas only 4.0 EE significantly decreased thymus to body weight ratios. Neither splenic weights, splenic cellularity, nor splenic cell phenotype were affected by either estrogenic compound regardless of route of exposure. Oral exposure of EE or E2 did not induce marked immunological effects.

Collectively, these data demonstrate that select thymic and reproductive endpoints are significantly altered following a 21-day subcutaneous exposure to either EE or E2 and that the thymus is a more sensitive target than the spleen with regard to subacute exposure to EE. In addition, EE at a comparable dose was more potent than E2 at exerting thymic and reproductive organ morphological alterations. Furthermore, route of administration is critical, as subcutaneous exposure induced far more dramatic thymic and reproductive morphological alterations than did oral administration. Future studies need to address the precise mechanism through which EE induces thymic atrophy and diminished thymus cellularity. Are these effects mediated directly through the thymus, perhaps through estrogen-induced increased thymocyte apoptosis or alterations to thymic epithelial cells? Or could EE be mediating alterations via bone marrow stem cells targeted for distribution to the thymus? Our novel findings regarding EE-induced effects on the thymus are of health significance and set the stage for future work.

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Appendix A

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List of Abbreviations

CD	Cluster of differentiation
Con-A	Concanavalin A
DES	Diethylstilbestrol
E2	17β-estradiol
EAE	Experimental allergic encephalomyelitis
EE	17α-ethinyl estradiol
ER	Estrogen receptor
ERT	Estrogen replacement therapy
FITC	Fluorescein isothiocyanate
FSH	Follicle-stimulating hormone
HRT	Hormone replacement therapy
IFN-γ	Interferon gamma
IL	Interleukin
LH	Luteinizing hormone
LPS	Lipopolysaccharide
NA	Norethindrone acetate
OC	Oral contraceptive
PE	Phycoerythrin
PHA	Phytohemagglutinin
SLE	Systemic lupus erythematosus

Chapter 1: Introduction and Rationale

The synthetic estrogen, 17α-ethinyl estradiol (EE), is an analog of natural estrogen 17β-estradiol (E2) and was first characterized in 1938 [1]. EE is a primary component in hormonal contraceptives which are one of the most commonly prescribed drugs in the United States, used by an estimated 16 million women in the United States [2] and 100 million women worldwide [3]. Since the 1980's, EE has become more frequently prescribed for non-contraceptive conditions such as acne, osteoporosis, and anti-aging. It has also shown efficacy as an estrogen replacement for polycystic ovarian syndrome [4]. Unfortunately, EE is known to be released into the environment as a waste byproduct of wastewater following metabolism and excretion. Natural and synthetic estrogens, as well as their metabolites have been detected in sewage wastewater, oceans, rivers, and lakes worldwide [5-9]. Additionally, human exposure to estrogenic compounds can occur through consumption of beef from cattle given subcutaneous estrogen implants for growth promotion [10]. Exposure to exogenous estrogens in the environment is of immunological and reproductive concern [11].

Sex-related differences in immune function are known to exist. Specifically, females have more marked humoral immune responses than males [12]. Females have higher immunoglobulin concentrations than males [13] and respond more vigorously to antigenic challenge from microorganisms [14] and immunization [15, 16]. In addition, cell-mediated immune response is stronger in females compared to males, as females are more efficient at rejecting allografts, female mice are more susceptible to T cell-mediated autoimmune diabetes, and gender differences have been observed in the number of circulating T cells [14, 17-19]. Women are more susceptible to development of autoimmune conditions such as lupus, Graves' disease, Hashimoto's thyroiditis and rheumatoid arthritis than men [20]. It has been suggested that physiological levels of estrogens stimulate the immune response, while male hormones suppress the immune response [21]. Therefore, both sexes were assessed for their response to treatment with EE or E2.

Further, immune function is altered with age, and there is a paucity of data with relation to EE or E2 exposure and immune function in the geriatric population. An impaired bone marrow progenitor cell proliferative response has been observed in the elderly [22]. Both aged mice and humans have been observed to have diminished T cell proliferative response, as well as a decline in anti-microbial and anti-tumor function [23-25]. An age-associated decrease in IL-2

and IL-2 receptor expression on activated splenocytes has also been observed in the mouse [26]. Further, thymus involution begins at puberty and continues throughout adult life [27]. The antibody response is impaired as human's age, with a decreased ability to produce specific antibody following antigenic challenge [28]. In addition, the secretion of cytokines such as IFN-γ has been reported to be increased with age in mice [29]. It was unknown if the response to estrogenic compounds in juvenile mice would be the same or similar to the response in geriatric mice.

Presently, the potential immune effects from exposure to low levels of estrogens have not been well characterized, nor are the immunological effects of pharmaceutical EE exposure well understood. Specifically, it is not known if EE is comparable to E2, an endogenous hormone whose immune-modulating capabilities have been extensively studied and characterized. Does EE possess similar immunomodulatory capabilities? If so, are these effects similar across juvenile and geriatric mice? How do the effects differ across gender? Will two different common routes of pharmaceutical administration reveal differences in immune changes? None of these questions have been adequately addressed in prior work and are what we sought to answer in this body of work.

A series of studies were conducted that examined the immune effects of EE on C57BL/6 mice across, age, gender, and route of exposure (oral and subcutaneous). In the first study, an environmentally relevant dose (10 ng/kg) was administered orally to male and female, juvenile and geriatric mice for a period of 21 days. In the subcutaneous (second study) and oral (third study) exposure studies, doses of 0.04, 0.4, or 4.0 µg/25 g body weight of EE or E2 were administered for 21 days, either via subcutaneous or oral dosing. Doses were within the upper range of environmental contamination up to pharmacologically relevant. The goals of these two studies were to attempt to address the questions that were put forth above.

We examined reproductive and immune organs, as *in vivo* bio-indicators of estrogenicity, of female and male C57BL/6 mice. The immune and reproductive systems are intricately linked, with prior studies demonstrating that E2 administration causes thymic involution [30], while removal of the thymus can induce pathologies in the ovaries, testes, and thyroid tissues over time [31, 32]. The assessment of immune changes related to the estrogenic compound exposure centered on the thymic and splenic endpoints. This area of investigation included a thorough analysis of phenotypic expression of each major immune cell subset in the immune organs via

flow cytometry. CD4 (a helper T cell marker) and CD8 (a cytotoxic T cell marker) expression markers were assessed in the thymus, as well as in the spleen. Further, B cells, natural killer cells, macrophages, and dendritic cells were assessed in the spleen. Interferon-gamma (IFN-γ, measured by ELISA) and nitric oxide (NO, measured by Griess assay) production levels were measured from Con-A stimulated splenocytes cultured at 24 or 48 hr. Non-specific functionality of the splenocytes was evaluated using the Alamar Blue™ proliferation assay.

In the first study, oral exposure of a very low environmentally relevant dose of EE or E2 showed no significant alterations in the reproductive organs, or in the immune endpoints examined. However, in the second and third studies, profound alterations in both the thymus and reproductive organs were observed when exposed to EE subcutaneously. Interestingly, EE-induced profound alterations of immune and reproductive organs were not noticed in mice given EE via oral route, suggesting that route of exposure dramatically influences immunological and reproductive changes manifested. Increased potency was demonstrated by EE at comparable doses to E2, with regard to causing increased uterine weight and seminal vesicle atrophy. For example, a 100-fold greater dose of E2 was needed in order to induce a similar magnitude of seminal vesicle atrophy as was caused by EE. Further, EE was more potent at inducing thymic atrophy relative to dose-matched E2. Our findings for 21-day exposures reveal that the thymus is most sensitive to EE, while secondary lymphoid organs (i.e. the spleen) are not overtly affected. This finding is not altogether surprising, as the thymus is a developmental immune organ housing largely immature T cells. These immature cells would be more vulnerable to modulation from estrogenic compounds, while those cells located in the spleen would already be fully mature and functional. Thus, it is possible that estrogen exposure greater than 21 days may still elicit changes in the secondary lymphoid organs. The direct comparison of EE to comparable doses of E2 has shown EE to be significantly more potent in causing thymic atrophy and reproductive organ morphological alterations in both male and female mice. Thymic effects mediated by EE include diminished thymic weight and decreased thymocyte cellularity, though percent expressions of thymocyte subsets were not significantly altered by treatment.

Future studies need to address the effects of chronic exposure of low, environmentally relevant, levels of EE to the thymus. Altered thymic development may have implications for susceptibility to viral infection, autoimmunity, allergies, and other immune abnormalities. The T cells which develop in this organ end up seeding the secondary immune sites including the

spleen and lymph nodes. This aspect needs to be studied in greater depth, especially with regard to molecular effects such as increased IFN-γ and nitric oxide, two important cytokines related to inflammation and autoimmunity. This series of studies provides valuable preliminary data with which to proceed in examining the effects of EE on the immune system. To our knowledge, these are the first studies which have compared the immunological effects of EE directly to E2, as well as examined these effects across age, gender, and two common routes of exposure (subcutaneous and oral).

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Chapter 2: Literature Review

2.1 17α-ethinyl estradiol: General Introduction

Historical Development and Mechanism of Biological Effect

The concept of pharmaceutical control of the reproductive cycle and fertility originated with the revelation that transplantation of ovaries from pregnant rabbits into fertile female rabbits suppressed ovulation and fertility [1]. The first combined hormonal oral contraceptive was not FDA-approved for use in the United States until 1960 [2]. Envoid® was composed of 150 μg mestranol, a methyl ether of 17α-ethinyl estradiol (EE), and 9.8 mg norethynodrel. The following year, the first EE-containing oral contraceptive, Anovlar®, became available in Europe and consisted of 50 μg EE and 4 mg norethisterone acetate [3]. EE (IUPAC name: 17α-ethinyl-1, 3, 5 (10)-estratrien-3, 17β-diol) was first synthesized and characterized by Hans Inhoffen and Walter Hohlweg in 1938 [4]. The addition of the ethinyl group to C17 of 17β-estradiol (E2) results in 17α-ethinyl estradiol. EE is a potent estrogenic molecule that is the most common estrogenic component of modern hormonal contraceptives. Natural estradiol is readily absorbed orally but is quickly inactivated by cytochrome P450 enzymes of the liver. The addition of the ethinyl group results in inhibited hepatic metabolism at C-16 and C-17 [5], increasing the molecule's bioavailability and half-life relative to natural estrogen [6].

The original hormonal contraceptives contained greater than 50 μg of EE or mestranol and a progestin component. The most widely reported side effect of high dose estrogen therapy was coronary thrombosis, or heart attack [7]. The second generation of hormonal contraceptives sought to decrease the amount of EE (less than 50 μg), as well as develop more progestin options in order to decrease undesirable side effects. The modern, so-called third-generation, hormonal contraceptives contain EE in much lower doses (typically 20-35 μg) and several progestin options have been developed which are believed to diminish cardiovascular side effects [8]. In addition, several new routes of hormone delivery have been developed in the past 10 years, including the transdermal hormone patch [9], vaginal ring [10], and monthly intramuscular injections [11]. When taken alone, estrogen can cause the cells in the uterine lining to become crowded or malformed and possibly cancerous. Therefore, when a woman still has a uterus,

estrogen is often prescribed in combination with progesterone, which controls this effect and protects from endometrial abnormalities [12]. Further progestin contributions to hormonal contraceptives include increasing thickness of cervical mucus, decreasing sperm motility, slowing movement of the ovum, inhibiting development of the uterine lining, suppressing midcycle peaks of luteininzing hormone (LH) and follicle-stimulating hormone (FSH), and reducing cilia activity in the fallopian tubes [13, 14].

The basic principle of hormonal contraceptives revolves around administering hormones that signal to the body that there is a pregnancy present [15]. This results in inhibition of ovulation. The pill works both centrally in the brain (hypothalamus and anterior pituitary) and peripherally in the ovaries, the Fallopian tubes, and the uterus. EE prevents release of follicle-stimulating hormone from the anterior pituitary, resulting in inhibition of ovarian activity and preventing development of a mature egg [16]. In addition, hormonal contraceptives inhibit the release of LH, which normally induces ovulation in the middle of the cycle [17]. The natural production of both hormones starts when the levels of progesterone and estrogen are low. But since both substances are contained in the pill, the FSH/LH production cycle cannot get started because the levels of estrogen and progesterone are not sufficiently decreased.

Metabolism of EE

Metabolism of EE is complicated, but a limited description follows. After absorption in the small intestine, EE is carried in the hepatic portal vein to the liver. A portion is directly absorbed into the bloodstream, but the majority is metabolized in conjugated forms that are released through the gallbladder back into the intestines. Liver metabolism creates conjugated metabolites of EE which are polar and soluble, making excretion easier. Approximately 60% of orally administered EE is inactivated by the liver via first-pass effect through enterohepatic circulation [18], a reduction in activity that does not occur in transdermal methods of hormonal administration. The major metabolic pathway of EE degradation is aromatic hydroxylation, but a wide variety of hydroxylated and methylated metabolites are formed. These are present as free metabolites and as conjugates with glucuronide and sulfate. CYP3A4 is the major enzyme in the liver responsible for the 2-hydroxylation which is the major oxidative reaction [19]. The 2-hydroxy metabolite is further transformed by methylation and glucuronidation prior to urinary and fecal excretion [20, 21]. Hydroxylation can also occur at the 4, 6, and 16α-positions of EE [22, 23], while removal of the ethinyl group is another possible oxidative reaction [24]. Sulfation and glurconidation also occur in the metabolism of EE. Most commonly, sulfation occurs at the 3-*O*-position but can also occur at the 17-*O*-position [25]. Similarly, conjugation of EE with glucuronic acid can occur at the 3- and 17-*O* position, forming EE glucuronides [26]. The sulfate and glucuronide metabolites re-enter the lumen of the bowel, where they can be excreted or the bowel flora can deconjugate the metabolites, which are reabsorbed causing an increase in circulating EE. Some of the major metabolic pathways of EE in humans can be found in Figure 2.1. As a matter of comparison, some of the major E2 metabolites in humans are presented in Figure 2.3.

EE is orally bioactive because it is removed more slowly from circulation than E2, an effect that is essential for improved oral effectiveness and biological efficacy. [5]. Urinary recovery of EE in humans is much less than that of E2, with substantial EE metabolites being found in human feces [27]. Transdermal methods of EE-administration (i.e. Ortho Evra contraceptive patch) avoid first-pass metabolism through the liver. This results in higher initial systemic concentrations of EE but overall more controlled steady-state levels of hormone than oral routes since the skin controls the rate of absorption at a much more consistent rate than if the

hormone traveled through the gastrointestinal tract [28]. Nonetheless, following systemic circulation, EE is eventually metabolized by the liver into various hydroxylated and conjugated metabolites [29] similar to oral routes. Blood levels of an oral dose of EE vary widely from patient to patient [18]. One important source of inter-individual variation is the first-pass enterohepatic circulation. Portions of an oral drug such as EE are inactivated shortly after passing through the liver, resulting in highly variable systemic distribution. EE is also susceptible to various drug-to-drug interactions which may affect the enzymes responsible for EE metabolism. Interpopulation variation in EE blood levels may be a result of ethnic, geographic, nutritional, or other causes [18]. Differences in absorption, distribution, metabolism, and elimination may affect blood reproducibility levels. A range of between 52-62% of EE is inactivated by the liver and intestinal wall in oral routes before reaching the systemic circulation [18].

The acute toxicity of EE in humans and rodents is considered low to moderate [30] with the LD_{50} of EE in rodents determined to range from 0.5 > 5 g/kg [31]. Maier et al. [32] performed a review of the literature regarding the toxicity of EE and norethindrone acetate (NA) and conclude that long term exposure to EE and NA formulations pose very little health risks to humans. However, no mention is made of any immunological consequences related to exposure to these compounds, an interesting fact to note as EE seems to be commonly overlooked with regard to its potential immunomodulating effects.

2.2 17α-ethinyl estradiol: Common sources of exposure

Hormonal Contraceptives

In the United States, there were an estimated 16 million women taking hormonal contraceptives in 1999, a number that has likely increased in the ensuing years [33]. Worldwide, there are over an estimated 100 million women taking hormonal contraceptives as of 2003 [34]. There are currently over 200 different formulations of oral contraceptive pills currently available, the majority of which contain EE. In addition, several relatively new products have been developed in the past 10 years which allow for non-oral delivery of hormonal contraception. The typical dosing cycle of a hormonal contraceptive involves 21-day administration of the

hormones, with a 7-day period without hormonal administration to allow menstrual bleeding to occur. Table 2.1 contains several examples of EE-containing hormonal contraceptives.

The only transdermal route of hormonal contraception currently approved for use in the United States is the Ortho Evra® contraceptive patch. The patch administers 20 μg of EE to the bloodstream every 24 hours and the half-life of EE is approximately 17 hours [29]. Use of the patch is for 21 days, followed by seven days without hormone administration. One cycle consists of a total of 28 days and the patch is changed three times (every 7 days) during the 21-day use cycle. The cycle is repeated as long as the woman desires to be using a hormonal contraceptive. The typical range of serum EE concentrations while using the patch is 25 to 75 pg/ml [35, 36]. In this method of hormone delivery, first-pass metabolism through the liver is avoided but normal hepatic metabolism still occurs due to enterohepatic circulation. The mechanism of action for transdermal EE/norelgestromin (Ortho Evra) is similar to that of oral contraceptives: inhibition of ovulation by suppression of gonadotropins. It suppresses follicular development, induces changes to the endometrium that reduce the probability of implantation, and increases the viscosity of cervical mucus, which may prevent sperm penetration into the uterus [37]. The vaginal ring (NuvaRing®) is another alternative to the oral contraceptive pill and yet another source of exposure to EE. Similar to the patch, the vaginal ring avoids first-pass metabolism and gastrointestinal interference with absorption of hormones, allowing lower doses of EE to be used. This product delivers 15 μg of EE daily. The half-life of EE in this product is 45 hr [38].

Environmental Contamination

Both EE and E2 have been identified in the environment most prominently in the aquatic environment, where the main sources of contamination are sewage treatment plants and agricultural runoff or discharge [39, 40]. Increased persistence in the human body, a desired biological effect of EE, leads to the inevitable effect of increased persistence in the environment. Incomplete absorption of EE leads to excretion of a significant proportion in both active and inactive forms.

EE tends to be present in the environment at much lower levels than E2 but it is more persistent and less volatile [41, 42]. Bacterial degradation is slower due to the presence of an

ethinyl group on C17 and metabolites released from human waste are not as well broken down compared to E2 metabolites [41]. A study examining removal of EE from sewage in Australia suggests that EE is resistant to biological degradation based on findings that 100% of the EE placed in a sequential reactor is recovered and remains non-degraded, whereas 6% of E2 was further broken down and more susceptible to bacterial degradation [43]. Tabak et al. [44] found that EE concentrations in wastewater were indeed lower than E2 but that only 20 to 40% of EE can be expected to be degraded in a sewage plant with primary (mechanical) and secondary (biological) treatment, whereas E2 would be expected to be degraded by 50 to 70% in a plant with primary and secondary treatment. Degradation of EE under both anaerobic [45] and aerobic [46, 47] conditions has been demonstrated to be poor.

Wastewater treatment plant effluents are common sources of estrogenic substances in the aquatic environment [48, 49] and thus are areas commonly studied for estrogen-induced abnormalities. In the United Kingdom, studies of wastewater effluents found concentrations of E2 ranging from 1 to 88 ng/l, while EE was found at concentrations ranging from <0.2 ng/l to 7.0 ng/l [48, 50]. In a flow-through system designed to assess the impact of a defined chemical exposure, an environmentally relevant EE concentration was determined to be 3 ng/l. This concentration caused arrested testicular development and transition from an early all-ovary stage in genetically male zebrafish [51]. These effects were reversible upon removal from the EE-contaminated system. A chemical fate model of EE in wastewater demonstrated possible routes through which the chemical may potentially enter the human food chain. [52]. EE was traced from human ingestion and excretion to treatment in a sewage treatment plant, discharge into a receiving river (effluent), and eventually into the sea. The model suggested that 30,000 portions of fish would need to be ingested by a human in order to achieve the dose of a typical contraceptive pill, but warn that the biochemical significance of even small exposures to lipophilic substances like EE must be determined. Furthermore, the persistence and potency of this molecule lends credence to the possibility of biologically significant levels of EE exposure via drinking water. Increased estrogen-like activity in fish tissues has been observed in edible fish removed from the Mediterranean Sea, as determined by an *in vitro* yeast reporter gene assay [53], though it was not determined what the precise contribution of EE was to this finding. Animals treated with sex steroids for growth hormone production have also been suggested as a

source of exposure to exogenous estrogens, a concern especially relevant in adolescents who may be more vulnerable to modulation by estrogenic compounds [54].

The effluent of four sewage treatment plants in Germany were found to contain EE concentrations of between 0.4 to 3.3 ng/l, while E2 was present at a range of 3.8-51 ng/l. Sampling of surface waters from the Rhine river determined EE to be present in concentrations ranging from 0.3 to 1.0 ng/l and E2 from 1.3 to 9.2 ng/l [55]. An extensive study of the aquatic environment in the Netherlands found EE in municipal (untreated) wastewater (median: 3.2 ng/l), sewage treatment plant (STP) effluent (median: 2.6 ng/l), industrial wastewater (3.8 ng/l), and surface water (0.4 ng/l) [56]. E2 was found in untreated wastewater at a median concentration of 36.5 ng/l but was not detectable in STP effluent, highlighting the fact that though greater amounts of E2 are released into the environment compared to EE, it is removed from wastewater much more effectively than EE and does not accumulate for long periods of time. In a sample of Canadian STP effluents, EE was detected in 9 out of 10 samples, with a maximum concentration of 42 ng/l and mean concentration of 9 ng/l [57].

In Australia, measurement of raw sewage concentrations of EE and E2 yielded values of 5 ng/l and 55 ng/l respectively, values that are consistent with previously published reports in other regions [57-59]. Concentrations in the sewage effluent were measured at <0.1 ng/l for EE and 0.95 ng/l for E2 [43]. Interestingly, measurement of three New Zealand sewage effluents yielded only trace amounts of EE, but the limitation of this study was the method detection limit, reported to be 10 ng/l, a concentration lower than what has been reported in several other regions. E2 was reported to be present in only trace amounts at two sewage effluents and 14.8 ng/l at another. These values are lower than what has been reported in many European and North American locations [60], possibly suggesting that New Zealand either has a lower sewage estrogenic hormone burden (i.e. smaller population, fewer hormonal contraceptive users) or perhaps better methods of removing estrogenic compounds from sewage. These aspects were not considered in the referenced work.

Both EE and E2 and their metabolites have been detected in ocean sediments [61, 62] associated with sewage treatment plant discharge points far downstream. EE has been detected in river and drinking water samples in the range of 20-200 pg/ml [63] and samples from two Nevada rivers revealed concentrations of EE ranging from non-detectable to 14 ng/ml [64]. In lakes, high-pressure liquid chromatography analysis revealed concentrations of EE from 5.7 to

30.8 ng/l, while E2 was detected at lower levels of 1.6 to 15.5 ng/l [65]. Further, EE has been found in concentrations above 1 ng/l in river samples taken 100 km away from the discharge point for a sewage effluent, suggesting that this hormone can be transported considerable distances from the initial source of introduction into the environment [66].

At environmentally relevant EE concentrations ranging between 10 to 1000 ng/ml, both EE and E2 have been observed to quickly be absorbed by soil. Further, soils were demonstrated to have a large capacity to bind estrogens. Interestingly, desorption from the soil in the aqueous phase was also shown to occur easily, demonstrating the ability of large concentrations of estrogens to be released from soil into water sources during rainfall [67]. EE and other estrogens have been previously demonstrated to be absorbed by soil samples even when present in very small sub-nanogram per liter levels [68].

Despite the fact that EE concentrations are detected at low levels, it is abundantly clear that these levels are sufficiently high enough to induce reproductive alterations in the aquatic environment [40, 69]. Further, mixtures of synthetic estrogenic compounds, including EE, have recently been observed to alter the function of hemocytes in the marine bivalve *Mytilus* [70]. Hemocytes are the immune cells of this marine animal and although the precise effect of EE in the synthetic estrogenic mixture was not determined, it is noteworthy that immune parameters were altered in an aquatic species at concentrations comparable with environmental exposure levels and in a mixture containing EE. Development of more sensitive and widely available assays for EE quantification [71, 72] may reveal the chemical's presence in even more environmental locations in which it had previously been non-detectable.

Hormone Replacement Therapy

The use of hormone replacement therapy (HRT) in the United States has dropped by 38% since the release of results from the Women's Health Initiative Study [73]. Nonetheless, there are currently an estimated 21 million women with prescriptions for HRT in the US, with millions more women worldwide currently on some form of hormone replacement. Following menopause, the ovaries produce less estrogen and replacement of that estrogen leads to improvement of menopausal symptoms. It is believed that estrogen protects post-menopausal women from bone fractures, heart attacks, stroke, and dementia, as well as improves skin tone,

and other symptoms of menopause [74]. The principal benefit of HRT is in treatment of post-menopausal osteoporosis [75]. Estrogen has been linked to the prevention of bone loss and possible diminished occurrence of bone fractures in post-menopausal women [76]. There are currently an estimated 40 million post-menopausal women in the United States and an additional 20 million are estimated to reach menopause in the next 10 years.

There are very few EE-containing HRT regimens currently in use, making this route of EE exposure relatively low. Nonetheless, there are currently more EE-containing HRT regimens being developed for clinical use. One of the few currently in use is FemHRT®, a low dose, continuous regimen of EE (5 μg) and the progestin NA (2.5 mg). FemHRT® is available in two combinations, the first being Low-Dose FemHRT® 0.5/2.5 provides half the dose of estrogen and progestin found in femhrt 1/5. Low-Dose FemHRT® 0.5/2.5 is the lowest effective dose approved by the FDA to treat menopausal symptoms with this combination of hormones. The second formulation contains twice as much EE and NA as the low-dose form [77]. Estinyl is an EE-only form of estrogen replacement therapy that has been discontinued in the United States but remains in use in Europe. More low dose EE-containing methods are currently being developed for hormone replacement therapeutic purposes [78]. As is the case with hormonal contraceptives, there is little to no information available with regard to the potential immunological effects of different HRT regimens.

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Other sources of exposure

Since the 1980's, there has been a dramatic increase in use of oral contraceptives for other therapeutic purposes such as hormone replacement therapy, acne vulgaris, osteoporosis, and anti-aging [32]. Therapeutic doses of EE have steadily declined in the past 30 years, resulting in fewer cardiovascular and metabolic side effects. Nonetheless, little research has been performed with regard to systemic effects of EE administration under these newly-indicated medical conditions. EE is no longer used strictly for hormonal contraception, indicating that the increasing number of uses also leads to a greater proportion of the human population being prescribed this compound.

2.3 17β-estradiol: A brief overview

17β-estradiol (E2)

The natural estrogen, 17β-estradiol (IUPAC name: 17β-estra-1,3,5(10)-triene-3,17-diol), or E2, is the primary reproductive hormone in females, and is the dominant estrogen in reproductively active women. The androgen testosterone is the precursor in the body to E2 formation, with E2 production primarily occurring from developing follicles in the ovaries, corpus luteum, and the placenta [17]. E2, in addition to the secondary estrogens, estriol and estrone, is responsible for the development of female secondary sex characteristics, and is also responsible for the regulation of the menstrual cycle. EE is capable of mimicking the endocrine properties of its natural hormonal counterpart E2 [32]. E2 also promotes proliferation and growth of specific cells in sex organs and other reproductive tissues such as the endometrium and initiates epithelial cell proliferation in the vagina, uterus, and breast [79]. Similar to all steroid hormones, E2 readily diffuses across the plasma membrane and binds to the nuclear-bound estrogen receptor (ER) α or β [80].

Classical Estrogen Receptor Signaling

The ER is a member of a superfamily of nuclear receptors that act as ligand-activated transcription factors [81]. It is typically confined to the nuclear compartment of the cell and is inactive when not bound by E2 or another estrogenic molecule. In an unbound state, the ER is associated with heat shock proteins (i.e. hsp90). When E2 binds, the heat shock protein dissociates and a nuclear translocation signal is revealed which guides the ligand-receptor complex to an estrogen response element (ERE), a specific DNA sequence that is located in the promoter region of target genes [80]. Binding of the steroid-receptor complex to the ERE results in gene activation (i.e. gene transcription) and increased mRNA synthesis of estrogen responsive-genes [32]. Evidence has emerged for the existence of alternate ER signaling pathways and it is now accepted that ERs can regulate gene expression via additional pathways beyond the classical pathway presented here [82].

Immunological Effects of E2

In addition to its function as a reproductive hormone, E2 has been extensively documented to possess immunomodulatory capabilities [83]. This capability stems largely from the presence of estrogen receptors (ERs) on most immune organs (i.e. thymus, spleen) in addition to ER expression on many cell subsets involved in the immune response [84]. These cellular subsets include thymic cells [85], T cells [86], and macrophages [87]. E2 has been observed to cause a ten-fold increase in plasma B cells, resulting in an increase in both immunoglobulin production and auto-antibody production in C57BL/6 mice [88]. In addition, E2 has been demonstrated to regulate the production of IgG, IgM, and IgA in the genital tract of female rats following immunization [89]. E2 has been demonstrated to decrease the cytotoxicity of natural killer (NK) cells in several mouse strains, with a concomitant increase in immunoglobulin-producing cells [90], suggesting that decreased NK cell function also resulted in a decrease in inhibitory signals toward B cells. E2 has also been observed to stimulate extrathymic T cell differentiation in the liver, an occurrence which could potentially lead to T cells with autoimmune reactivity [91]. E2 also has the ability to modulate uterine eosinophils and macrophages in ovariectomized and sexually immature mice [92].

E2 has been extensively documented to cause increased IFN-γ mRNA and increased secretion of IFN-γ production in stimulated splenic lymphocytes [93-95]. This cytokine has been linked to the development of autoimmune conditions, especially lupus [96]. Chronic E2 administration via subcutaneous silastic implants has demonstrated the marked influence on the secretion of chemokines [97] and nitric oxide [98] by activated splenocytes, in addition to causing an increase in NF-κB signaling [99].

Another well-documented aspect of E2 and immune system interaction is in the area of the gonadal-thymic axis. Gonadectomy of either female or male rats leads to an increase in thymic growth [100] while periods of high sex hormones (i.e. puberty and pregnancy) are known to cause thymic involution in the mouse [101]. In addition, E2 treatment is known to lead to thymic involution as well as a loss in thymocyte cellularity [102]. There have been several proposed mechanisms for estrogen-mediated thymic-involution, including an E2-induced blockage in T cell development in the thymus. This mechanism was postulated based on the observation that E2-treated mice lost a significant number of immature $CD4^{+}CD8^{+}$ double

positive thymocytes, as well as $CD3^-/CD4^-/CD8^-$ early precursor cells. Further analysis revealed that the triple negative cells were almost entirely $CD44^+CD25^-$ cells, while the rest of the maturation stages ($CD44^+CD25^+$, $CD44^-CD25+$, $CD44^-CD25^-$) were not present at all [102]. Several alternatives to this theory have been suggested, including an alteration in bone marrow prothymocyte trafficking to the thymus [103], increased apoptosis of thymocytes [104], or damage to thymic epithelial cells [105] with the result being a decrease in the production of thymocyte-maturation and proliferation signals. The precise mechanism of E2-induced thymus involution, although well-studied, remains undetermined.

ER Binding and Relative Potency of 17α-ethinyl estradiol to 17β-estradiol

EE has been demonstrated to have greater than 2-fold affinity for the ER compared to E2 [106]. EE vapor pressure, an indication of volatility, has been measured at 4.5×10^{-11} mm Hg. In comparison, E2 has been demonstrated to be a lot more volatile with a reported vapor pressure of 2.3×10^{-10} [107]. Further, EE has been observed to be significantly more potent than E2 at inducing nuclear translocation of the estrogen receptor in the liver, with a concentration of E2 100-fold greater than EE needed to induce the same hepatic response [108]. Median effective concentrations for inducing vitellogenin synthesis in rainbow trout are 19-26 ng/l E2 and 0.95-1.8 ng/l EE [49]. These data suggest that with regard to estrogen-dependent vitellogenin production in aquatic species, EE is 11 to 27 times more potent compared to E2. Increased reproductive potency of EE relative to E2 has been observed in numerous studies [49, 109, 110]. Nonetheless, the comparative potency of EE to E2 on the immune system is undetermined.

2.4 Metabolic, Cardiovascular, and Non-Immune-Mediated Parameters Altered by EE

Hormonal contraceptive use has been linked to the alteration of several metabolic and vascular parameters, increased risk of certain cancers, and other negative side effects. EE stimulates or inhibits the synthesis or metabolism of many hepatic proteins, including the renin substrate angiotensinogen, clotting factors, corticosteroid-binding globulin, and throxine-binding globulin [111]. EE is considered to be carcinogenic to humans [112], though hormonal contraceptive use is considered to reduce the incidence of cancers of the ovary and endometrium

[113, 114]. EE is genotoxic to mammalian cells treated *in vitro* with 5 and 10 μM concentrations of EE [115]. Upon cellular activation, EE-treated cells exhibited an increase in mitotic activity and decreased sister chromatid exchanges but this effect was not observed unless cells were stimulated, indicating that EE is not potent enough to induce such effects without an additional activating stimulus. Chronic, 10-year administration of EE-containing oral contraceptives alters estrogen metabolism in rhesus monkeys [116]. These data suggest that endogenous plasma E2 concentrations were reduced partially because OCs enhanced the biotransformation of E2 into metabolites that could be more readily eliminated. EE also decreases liver cytochrome P450 enzymes in the liver of rats, further suggesting the metabolic influence of EE [117]. Oral contraceptives increase the ability of the liver to produce clotting factors, leading to an increased risk of blood clots [118]. Estrogen exposure has been shown to increase the risk of blood clots in the legs and lungs, and to cause clotting problems such as strokes and heart attacks [119]. Headache, nausea, breast discomfort, application site reactions, dysmenorrheal, and abdominal pain are the most commonly reported side effects of hormonal contraceptive patch use [120]. An increase in C-reactive protein, an important marker of systemic inflammation and potential cardiovascular risk, has been observed to be increased by oral but not transdermal use of contraceptives [121]. In addition, serum amyloid A, another marker of inflammation, has also been found to be increased by oral but not transdermal contraceptive use [122]. Oral but not transdermal EE caused a significant decrease in serum IGF-I and increase in growth hormone (GH) in post-menopausal women, as well as reduced lipid oxidation, increased fat mass, and reduced lean body mass compared to women administered EE transdermally [123]. Oral, but not transdermal, EE administration also decreases plasma levels of tissue-type plasminogen activator (tPA), a thrombolytic agent that can dissolve blood clots [124].

Nonethless, hormonal contraceptive use has also been linked to several noncontraceptive benefits, such as increased serum sex-hormone binding globulin (SHBG) [125] and high density lipoprotein (HDL) [126]. Increased SHBG, found only in women administered EE transdermally, can lead to a decrease in androgenic hormones such as testosterone and DHEA-S, indicating the potential for OC use as a treatment for women with hormonal disorders such as polycystic ovarian syndrome [127]. Further, improvement of acne, irregular menstrual cycles, anemia, and decreased incidence of ovarian cysts are some of the several reported benefits of hormonal contraceptive use [16, 128-131].

2.5 Immunological studies on 17α-ethinyl estradiol: Human and Animal Studies

Studies in Humans

It is evident from studies examining the effects of orally-delivered hormonal contraceptives in humans that there are several immune parameters affected by EE. Klinger et al. [132] compared the effects of two combination oral contraceptives on the human immune system. Lovelle®, an oral contraceptive (OC) containing 20 μg EE, and Valette®, an OC containing 30 μg EE were compared over one treatment cycle and the overall conclusion was that no immunosuppression occurred. However, Lovelle® increased peripheral $CD4^+$ cells, lymphocytes, monocytes, and granulocytes, while lowering serum IgA, IgM, and IgG. Valette® caused a decrease in $CD4^+$ lymphocytes during the treatment cycle which returned to normal by the end of the treatment. These results indicate a possibility of immunomodulation by EE, and may also suggest an upregulation of the immune response. This would typically be considered positive, however, long-term upregulation could conceivably result in pathological autoimmune conditions (i.e. lupus) which would otherwise remain latent or never occur. An alteration in $CD4^+$ cell subsets has been demonstrated to play both an inducing and preventative role in autoimmunity [133]. Reproductive hormones have been demonstrated to influence mucosal immunity [134]. An assessment of EE use on reproductive tract immunity examined IgA, IgG, IL-1β, IL-6, and IL-10 levels in cervical mucus samples from women on OCs and those not on OCs. Women on an OCs containing 35 μg of EE had significantly increased vaginal IgA concentrations in comparison to women using OCs. Auerbach et al. [135] investigated whether levels of lymphocyte subsets in the blood vary in women using OCs versus those not taking OCs at different stages of the menstrual cycle. Oral contraceptive use caused a significant increase in peripheral cytotoxic T cells ($CD3^+CD8^+$ cells) for two different OCs, one containing 35 μg EE (Cileste®) and the other containing 30 μg EE (Marvelon®). Several studies have shown an increase in serum levels on IgG and IgM in women taking oral contraceptives compared to women not taking oral contraceptives [136, 137]. Natural killer (NK) cell activity has been determined to be altered in oral contraceptive users, with Baker et al. finding that NK cell numbers were reduced and cytotoxic activity of NK cells was reduced at three months of use but

returned to similar cytotoxic capacity compared to non-OC users after six months [138]. Scanlan et al. [139] found NK cell cytotoxicity to be reduced both at three months and six months in a population of women who had been taking OCs for a mean duration of 36 months, compared to six months in Baker's study. This suggests that duration of OC use impacts the extent of immune alterations induced by EE. Anti-nuclear antibodies in patients with rheumatoid arthritis were increased in women taking oral contraceptives [140] and may contribute to the development of various autoimmune conditions such as systemic lupus erythematosous (SLE) but this has not been firmly established [141].

Conversely, several studies have determined that oral contraceptive use does not exert an effect on immunity [142-144]. Women on oral contraceptives containing from 30-50 μg of EE were compared to women not taking oral contraceptives and it was determined that there was no alteration in plasma levels of IgG and IgM [145]. Cell-mediated immune reactivity to T cell mitogens Concanavalin-A and phytohemagglutinin were demonstrated to be similar in women taking combined oral contraceptives compared to women who were not. The lymphocyte proliferative response was measured in women taking various forms of hormonal contraceptives and determined to be similar to that of age-matched controls [146]. However, when skin reactivity of women on combined oral contraceptives was tested with the contact allergen 2,4-dinitrochlorobenzene (DNCB), skin reactivity was demonstrated to be significantly increased [147].

Anti-EE antibodies have been suggested to play a role in the development of vascular thrombosis [148, 149]. This may be promoted by the infiltration of circulating immune complexes into the vascular wall. Vascular lesions are commonly observed in women using oral contraceptives [150], while anti-EE antibodies are also commonly observed in women taking OCs. Anti-EE antibodies have been observed in both healthy OC users and OC users with SLE [151, 152].

There are many often conflicting results of steroid-based oral contraceptives on plasma Ig levels [145]. The available literature is filled with conflicting and confounding results, with little attempt currently to investigate the effects of oral contraceptives or HRT on immune response. Numerous studies from the 1970s and 1980s have produced conflicting results. As technology has advanced and the number of uses/target population for EE-containing drugs has increased, it is imperative that immunological alterations be investigated more vigorously. More advanced

immunological and molecular assays now exist which can more accurately assess precisely what the effects of hormonal therapies are on the immune system. Formulations of hormonal contraceptives and routes of delivery have varied greatly in the ensuing years [153] and many of the immunological studies from the 1960, 1970s, and 1980s may not be applicable to modern formulations. Immunological studies have investigated only oral formulations of hormones and even those studies do not adequately address immunological effects and function. There are no studies which examine the immunological effects of transdermal, subcutaneous, or intramuscular injection of hormonal contraceptives or estrogen replacement therapy, an aspect which warrants intense investigation.

Studies in Animals

Studies with regard to the effect of EE on induced experimental allergic encephalomyelitis (EAE) in female Lewis rats have determined that EE injected subcutaneously in doses comparable with estrogen serum levels in the final term of human pregnancy (0.767 ± 0.427 μg/l EE) significantly suppressed the clinical signs of EAE [154]. Treatment with EE at the 29 μg/kg BW level caused a significant decrease in spleen and thymus weight in diseased rats, relative to vehicle-treated EAE-induced animals, as well as decreased the number of cellular infiltrates into the central nervous system, further diminishing clinical signs of EAE (an animal model of human multiple sclerosis). Oral feeding of EE to SJL mice resulted in decreased secretion of proinflammatory cytokines IFN-γ, TNF-α, and IL-6 by activated T cells [155]. There was also an inhibition of infiltrating lymphocytes into the central nervous system similar to what Trooster et al. had previously observed [154]. Oral doses of 50 μg and 200 μg EE were demonstrated to inhibit the production of TNF-α and IL-1β in collagen-induced arthritis-susceptible mice [156]. EE treatment significantly reduced secretion of pro-inflammatory factors, chemokines, and chemokine receptors in joint tissue. These studies suggest that EE is a potential therapeutic agent for treating rheumatoid arthritis in humans [156]. Experiments in lupus-prone NZB x NZW mice demonstrated that longevity of mice treated with EE or E2 was significantly reduced [157]. Furthermore, EE and E2 induced bladder distension, urine stasis, endometritis, and neoplasms, contributing to their early death relative to controls.

Two to four-week treatment of EE has been observed to cause marked but reversible thymus involution in Wistar rats injected with 10 μg EE daily. However, it could not be concluded whether involution of the rat thymus results in impairment in immune response [158]. In another study, a 40 μg dose of EE was observed to inhibit thymic growth by 26% in 5-week-old male rats but only 4% in 4-week old female rats [100]. EE has been observed to decrease numbers of both circulating peripheral lymphocytes and total spleen cell numbers in the lizard *Sceloporus occidentalis* [159]. However, spleen cell proliferation was increased following a single IP injection of either 0.01, 0.10, or 1.0 mg/kg BW of EE. It has been suggested that the association between females and higher rates of autoimmune disease is actually related to their lack of protective androgens, rather than due to estrogens accelerating the disease process [160]. Administration of both supraphysiological and physiological doses of EE to two spontaneous autoimmune disease mouse models, NZB/NZW F1 and NOD mice, was performed. Daily administration of 70 μg of EE resulted in an acceleration of the disease process, earlier death, decreased body weights and severe reduction of several organ weights. Mice receiving physiological doses of 0.1, 1.0, and 8 μg EE/day did not demonstrate increased mortality or accelerated disease progression, leading to the conclusion that accelerated disease processes were a function of EE toxicity and that females experience exacerbated autoimmune conditions because they lack the protective effects of androgens.

As can be inferred from the above studies, fundamental endpoints regarding EE effects, especially in direct comparison to E2, on a primary (thymus) and a mature (spleen) lymphoid organ still needs to be studied. The following studies attempt to address unanswered questions with regard to environmental exposure of EE on the immune system across age and sexes, as well as address potential differences due to route of exposure.

Figure 2.1: Primary Metabolic Pathways of 17α-ethinyl estradiol (EE) in humans

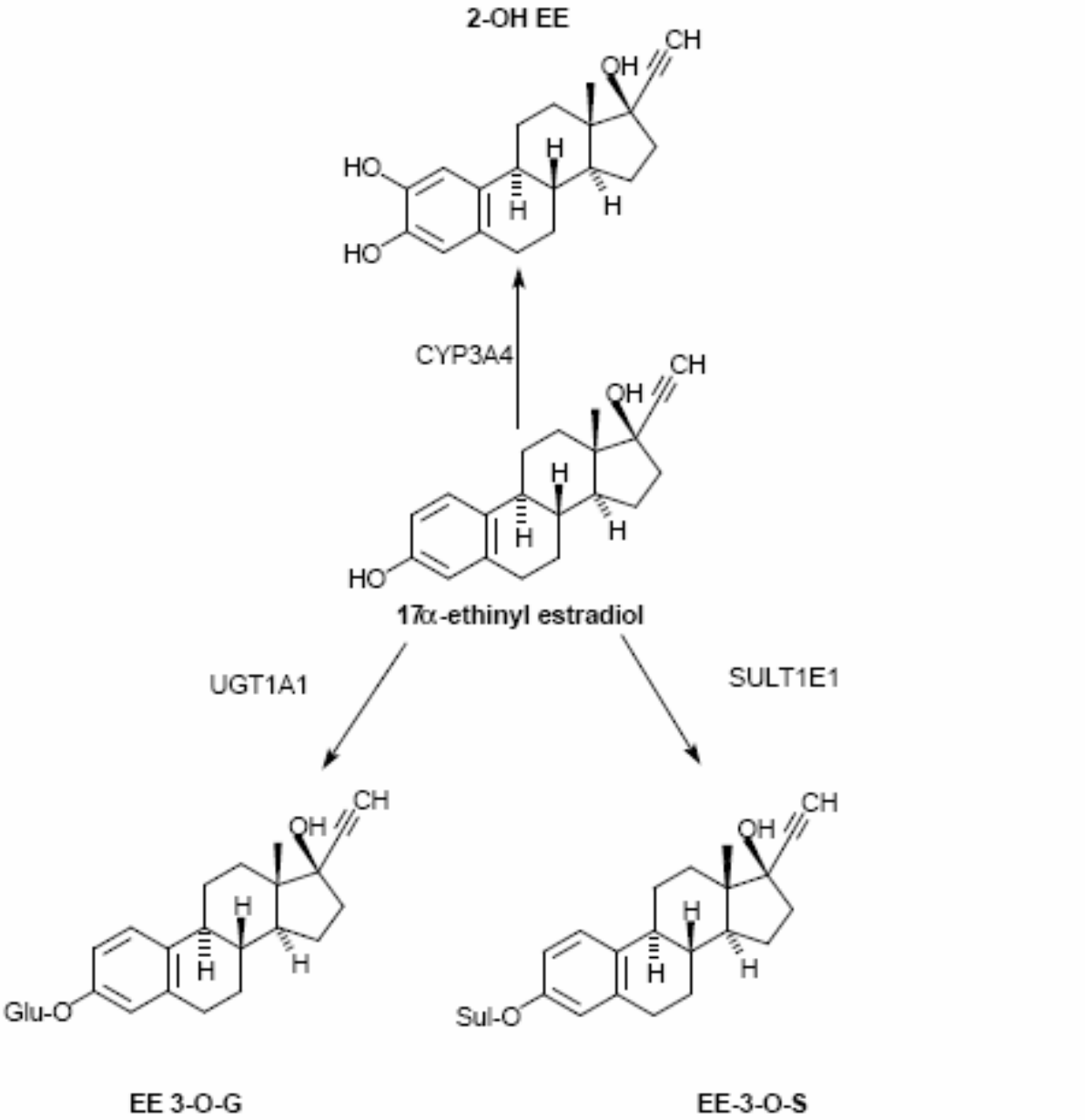
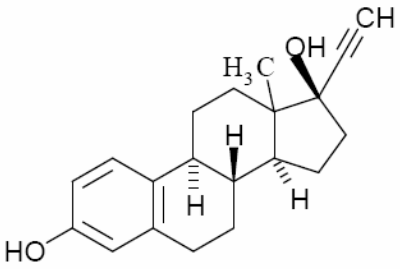


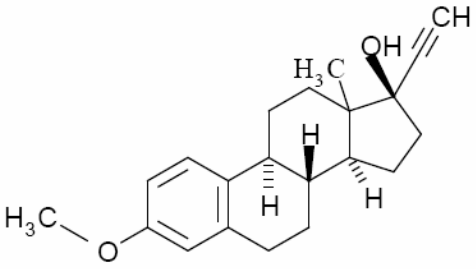
Figure from Zhang, H. et al., 2007. Pharmacokinetic Drug Interactions Involving 17α-Ethinylestradiol. *Clinical Pharmacokinetics*. 46 (2): 133-157.

Figure 2.2: Common Synethetic and Natural Estrogenic Compounds

A: Synthetic Estrogens Used in Hormonal Contraceptives

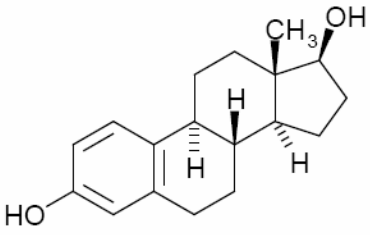


(i) 17α-ethinyl estradiol (EE)

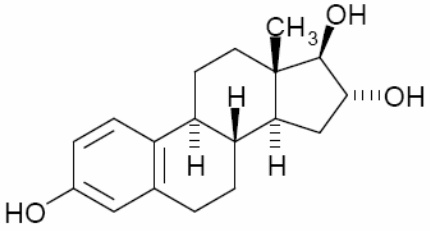


(ii) Mestranol

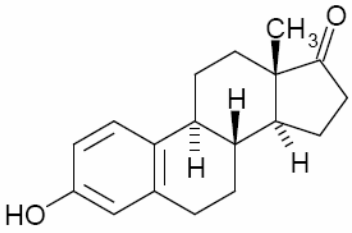
B: Three Main Estrogens Produced by the Human Body



(i) 17β -estradiol

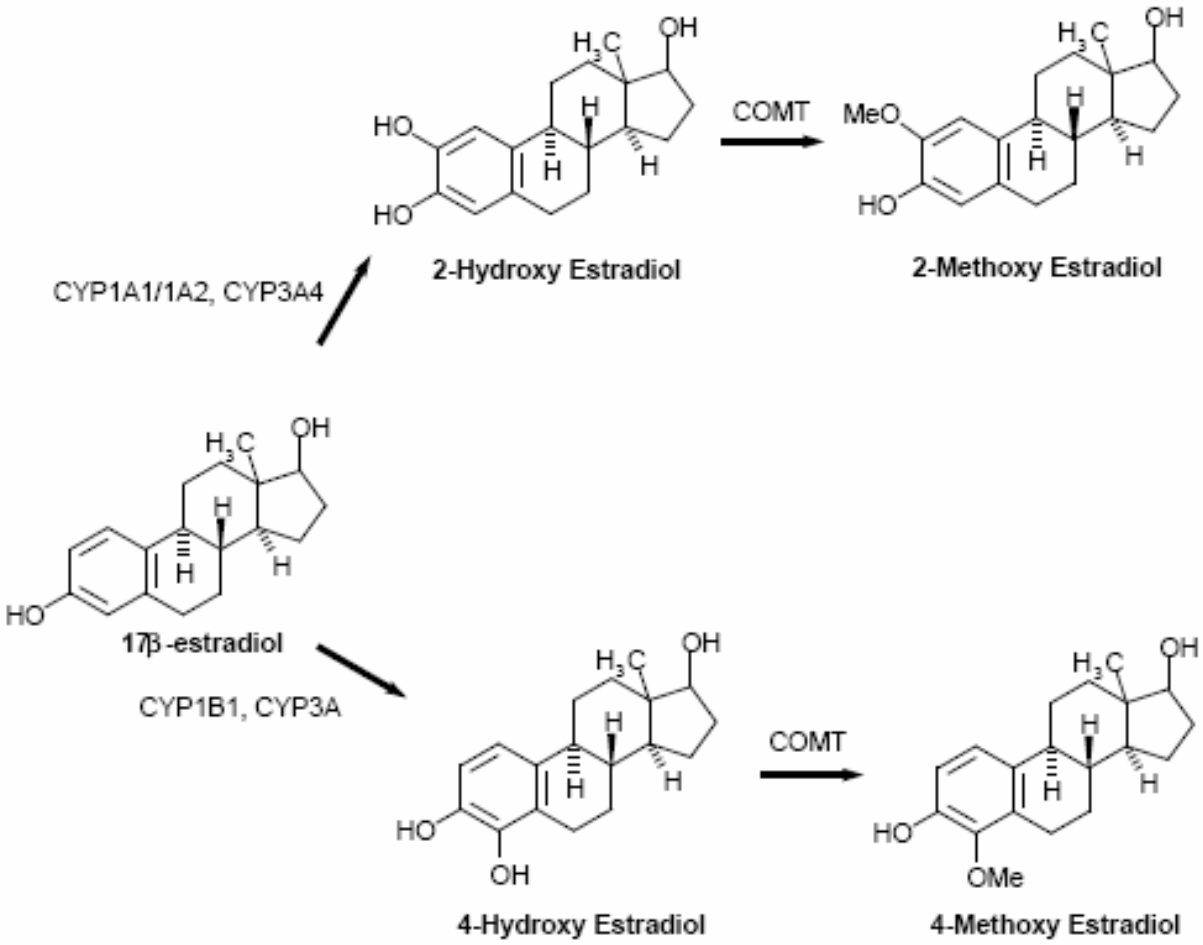


(ii) Estriol



(iii) Estrone

Figure 2.3: Common Metabolic Pathways of 17β-estradiol (E2) in humans



The main route of E2 metabolism consists of the formation of 2-hydroxy estradiol or 4-hydroxy estradiol. Catechol-*O*-methyl transferase (COMT) further metabolizes these products to form 2-methoxy estradiol and 4-methoxy estradiol.
Figure from Nester, RA. 2003. Characterization of CYP450 Enzymes and Estradiol Metabolism. *Master's Thesis*. Virginia Commonwealth University. Richmond, Virginia.

Table 2.1: 17α-ethinyl estradiol containing hormonal contraceptives

Monophasic Products

Brand Name	17α-ethinyl estradiol (μg)	Progestin (μg)
Alesse-28	20 μg ethinyl estradiol	100 μg levonorgestrel
Apri	30 μg ethinyl estradiol	150 μg desogestrel
Aviane	30 μg ethinyl estradiol	100 μg levonorgestrel
Brevicon	35 μg ethinyl estradiol	500 μg norethindrone
Demulen 1/35-21	35 μg ethinyl estradiol	1000 μg ethynodiol diacetate
Demulen 1/35-28	35 μg ethinyl estradiol	1000 μg ethynodiol diacetate
Demulen 1/50-21	50 μg ethinyl estradiol	1000 μg ethynodiol diacetate
Demulen 1/50-28	50 μg ethinyl estradiol	1000 μg ethynodiol diacetate
Desogen	30 μg ethinyl estradiol	150 μg desogestrel
Lessina	20 μg ethinyl estradiol	100 μg levonorgestrel
Levlen 21	30 μg ethinyl estradiol	150 μg levonorgestrel
Levlen 28	30 μg ethinyl estradiol	150 μg levonorgestrel
Levlite 28	20 μg ethinyl estradiol	100 μg levonorgestrel
Levora 0.15/30-21	30 μg ethinyl estradiol	150 μg levonorgestrel
Levora 0.15/30-28	30 μg ethinyl estradiol	150 μg levonorgestrel
Loestrin 21 1.5/30	30 μg ethinyl estradiol	1500 μg norethindrone acetate
Loestrin 21 1/20	20 μg ethinyl estradiol	1000 μg norethindrone acetate
Loestrin FE 1.5/30	30 μg ethinyl estradiol	1500 μg norethindrone acetate
Loestrin FE 1/20	20 μg ethinyl estradiol	1000 μg norethindrone acetate
Lo-Ovral 28	30 μg ethinyl estradiol	300 μg norgestrel
Low-Ogestrel 28	30 μg ethinyl estradiol	300 μg norgestrel
Microgestin 1.5/30	30 μg ethinyl estradiol	1500 μg norethindrone acetate
Microgestin 1/20	20 μg ethinyl estradiol	1000 μg norethindrone acetate
Microgestin FE 1/20	20 μg ethinyl estradiol	1000 μg norethindrone acetate
Modicon	35 μg ethinyl estradiol	500 μg norethindrone
Necon 0.5/35-21	35 μg ethinyl estradiol	500 μg norethindrone
Necon 0.5/35-28	35 μg ethinyl estradiol	1000 μg norethindrone
Necon 1/35-21	35 μg ethinyl estradiol	1000 μg norethindrone
Necon 1/35-28	35 μg ethinyl estradiol	1000 μg norethindrone
Necon 1/50-21	50 μg ethinyl estradiol	1000 μg norethindrone
Necon 1/50-28	50 μg ethinyl estradiol	1000 μg norethindrone
Nelova 1/35	35 μg ethinyl estradiol	1000 μg norethindrone
Nelova 1/50	50 μg ethinyl estradiol	1000 μg norethindrone
Nordette 28	30 μg ethinyl estradiol	150 μg levonorgestrel
Norinyl 1/35	35 μg ethinyl estradiol	1000 μg norethindrone
Nortrel 0.5/35	35 μg ethinyl estradiol	500 μg norethindrone
Nortrel 1/35	35 μg ethinyl estradiol	1000 μg norethindrone
Ogestrel 0.5/50-28	50 μg ethinyl estradiol	500 μg norgestrel
Ortho-Cept	30 μg ethinyl estradiol	150 μg desogestrel

Monophasic Products (continued...)

Brand Name	17α-ethinyl estradiol (μg)		Progestin (μg)	
Ortho-Cyclen	35 μg	ethinyl estradiol	250 μg	norgestimate
Ortho-Novum 1/35	35 μg	ethinyl estradiol	1000 μg	norethindrone
Ovcon 35	35 μg	ethinyl estradiol	400 μg	norethindrone
Ovcon 50	50 μg	ethinyl estradiol	1000 μg	norethindrone
Ovral 28	50 μg	ethinyl estradiol	500 μg	norgestrel
Portia	30 μg	ethinyl estradiol	150 μg	levonorgestrel
Tri-Norinyl 28	35 μg	ethinyl estradiol	500, 1000 μg	norethindrone
Yasmin 28	30 μg	ethinyl estradiol	3000 μg	drospirenone
Zovia 1/35E	35 μg	ethinyl estradiol	1000 μg	ethynodiol diacetate
Zovia 1/50E	50 μg	ethinyl estradiol	1000 μg	ethynodiol diacetate
Biphasic Products				
Jenest 28	35 μg	ethinyl estradiol	500, 1000 μg	norethindrone
Kariva	10, 20 μg	ethinyl estradiol	150 μg	desogestrel
Mircette	20, 100 μg	ethinyl estradiol	150 μg	desogestrel
Necon 10/11-21	35 μg	ethinyl estradiol	500, 1000 μg	norethindrone
Ortho-Novum 10/11	35 μg	ethinyl estradiol	500, 100 μg	norethindrone
Triphasic Products				
Cyclessa	25 μg	ethinyl estradiol	100, 125, 150 μg	desogestrel
Enpresse	30. 40. 30 μg	ethinyl estradiol	50, 75, 125 μg	levonorgestrel
Estrostep 21	20, 30, 35 μg	ethinyl estradiol	1000 μg	norethindrone
Estrostep FE	20, 30, 35 μg	ethinyl estradiol	1000 μg	norethindrone
Ortho Tri-Cyclen	35 μg	ethinyl estradiol	180, 215, 250 μg	norgestimate
Ortho Tri-Cyclen LO	25 μg	ethinyl estradiol	180, 215, 250 μg	norgestimate
Ortho-Novum 7/7/7	35 μg	ethinyl estradiol	750 μg	norethindrone
Tri-Levlen 21	30, 40, 30 μg	ethinyl estradiol	50, 75, 125 μg	levonorgestrel
Tri-Levlen 28	30, 40, 30 μg	ethinyl estradiol	50, 75, 125 μg	levonorgestrel
Tri-Norinyl 28	30, 40, 30 μg	ethinyl estradiol	30, 40, 30 μg	norethindrone
Triphasil 28	30, 40, 30 μg	ethinyl estradiol	50, 75, 125 μg	levonorgestrel
Trivora 28	30, 40, 30 μg	ethinyl estradiol	50, 75, 125 μg	levonorgestrel
Alternate Hormonal Contraceptives				
Nuva Ring (Vaginal Ring)	15 ug	ethinyl estradiol	120 ug	etonogestrel
Ortho Evra (Patch)	20 ug	ethinyl estradiol	150 μg	norelgestromin

Hormonal contraceptives can be classified by schedules of administration (Monophasic, Biphasic, Triphasic). These schedules involve alternating the dose of either EE or the progestin component. The theory behind phasic preparations is that it would decrease the amount of total progestin administered in an attempt to reduce adverse metabolic effects.

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Chapter 3: Subacute oral administration of low dose 17β–estradiol or 17α–ethinyl estradiol does not markedly alter the immune system of young adult and aged C57BL/6 mice

Tyson P. Brummer, Rebecca A. Phillips, Robert M. Gogal Jr., S. Ansar Ahmed

Center for Molecular Medicine and Infectious Diseases

Department of Biomedical Sciences and Pathobiology

Virginia-Maryland Regional College of Veterinary Medicine

Virginia Polytechnic Institute and State University

1410 Prices Fork Road

Blacksburg, VA 24061

USA

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Address correspondence and reprint requests to: Dr. S. Ansar Ahmed, Professor and Director, Center for Molecular Medicine and Infectious Diseases, 1410 Prices Fork Road, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA 24061-0342. Phone: (540) 231-5591 Fax: (540) 231-3426 Email: ansrahmd@vt.edu

Running Head: Immune effects of oral estrogen exposure
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Abstract

Due to growing awareness that estrogens and their metabolites are leaked into the environment at low concentrations through wastewater and agricultural run-off, it is increasingly important to examine the potential adverse health effects of environmental exposure to estrogens. Little attention has been paid to the immunological effects of 17β-estradiol (E2) and 17α-ethinylestradiol (EE), potent modulators of the immune system. Does exposure to E2 or EE at low concentrations for a subacute duration affect this system? Are the effects similar for both hormones and between genders? Are these effects similar in young and aged mice? These questions were addressed here, where 10 ng/kg BW of E2, EE, or vehicle were orally administered every other day for 21 days to young (6 week-old) and aged (>15 month-old) C57BL/6 mice. As expected, significant gender and age-related differences were noted with regard to thymus weight, thymocyte recovery, spleen weight, and splenocyte recovery. However, low dose treatment of E2 or EE produced no marked effects on the thymus or spleen organ to body weight ratios, cell numbers, or lymphocyte subsets. Low dose oral estrogens did not alter the ability of activated splenocytes to induce interferon-γ or nitric oxide. In summary, our studies of young and aged C57BL/6 mice suggest that low dose subacute exposure to E2 or EE did not markedly affect lymphoid organs (thymus or spleen weight) or lymphocyte subsets.

Introduction

Estrogens are biologically active steroid hormones that originate from androgen precursors and have a common cyclopenta-*o*-perhydrophenanthrene ring [1]. Natural estrogens are produced by the ovaries and are responsible for stimulating female secondary sex characteristics and systemic effects such as long bone growth and maturation. Synthetic estrogens are the second most prescribed drugs in the United States. They are widely used as oral contraceptives and as a treatment for post-menopausal conditions, cancer, osteoporosis, and other medical conditions (i.e. hormonal imbalances) [2]. Both natural and synthetic estrogens were found as environmental contaminants primarily due to incomplete removal from municipal or animal wastewater treatment facilities, and the use of livestock manure as fertilizer [3]. The presence of 17β-estradiol (E2) and 17α-ethinylestradiol (EE) and their metabolites in the environment raises concern because these compounds are known to be endocrine disrupting chemicals (EDCs) [4-6]. They are bioactive at very low concentrations and lipid soluble with the potential to bioaccumulate in body fat. EDCs mimic or block the function of endogenous hormones. Furthermore, estrogens are capable of altering immune system function [7-9]. Currently, it is not known at what levels they pose a threat to human health [5, 10].

E2 and EE are the two most common circulating forms of estrogen in sewage treatment water effluents [11]. These estrogens were found in ocean sediments [12], wastewater [13], agricultural soils [14, 15], lakes, and rivers worldwide [1]. Estrogens have been consistently found in sewage treatment plant discharges in the ng/l range due to incomplete removal from wastewater [16]. The natural estrogen, E2, was documented to be present in freshwater at concentrations as low as 1 ng/l and demonstrated to induce feminization of fish, indicating the ability of environmental estrogens to adversely affect the endocrine system even at very low levels [17, 18]. Concentrations of E2 in sewage treatment discharge were found to range anywhere from 1 ng/l all the way up to the μg/l range [19]. Interestingly, these authors have found concentrations of EE are somewhat lower. However, EE is more persistent because the ethinyl group prevents oxidative attack at C17. Therefore, bacterial degradation of EE is slower than degradation of E2 [20].

In aquatic systems, EE was demonstrated to be significantly more potent at inducing vitellogenin (Vtg) in juvenile rainbow trout than E2. Thorpe et al. [21] found that an E2

concentration 11 to 27 times greater than EE was needed to induce the same Vtg response . Additionally, EE at a concentration of 25 ng/l was found to produce vitellogenesis in male trout to levels comparable to that of mature female trout. Concentrations as low as 0.1 ng/l were reported to produce a significant rise in Vtg in trout [22]. Furthermore, in the rat, EE was shown to be up to 10 times more potent in terms of increased uterine epithelial cell hypertrophy [23]. Although these studies investigate the effects of E2 and EE on reproductive function in fish, the effects of EE or its relative potency are not investigated, nor well known with respect to the immune system. With regard to EE, this is especially true when compared to the effects known to be produced by the more well-studied E2.

Gender-related differences in immune function are known to exist. In general, the female human immune system is regarded as more responsive, especially in B-cell-mediated immunity, where females tend to have higher levels of serum immunoglobulins [24-26]. Females tend to be more immunologically responsive than males to both self and non-self antigens and tend to be more resistant to certain infections. In addition, at the cell-mediated level, females have increased $CD4^+$ cells and elevated $CD4^+/CD8^+$ ratio [27]. Estrogens are known to affect all major cell subsets of the immune system including T cells, B cells, synovial macrophages, and endothelial cells [25, 28-30]. In addition, there is a strong bi-directional relationship between reproductive hormones and the immune system. For example, estrogen administration leads to thymic involution while neonatal thymectomy leads to abnormal function of ovaries, testes, and thyroid gland [29]. Furthermore, estrogens were shown to alter many experimental autoimmune conditions, which tend to be female-predominant [31].

There are several known gender and age-related effects attributable to environmental estrogen exposure. This exposure can occur via food, soil, air, and water and can include compounds as varied as plastics (bisphenol A), detergents (octylphenol), pesticides (methoxychlor, DDT) and many others. Environmental estrogens have been implicated in contributing to increased rates of autoimmune disease development in females [32]. Chlordane, a pesticide that mimics estrogen function, was shown to significantly alter myeloid cell development in female mice prenatally exposed, yet males were unaffected [33]. Furthermore, chlordane exposure during fetal life was demonstrated to alter natural killer cell activity, as well as impair the mixed lymphocyte reactivity of spleen cells in males but unaffecting female reactivity [34, 35]. Interestingly, these effects were not seen during exposure as adults. These

observations demonstrate the differential response of the immune system to environmental estrogen exposure, varying with the immune parameter being analyzed, as well as across age and gender.

It is well established that an individual's immune function changes with age. The phagocytic ability of neutrophils was demonstrated to decrease with age, resulting in diminished non-specific immunity [36]. In addition, T helper cells and NK cells were found to decrease with age, suggesting a decreased efficacy in T-cell-mediated immunity [37]. The most striking differences across age ranges are related to the involution of the thymus, which begins following puberty and continues into geriatric age [38].

It is unknown whether immunomodulation via environmental estrogens occurs equally with regard to gender. In addition, the potential effects of estrogenic compounds on the geriatric population are a concern due to increasing lifespan and aging of the "Baby Boomer" population. In this study, the effects of a subacute low-level exposure of a natural estrogen (E2) were compared to a similar exposure of a synthetic estrogen (EE), on the immune system of young (6-week-old) and aged adult (>15 months-old) male and female C57BL/6 mice.

Materials and Methods

Mice

Young (6 weeks) and aged (> 15 months) male and female mice were randomly divided into treatment groups: (i) control (corn-oil only, young females weighing 13.33 ± 0.11 g, young males weighing 16.67 ± 0.81 g, aged females weighing 29.21 ± 1.81 g, aged males weighing 34.52 ± 3.65 g.) , (ii) E2, (young females weighing 13.39 ± 0.29 g, young males weighing 16.49 ± 0.42 g, aged females weighing 27.58 ± 1.92, aged males weighing 41.42 ± 1.35 g). and (iii) EE (young females weighing 12.72 ± 0.43 g, young males weighing 15.66 ± 0.56 g, aged females weighing 28.28 ± 1.49 g, aged males weighing 38.02 ± 4.93 g). Each treatment group was comprised of 5-7 mice for a total of 81 animals. Mice were originally obtained from Charles River Laboratories (Wilmington, MA). Aged mice were obtained as juveniles and allowed to mature at our facility. All mice were maintained at the Center for Molecular Medicine and Infectious Diseases Animal Laboratory facility, fed a commercial pellet diet devoid of estrogenic

hormones (7013 NIH-31 Modified 6% Mouse/Rat Sterilizable Diet, Harlan Teklad, Madison, WI), given water *ad libitum*, and housed 2-5 mice per cage in traditional polycarbonate plastic cages. Mice were maintained in a 12/12 hr light/dark cycle at 23^{o}C and sacrificed within 24 hr of the last oral dose of E2 or EE. All animal procedures including housing, dosing, and euthanasia were in accordance with the guidelines of the Animal Care Committee at Virginia Polytechnic Institute and State University.

Estrogen dosing

Mice were orally dosed, as per our previously described methods [39] every other day for a period of 21 days with one of the following: oil only, E2 (10 ng/kg), or EE (10 ng/kg) in 10 µl of tocopherol-stripped-corn oil (ICN, Aurora, Ohio). The corn oil chemical composition was as follows: glycerides 98.8 wt.%, unsaponifiable matter 1.2 wt.%, and free fatty acids 0.07 wt.%. It contained only trace amounts of squalene and carotenoids, as well as 0.02% of ubiquinone, stigmasterol 0.07%, and campestrol 0.2%. Dosing with a micropipette ensured that each mouse received 10 µl of their assigned treatment. Each mouse was weighed 24 hr before their first dosing and at the end of each week during the 21-day dosing period. Mice were euthanized by cervical dislocation within 24 hr of the last oral dosing.

Organ and cell collection

The thymus and spleen were collected from each mouse. Spleens were collected under sterile conditions and lymphocytes were isolated according to our previously published procedures [40]. Briefly, lymphoid organs were passed over a sterile 60-mesh steel screen and the cells were washed in RPMI-1640 media devoid of estrogenic phenol red (Cell Gro, Mediatech, Herndon, VA). Spleen cells were treated with Tris-ACK-NH_4Cl lysis buffer (pH 7.2) and washed twice. Cells were then suspended in RPMI-1640 media supplemented with estrogen-devoid 10% charcoal-stripped fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine (ICN, Costa Mesa, CA), 50 IU/ml penicillin (Mediatech, Herndon, VA), 50 µg/ml streptomycin (Mediatech, Herndon, VA), and non-essential amino acids (Fisher, Pittsburgh, PA). Lymphocyte numbers were determined by a Beckman Coulter Multisizer^{TM} 3 Cell Counter (Miami, FL) and adjusted to 5 x 10^6 cells/ml prior to culture.

Flow cytometry

Thymocytes and splenocytes were stained and subjected to dual color flow cytometric analysis per our previously reported work [40]. Briefly, 100 μl of freshly enriched thymocytes or splenocytes at a concentration of 5 x 10^6 cells per ml (5 x 10^5 cells per well) were plated in Corning 96-well round-bottom tissue-culture plates (Corning, NY). Splenic lymphocytes were stained with PE-anti-CD45R (B220, a pan B cell marker) and FITC-anti-CD11b (a macrophage, natural killer cell, and neutrophil marker), in addition to FITC-anti-CD4 and PE-anti-CD8 antibodies (all from eBioscience, San Diego, CA). Single staining was performed with anti-CD45R and anti-CD11b (eBioscience, San Diego, CA). Thymocytes were dual stained with FITC-anti-CD4 and PE-anti-CD8 antibody cocktail. Cells were incubated with the antibodies on a shaker at 4^{o}C for 30 min. Phosphate buffered saline (PBS, pH 7.4) (100 μl, Fisher Scientific, Pittsburgh, PA) was then added to each well and the plates were centrifuged at 250g for 10 min. Supernatants were aspirated and 200 μl of 2% paraformaldehyde were added to each well to fix the cells. Cells were analyzed the following day on a Coulter Epics XL/MXL flow cytometer (Hialeah, FL).

Cell culture

Splenic lymphocytes (2.5 x 10^6 cells) were stimulated with either an optimal concentration of concanavalin A (Con-A) (5 μg/ml, Sigma, St. Louis, MO), anti-CD40 (10 μg/ml, eBioscience), or left unstimulated in media only. Cells were cultured in 24-well plates for 24 or 48 hr. At the end of the culture period, cells and supernatants were collected and frozen at –80^o C.

Interferon-γ (IFN-γ) ELISA

The levels of IFN-γ in supernatants from splenic lymphocytes cultured for 24 or 48 hr were measured by an ELISA, as in our previously published studies [7, 41]. Plates were read on an optical density plate reader at a wavelength of 450 nm (Molecular Devices, Sunnyvale, CA). A linear regression line, used to determine the level of IFN-γ in the supernatants, was calculated using SoftMax Pro software, based on the concentration of the recombinant standards versus optical density.

Griess assay

Nitric oxide levels were measured in supernatants derived from splenic lymphocytes, with a Griess assay which measures nitrite, an end product of oxidation of nitric oxide, as per our previously published procedures [41, 42]. Briefly, 50 µl of nitric oxide standards, with serial dilutions from 1000 µM to 0.5µM nitric oxide, or supernatants were added to a 96-well optical density plate. Equal volumes of Griess assay reagents, 50 µl 1% (w/v) sulfanilamide (Sigma St. Louis, MO) and 50 µl 0.1% (w/v) naphthylethylenediamine dihydrochloride (Sigma, St. Louis, MO) were then added to each well and the color change was immediately measured at 550 nm with an ELISA reader (Molecular Devices, Sunnyvale, CA). The nitric oxide levels in the supernatants were calculated using SoftMax Pro software from Molecular Devices Inc. A standard curve was generated by linear regression.

Statistical Analysis

Dr. Stephen Werre and Daniel Ward conducted all statistical procedures at the Laboratory for Study Design and Statistical Services, Virginia-Maryland Regional College of Veterinary Medicine. The GLM procedure of the SAS system (version 8.1, SAS Institute Inc., Cary, NC 27513) was used to perform an analysis of variance to test for treatment effects. Treatment means were separated using Tukey's HSD ($p < 0.05$). Violations of model assumptions were assessed using residual plots. Each response variable was analyzed separately. The null hypotheses tested consisted of equality of the treatment means. The experiment was analyzed as a randomized complete block design, where blocks were experiment days to correct for day-to-day variation.

Results

Body weights were unaffected by treatment

Neither E2 nor EE at 10 ng/kg significantly altered the rate of weight gain or loss in males or females (data not shown).

Thymus weight, recovery, and major cellular subsets were unaffected by treatment

Thymus to body weight ratio and thymocyte recovery (Table 1A) were unchanged by treatment with E2 or EE. Thymus to body weight ratio and thymocyte recovery were significantly less in aged adults (across gender and across all treatment groups) compared to young adults, with the young adult ratio being 2.4 times greater than the old adult ratio and young thymocyte recovery being 3.4 times greater than that of old adults. This finding is a result of aging and the involution of the thymus, a process which begins shortly after puberty and continues throughout adulthood. In regards to analysis of thymus to BW ratio across gender, both young and aged adult females were significantly greater in comparison to the male thymus to BW ratio of both young and aged. The female ratio was 1.6 times greater than that of males, regardless of age. Treatment with E2 or EE had no significant effect on the T cell maturation markers CD4 or CD8 regardless of gender or age. The % of mature $CD4^+CD8^-$ and $CD4^-CD8^+$, as well as immature $CD4^+CD8^+$ and $CD4^-CD8^-$ cells was not significantly changed (Table 1).

Spleen weight, recovery, and major cellular subsets were unaffected by treatment

The spleen was largely unaffected by either estrogen treatment; weight and splenocyte cellularity were not significantly changed (data not shown). Phenotypic expression of the T helper cell marker CD4 and the cytotoxic T cell marker CD8 was unaffected by treatment (Table 2). Aged adult male mice exhibited a 67% decrease in $CD4^+$ cells compared to young males, an observation that was statistically significant. Females did not demonstrate this trend, though both the aged male and female adults had significantly fewer $CD8^+$ spleen cells compared to the young mice of each gender, regardless of treatment. This was a decrease of almost 60%. Additionally, the B cell marker $CD45R_{B220}$, and the macrophage, natural killer cell, and neutrophil marker CD11b were unaffected by treatment (Table 2). B cell numbers were significantly increased due to age in both genders, an increase of 21% in aged adults compared to young adults, regardless of treatment group. CD11b expression was significantly greater in aged adult male and female mice compared to young mice, an increase of 93%. Furthermore, aged females demonstrated a significant increase in CD11b expression compared to aged males, demonstrating an increase of 1.96 times % expression..

IFN-γ at 24 h and nitric oxide at 48 h were not altered by treatment

IFN-γ levels (Figure 1) were not significantly changed by treatment. IFN-γ levels showed an increasing non-significant trend due to age. IFN-γ production was evident only in Con-A-activated splenocytes at 24 hours, while media controls and anti-CD40 stimulated little or no detectable levels of IFN-γ. There were no noticeable changes in the levels of nitric oxide produced by splenocytes stimulated with Con-A or anti-CD40, or cultured in media alone (data not shown).

Discussion

The presence of estrogens and estrogen-like substances in the environment has been extensively documented [11, 43]. E2 and EE are two of the most commonly found and most abundant estrogenic environmental contaminants. Exposure to estrogens was linked to both diminished reproductive system function and dysregulated immune system function [6, 28, 29]. However, little information is available regarding the effects of short-term exposure to low levels of natural and synthetic estrogens on the immune system. A dose believed to be comparable to environmental contaminant levels was used [14, 15, 44, 45]. Oral exposure was chosen to mimic exposure via contaminated food and drinking water, as well as to mimic exposure to synthetic estrogens such as oral contraceptives, though at a much lower dose. An identical dose (10 ng/kg) was used for both the natural and pharmacologic synthetic estrogen in order to determine whether or not the compounds exhibited comparable potency on the immune system. The results from this prefatory study in our experimental system suggest that subacute low level estrogen exposure, whether natural or synthetic, had no profound effects on thymic or splenic weights or major lymphocyte subsets.

Body weight was monitored throughout the study for two purposes: to a) ensure the doses used were not toxic and b) determine if there were any differential effects on weight change between the two compounds. Body weight change and food consumption are important indicators of toxicity and neither were adversely affected by treatment [46]. Estrogenic substances in fish were found to increase weight gain and have an anabolic effect [47], increasing food consumption and food conversion [48], but the opposite was found in chicks,

[49] indicating that there may be a physiologic difference in the effect of estrogens between species.

The thymus is a central T cell developmental organ from which mature T lymphocytes exit. It is a very sensitive target of estrogenic compounds, as estrogen administration leads to thymic involution, possibly due to increased apoptosis of immature $CD4^+/CD8^+$ cells [50]. Zoller et al. [51] demonstrated that E2 produced a significant decline in bone marrow T cell progenitors, as well as apoptosis of early thymus progenitors, markedly reducing cellular recovery and weight in the thymus. The thymus to BW ratio was not altered by treatment but was significantly increased due to both age and gender. Increasing levels of sex hormones following puberty contribute to the diminished thymus weight with advancing age [52]. Females were shown to have a larger thymus compared to males [40, 53].

No differences in resident thymocyte $CD4^+CD8^-$ cells, $CD4^-CD8^+$ cells, $CD4^-CD8^-$, or $CD4^+CD8^+$ cells due age, gender, or treatment were observed. However, thymocyte recovery was significantly increased in females compared to males. This is supportive of the increased thymus to BW ratio finding in females. Decreased thymocyte recovery in males may be an indication of androgens accelerating the rate of apoptosis, as previously reported [54].

Our analysis of the spleen supports previous reports that females have a greater spleen to BW ratio than males [55, 56]. Splenocyte recovery in the young females supports this trend, as they tended to yield greater splenocyte recovery than the young males. Aged adults (male and female) yielded similar recoveries but aged females exhibited a tendency for an increased spleen to BW ratio. One aspect to consider further is the fact that both aged adult male and female E2-treated mice exhibited a quantitative increase in splenocyte recovery (Figure 4). A greater number of mice per treatment group may have increased the statistical power and clarified whether this observation was due to treatment.

Spleen T cell, B cell, and CD11b expression were unaffected by treatment. Aged adult males demonstrate a significantly decreased T helper cell subset ($CD4^+CD8^-$) when compared to young adult males. This may indicate a reduced immune capability due to age in males. Similarly, cytotoxic T cells ($CD4^+CD8^-$) are significantly reduced by 50% in the aged adults. The aged males seem to compensate for this deficiency in T cell immunity with an increase in B cells and macrophages, both of which increase by as much as 25% in aged males. Interestingly, females do not exhibit a decrease in $CD4^+$ cells with respect to age, but aged adult controls

exhibited a decrease of almost 50% in $CD8^+$ cells. This decrease is similar to that seen in the aged males, indicating a general age-related decrease in $CD8^+$ cells which are presumed to be cytotoxic. This is consistent with the fact that advancing age is associated with declining health. Similar to the aged males, the aged females exhibit significantly increased B cells and macrophages with respect to young females.

Analysis of organ weights and cellularity indicated that treatment did not exert an effect on the immune system in our experimental system. However, in order to further confirm this, we assessed the functionality of cultured splenocytes to ensure there were no changes at the functional level. IFN-γ, a key cytokine, which regulates the activity of the major immune cell subsets, was measured in spleen cell supernatants cultured for 24 hr. *In vivo* estrogen treatment via silastic implant upregulates IFN-γ production by stimulated splenocytes [7]. However, the doses of estrogen employed were much higher than the low dose used in the present study. Nakaya et al. [57] found that *in vitro* E2 treatment of mouse splenocytes with concentrations up to 10^{-8} M upregulated production of IFN-γ. No differences in IFN-γ levels were found due to low dose subacute exposure of either E2 or EE, though there was an elevation in IFN-γ production with advancing age, similar to what was reported previously [58, 59].

Nitric oxide (NO) is a key immune system reactive oxygen species that is stimulated by IFN-γ, primarily produced by macrophages and important in controlling pathogenic infections [60]. When dysregulated, it has been linked to the development of autoimmune conditions. Predictably, since IFN-γ was unaltered, NO was also not altered by treatment.

In this study, experiments sought to ascertain whether subacute exposure to low dose E2 or EE modulates the mouse immune system. Our findings suggest that a short-term subacute exposure of either E2 or EE yielded no marked overt effects on the primary T cell developmental organ (thymus) or mature lymphoid organ (spleen) of normal, non-autoimmune prone mice. It is possible that E2 or EE may have induced other immunological changes, especially at the molecular level, aspects that were not assessed in the present studies. It is important to consider the possibility that *in vivo* antigenic challenge combined with E2 or EE treatment may have altered the results observed in this study. In addition, increased treatment duration or treatment frequency may have resulted in immune system alterations at this low dose. Furthermore, species and strain differences exist which may affect the sensitivity to estrogenic compounds, in addition to the timing of exposure to E2 or EE [61]. This lab has previously studied the effect of

prenatal exposure of diethylstilbestrol (DES) on the immune system of adult mice [62]. This *in utero* exposure resulted in long-term immunological alterations in the thymus 14 months after birth following an additional exposure to DES at 12 months of age. Thymus weight and thymocyte recovery were decreased in female but not male mice, in addition to increased dexamethasone-induced apoptosis of $CD4^+CD8^+$ thymocytes. This aspect highlights potential gender differences in immune modulation which may result from exposure to estrogenic compounds. A further caveat to consider in this study is the fact that mice were housed in polycarbonate plastic cages. Howdeshell et al. [63] found that regular autoclaving and re-use of plastic cages results in the leaching of Bisphenol A, an estrogenic compound, into the mouse environment. However, this is not believed to be of major concern as no classical E2-induced changes (i.e. thymic atrophy, IFN-γ increase, nitric oxide increase) were observed in the control groups. To our knowledge, this is the first study to investigate the immunological effects of EE and E2 in the context of age and gender in mice. Future studies aim to more specifically address the impact of EE treatment on serum immunoglobulins, NK cell activity, and the production of thymic cytokines relative to E2.

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Table 3.1: Thymus weight, thymus:BW ratio, cellularity, and thymocyte subsets

Table 3.1A: Female Thymus Weight, Thymus:BW Ratio and Cellularity

		Thymus Weight (Grams)	Thymus:Body Weight (Ratio)	Thymocyte Recovery Cells (Millions)
Young	Control	0.082 ± 0.008	0.0042 ± 0.0003	105.68 ± 19.26
	E2	0.079 ± 0.005	0.0041 ± 0.0002	105.04 ± 13.21
	EE	0.085 ± 0.011	0.0047 ± 0.0006	110.99 ± 24.72
Aged	Control	0.050 ± 0.006	0.0017 ± 0.0002	29.61 ± 7.14
	E2	0.040 ± 0.009	0.0015 ± 0.0003	26.42 ± 6.08
	EE	0.052 ± 0.006	0.0019 ± 0.0003	28.08 ± 6.31

Table 3.1B: Male Thymus Weight, Thymus:BW Ratio and Cellularity

		Thymus Weight (Grams)	Thymus:Body Weight (Ratio)	Thymocyte Recovery Cells (Millions)
Young	Control	0.069 ± 0.004	0.0028 ± 0.0001	79.01 ± 6.78
	E2	0.060 ± 0.004	0.0025 ± 0.0002	79.31 ± 13.80
	EE	0.062 ± 0.005	0.0026 ± 0.0003	65.42 ± 7.23
Aged	Control	0.037 ± 0.005	0.0011 ± 0.0001	21.20 ± 5.70
	E2	0.043 ± 0.010	0.0011 ± 0.0003	25.28 ± 7.79
	EE	0.038 ± 0.009	0.0010 ± 0.0001	26.63 ± 5.39

Young adult (6 weeks) and aged (<15 months) C57BL/6 mice were orally exposed to either E2 or EE (10 ng/kg BW) or vehicle-control corn oil every other day for 21 days. The data are presented as mean ± standard error of the mean. For each treatment group, $n \geq 5$. There were no significant differences due to treatment ($p < 0.05$). However, thymus:BW ratio and thymocyte recovery were significantly different due to age (Thymus:BW ratio; Young: 0.00347 ± 0.00023, Old: 0.00141 ± 0.00011, Thymocyte recovery or total thymocyte count; Young: 90.4 ± 6.21 x 10^6 cells, Old: 26.26 ± 2.44 x 10^6 cells). These comparisons were performed across age, regardless of gender or treatment group.

Table 3.2C: Female Thymic Cellular Subsets

		CD4- CD8- (% Expression)	CD4+ CD8+ (% Expression)	CD4+ CD8- % Expression	CD4- CD8+ (% Expression)
	Control	4.36 ± 1.93	81.06 ± 3.03	10.96 ± 0.72	3.57 ± 0.70
Young	**E2**	4.50 ± 1.67	79.50 ± 3.26	12.25 ± 0.91	3.75 ± 0.90
	EE	4.09 ± 1.50	78.33 ± 3.96	13.82 ± 1.88	3.77 ± 0.74
	Control	3.48 ± 0.40	67.70 ± 13.58	11.82 ± 0.83	2.85 ± 0.21
Aged	**E2**	3.50 ± 0.47	81.20 ± 1.66	12.02 ± 0.96	3.30 ± 0.52
	EE	3.50 ± 0.74	78.78 ± 1.83	14.33 ± 0.80	3.43 ± 0.73

Table 3.2D: Male Thymic Cellular Subsets

		CD4- CD8- (% Expression)	CD4+ CD8+ (% Expression)	CD4+ CD8- % Expression	CD4- CD8+ (% Expression)
	Control	2.36 ± 0.06	83.04 ± 1.25	11.76 ± 1.00	2.78 ± 0.28
Young	**E2**	2.90 ± 0.67	80.46 ± 2.37	13.20 ± 1.19	3.46 ± 0.54
	EE	2.05 ± 0.17	82.92 ± 1.03	12.10 ± 0.55	2.94 ± 0.43
	Control	3.68 ± 0.67	79.85 ± 3.06	12.57 ± 1.58	3.90 ± 1.01
Aged	**E2**	3.76 ± 0.61	80.43 ± 2.68	12.49 ± 1.43	3.32 ± 0.84
	EE	2.42 ± 0.26	82.53 ± 1.89	12.26 ± 1.64	2.78 ± 0.30

Table 3.1: Young adult (6 weeks) and aged (<15 months) C57BL/6 mice were orally exposed to either E2 or EE (10 ng/kg BW) or vehicle-control corn oil every other day for 21 days. Thymocytes were stained for T-cell subset markers CD4 and CD8 immediately after harvest. The data are presented as mean ± standard error of the mean. For each treatment group, $n \geq 5$. There were no significant differences due to treatment ($p < 0.05$).

Table 3.2: Splenic Cellular Subsets

Table 3.2A: Female Splenic Cellular Subsets

		CD4+ CD8- (% Expression)	CD4- CD8+ (% Expression)	B220 % Expression	CD11b (% Expression)
Young	Control	24.77 ± 0.55	14.05 ± 0.39	40.85 ± 2.41	6.82 ± 0.54
	E2	26.66 ± 0.74	11.85 ± 0.68	38.01 ± 2.59	7.27 ± 0.61
	EE	24.93 ± 0.39	12.13 ± 0.60	40.27 ± 2.16	10.44 ± 1.86
Aged	Control	30.80 ± 6.04	5.87 ± 1.57	50.50 ± 3.60	19.28 ± 5.00
	E2	22.67 ± 2.15	6.16 ± 0.74	39.87 ± 7.31	26.59 ± 5.19
	EE	25.57 ± 1.07	8.52 ± 1.44	48.47 ± 3.19	20.91 ± 4.62

Table 3.2B: Male Splenic Cellular Subsets

		CD4+ CD8- (% Expression)	CD4- CD8+ (% Expression)	B220 % Expression	CD11b (% Expression)
Young	Control	29.63 ± 0.59	14.51 ± 1.25	42.55 ± 2.46	8.76 ± 1.13
	E2	28.89 ± 1.71	12.95 ± 1.06	44.07 ± 1.09	9.50 ± 1.74
	EE	28.03 ± 0.89	12.84 ± 0.38	45.63 ± 1.06	8.05 ± 0.58
Aged	Control	19.70 ± 1.18	7.66 ± 0.96	55.57 ± 3.47	12.37 ± 2.31
	E2	19.30 ± 2.27	7.81 ± 0.88	61.18 ± 1.28	7.88 ± 0.51
	EE	19.00 ± 1.38	9.56 ± 0.45	53.20 ± 2.60	10.95 ± 1.18

Table 3.2: Young adult (6 weeks) and aged (<15 months) C57BL/6 mice were orally exposed to either E2 or EE (10 ng/kg BW) or vehicle-control corn oil every other day for 21 days. Splenocytes were stained for T-cell subset markers CD4 and CD8, B-cell marker B220, and macrophage, natural killer cell, and neutrophil marker CD11b immediately after harvest. The data are presented as mean ± standard error of the mean. For each treatment group, $n \geq 5$. There were no significant differences due to treatment ($p < 0.05$). However, across age, aged males, regardless of treatment, exhibited significantly fewer $CD4^+/CD8^-$ splenocytes compared to young males (Young males: 28.85 ± 0.65%, Aged males: 19.46 ± 0.46%). In addition, across age, regardless of gender or treatment group, both young male and female mice had significantly greater CD4-/CD8+ splenoyctes compared to aged males and females (Young: 13.03 ± 0.36%, Aged: 7.81 ± 0.45%). B cell numbers were significantly increased due to age in both genders, regardless of treatment (Young: 42.47 ± 1.01%, Aged 51.71 ± 1.90%).

Figure 3.1: Interferon-γ produced at 24 hours by Con-A-stimulated splenocytes

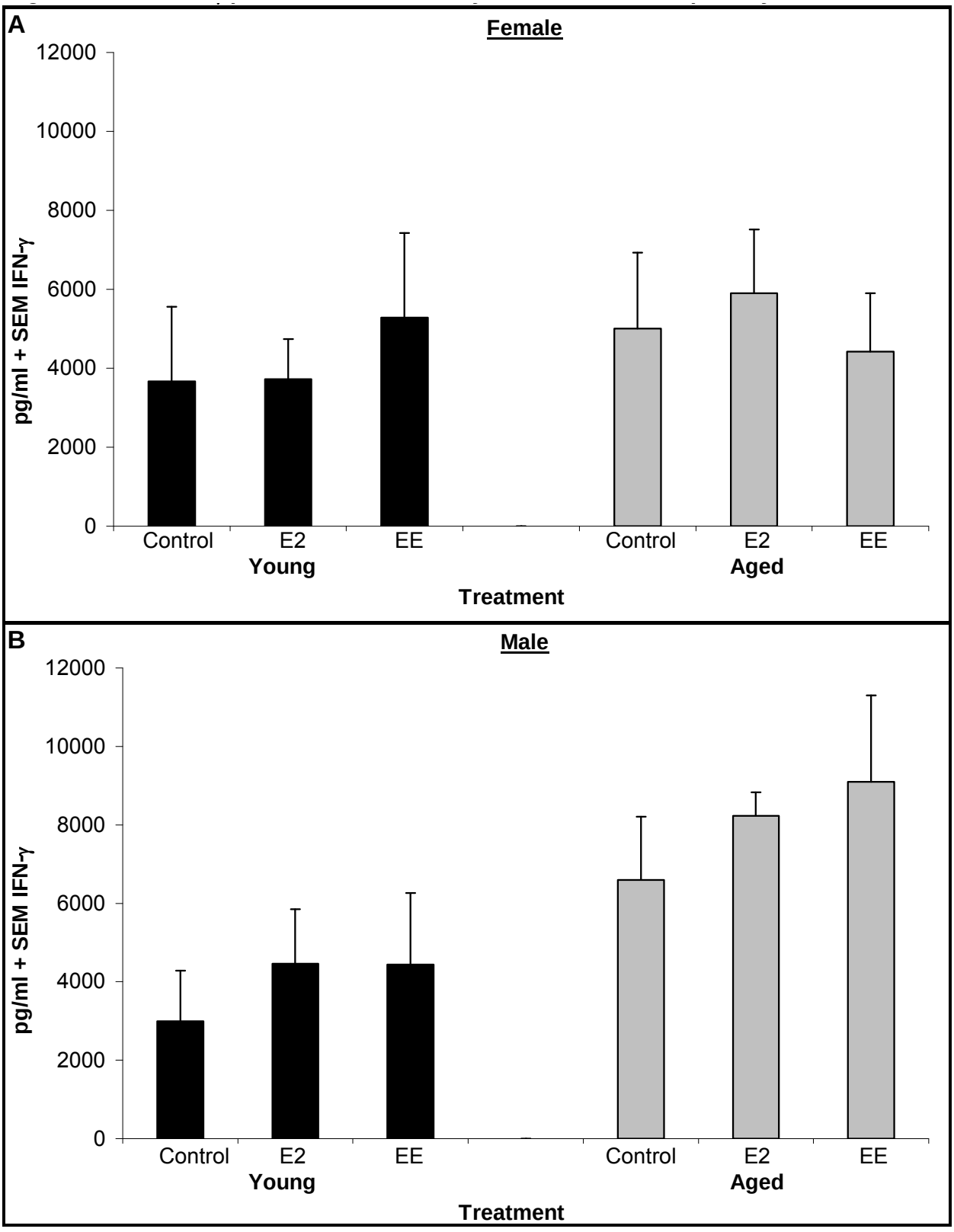

Figure Legend

Figure 3.1. Extracellular IFN-γ levels expressed in pg/ml (mean + SEM). Splenocytes adjusted to a concentration of 5 x 10^6 cells/ml were cultured for 24 h in media only, Concanavalin A, or anti-CD40. (**A**) Young and aged female IFN-γ levels. (**B**) Young and aged male IFN-γ levels. Media control splenocytes did not produce any detectable levels of IFN-γ and anti-CD40 produced little or no detectable levels of IFN-γ.

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Chapter 4: Subcutaneous, but not oral, administration of 17α-ethinyl estradiol markedly alters the thymus and reproductive organs of juvenile C57BL/6 mice relative to 17β-estradiol

Tyson P. Brummer, Rebecca A. Phillips, Richard P. Kerr, Robert M. Gogal Jr., D. Phillip Sponenberg, S. Ansar Ahmed

Center for Molecular Medicine and Infectious Diseases

Department of Biomedical Sciences & Pathobiology

Virginia-Maryland Regional College of Veterinary Medicine

Virginia Polytechnic Institute and State University

1410 Prices Fork Road

Blacksburg, VA 24061

USA

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Address correspondence and reprint requests to: Dr. S. Ansar Ahmed, Professor and Director, Center for Molecular Medicine and Infectious Diseases, 1410 Prices Fork Road, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA 24061-0342. Phone: (540) 231-5591 Fax: (540) 231-3426 Email: ansrahmd@vt.edu

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Abstract

Exposure to exogenous estrogens such as synthetic 17α-ethinyl estradiol (EE) occurs via multiple sources (i.e. environmental contamination, hormone replacement therapy, hormonal contraceptives). Presently, little data exist regarding the immune effects of EE via different routes of exposure and compared to 17β-estradiol (E2). To address this, two separate studies were performed in which intact juvenile female and male C57BL/6 mice were given daily subcutaneous or oral doses of either EE or E2 (0.04, 0.4, or 4.0 μg per 25 g BW) for 21 days. In the subcutaneous exposure study, both EE and E2 morphologically altered uterine and seminal vesicle weights. However, EE had a more pronounced effect compared to E2, especially in males, even at the lowest dose administered. Additionally, like E2, EE induced thymic atrophy in both sexes, but had a more profound effect than E2 in females. In female mice, thymic atrophy and thymic cellularity were decreased by subcutaneous EE and E2 at doses of 0.4 and 4.0 μg/25 g body weight. EE elicited more pronounced thymic atrophy compared to E2 at the 4.0 μg/25g dose. In males, thymocyte cellularity was decreased by both subcutaneous EE and E2 only at the highest dose tested (4.0 μg/25 g body weight), whereas only 4.0 EE decreased thymus to body weight ratios. Neither splenic weights, splenic cellularity, nor splenic cell phenotype were markedly affected by either estrogenic compound regardless of route of exposure. Collectively, these data demonstrate that select thymic and reproductive endpoints are altered following a 21-day subcutaneous exposure to either EE or E2 and that the thymus is a more sensitive target than the spleen with regard to subacute exposure to EE. In addition, EE at the same concentration, was more potent than E2 at exerting thymic and reproductive effects. Furthermore, route of administration proved to be critical in this exposure model, as subcutaneous exposure induced far more dramatic thymic and reproductive morphological alterations than did oral administration.

Introduction

The synthetic hormone 17α-ethinyl estradiol (EE) is an analog of 17β-estradiol (E2) and is used in almost all modern formulations of hormonal contraceptives. E2 is rapidly absorbed orally but is not lipophilic enough to be absorbed by the digestive tract so it is not suitable for use in oral contraceptives. Furthermore, since E2 is metabolized rapidly, this hormone is not highly efficient at inhibiting the release of follicle stimulating hormone from the anterior pituitary. The addition of an ethinyl group at C17 makes EE much more resistant to degradation than E2 so it can exert the desired biological reproductive effect, inhibition of maturation and release of a dominant follicle from the ovary [1].

It is known that E2 not only affects reproductive tissues, but also affects lymphoid tissues, particularly the thymus. Several studies have reported the bi-directional interactions between the thymus and reproductive tissues [2-4]. Surprisingly, very few studies have evaluated the potential immune effects of EE in general and effects on the thymus in particular. This aspect warrants study since EE is a major component of hormonal contraceptives [5], an environmental contaminant [6], and sometimes used in hormone replacement therapy (HRT) [7]. Additionally, the health effects of exposure to low levels of EE have not been determined, especially in males, where it may have adverse reproductive effects similar to E2 [8]. This is of concern given the influence of the reproductive system function on the immune system [9, 10]. EE-containing oral contraceptive administration to women has been demonstrated to increase $CD4^+$ T cells, lymphocytes, monocytes, and granulocytes, as well as vaginal IgA and peripheral cytotoxic T cells [11-13]. Furthermore, oral contraceptives (OCs) have been observed to lower serum IgA, IgM, and IgG [13].

In addition to pharmaceutical exposure to EE, studies have shown that EE has been commonly detected in aquatic environments, including sewage treatment plant (STP) effluents, rivers, ocean sediments, and drinking water [14-17]. EE and other estrogenic compounds are readily absorbed by soils with high clay content and organic carbon [18]. Estrogens are then readily extracted from soil by water. Furthermore, manure from agriculture and poultry litter is routinely applied to farmland, leading to the leaching of estrogenic hormones from this waste and into aquatic ecosystems [19]. In Germany, median concentrations of E2 and EE in surface waters were found to be 4.0 ng/l, and 0.7 ng/l, respectively [6]. In STP effluents, the median

concentration observed was 11.5 ng/l for E2 and 1.8 ng/l for EE. Tap water samples from these studies also yielded concentrations of E2 around 0.03 ng/l, while EE was measured at 2.44 ng/l. In the Netherlands, E2 was found in municipal wastewater at a median concentration of 36.5 ng/l, in industrial wastewater at a median concentration of 31 ng/l and in STP effluent at <0.8 ng/l. EE was found in municipal wastewater at a median concentration of 3.2 ng/l, 3.8 ng/l in industrial wastewater and 2.6 ng/l in STP effluent [16]. In a study conducted on a New Zealand dairy farm, E2 levels from sewage effluent were as high as 1360 ng/l [20], while EE has been detected at concentrations as high as 42 ng/l in Canadian STP effluents [21]. Aquatic studies have shown that EE can induce vitellogenin synthesis in rainbow trout at very low levels (0.1-0.3 ng/l), demonstrating that EE can dysregulate aquatic reproduction [22]. Estrogenic compounds have a strong potential to bioaccumulate in the food chain. Human exposure to bioaccumulated EE occurs via consumption of seafood or drinking water which may contain low levels of estrogenic compounds [23, 24].

The potential health hazards of exposure to environmental estrogens (xenoestrogens), are of concern, especially with respect to the immune system [25, 26]. Estrogens such as E2 exert a wide range of effects on the immune system. Physiologic thymic involution occurs during periods of high sex hormone (i.e. estrogen) production during puberty or during pregnancy [27]. In mice, E2 treatment has been shown to cause significant thymic atrophy and a decrease in thymocytes [28]. This is linked to the presence of estrogen receptors (ER) that are expressed on thymic epithelial cells and thymocytes [29, 30]. Further, these receptors have also been detected on developing B cells, bone-marrow derived stromal cells [31], and mature peripheral B and T lymphocytes [32]. In the spleen, E2 modulates T cell, B cell, and macrophage functions, as evidenced by alterations in cytokine levels (such as IFN-γ, IL-1, IL-6, TNF-α), autoantibodies, and nitric oxide. [33-37]. Furthermore, E2 has also been shown to regulate the secretion of chemokines from activated splenocytes [38].

Although estrogenic compounds have been shown to be potent immunomodulators, the level or type of immune changes may differ among sexes and routes of administration (i.e. subcutaneous vs. oral). These aspects have not been well-studied thus far, particularly with regard to EE. In contrast to extensive documentation of the immunoregulatory properties of E2, data regarding the immunmodulatory effects of EE are sparse despite its extensive use. To our knowledge, no studies exist that directly compare EE with E2 to address the following key

questions: (1) Is EE a thymic and reproductive system-altering hormone like E2? (2) If so, are these effects similar or dissimilar with regard to immunological and reproductive potency? (3) Do two common routes of exposure elicit differing responses with regard to immune effects? The purpose of this paper is to present results from two companion studies designed to explore the immune effects of exposure to two different types of estrogens, EE and E2, by administering these hormones subcutaneously and orally and comparing the effects of EE to the more well-studied E2.

Materials and Methods

Mice

Mice (C57BL/6C, 6-weeks-old, n = 6-8 mice/treatment) were maintained at the Center for Molecular Medicine and Infectious Diseases Animal Laboratory facility, fed a commercial pellet diet devoid of synthetic estrogenic hormones (7013 NIH-31 Modified 6% Mouse/Rat Sterilizable Diet, Harlan Teklad, Madison, WI), given water *ad libitum*, and housed 2-3 mice per cage in traditional polycarbonate plastic cages. All animal procedures including housing, dosing, and euthanasia were in accordance with the guidelines of the Animal Care Committee at Virginia Polytechnic Institute & State University. Mice were maintained in a 12/12 light/dark cycle at 23^{o}C and euthanized within 24 h of the last subcutaneous or oral dose of E2 or EE. Each mouse was weighed 24 h before their first dosing (referred to as "initial body weight") and at the end of each week during the 21-day dosing period (final weight referred to as "terminal body weight"). The difference between terminal weight and initial weight is referred to as "body weight gain" (Table 1). These studies were performed in two separate experiments. In the first, mice were exposed to EE or E2 via subcutaneous route of administration and in the second mice were orally exposed to EE or E2.

Estrogen dosing

Male and female mice (6-weeks-old) were randomly divided into the following 7 treatment groups: i) control (tocopherol-stripped corn-oil only, ICN, Aurora, Ohio), E2 at ii) 0.04 µg/25 g BW, iii) 0.4 µg/25 g BW, iv) 4.0 µg/25 g BW, and EE at v) 0.04 µg/25 g BW, vi) 0.4 µg/25 g BW, vii) 4.0 µg/25 g BW. Thus, there were a total of 28 treatment groups (14

subcutaneously exposed groups and 14 orally exposed groups, 7 treatments/gender). Mice were subcutaneously or orally dosed, as per our previously described methods [39, 40] daily for a period of 21 days with one of the following: oil only, EE, or E2 in 100 µl of tocopherol-stripped-corn oil. The dosing protocol of this study consisted of daily subcutaneous injections or oral administration of either EE or E2 at levels of 0.04, 0.4, or 4.0 µg/25 g BW. This treatment duration was based on the typical administration of oral contraceptives during a cycle for a period of 21 days. Furthermore, this lab previously performed 21-day exposure studies using other estrogenic compounds (diethylstilbestrol, genistein, α-zearalanol) and observed immune system alterations [39].The vehicle tocopherol-stripped corn oil chemical composition was as follows: glycerides 98.8 wt.%, unsaponifiable matter 1.2 wt.%, and free fatty acids 0.07 wt.%. It contained only trace amounts of squalene and carotenoids, as well as 0.02% of ubiquinone, stigmasterol 0.07%, and campestrol 0.2%.

Histopathology

The weights of female (ovaries, uterus) and male reproductive organs (seminal vesicles, testes) were determined. These functioned as positive *in vivo* bioindicators of estrogenicity. The reproductive organ weights were then related to the terminal body weight and referred to as "organ to BW ratio" (i.e. uterus to BW ratio, seminal vesicle to BW ratio, Figures 1-4). In order to assess the morphological effects of E2 and EE on the reproductive system, the uteri, ovaries, seminal vesicles, and testes were collected, weighed, and preserved in formalin. The tissues were processed for paraffin sections, sectioned at 6 µm, and stained with hematoxylin and eosin. From each slide, six glandular lumina were evaluated for height of epithelium. The height of the uterine epithelial cells was measured with a micrometer. The slides were read in a coded fashion and decoded later for statistical analysis.

Thymus and spleen

Immediately upon termination of life, the thymus and spleen were collected from each mouse. Organs were weighed and these weights were related to individual body weight for normalization and referred to as thymus to BW and spleen to BW ratios. Spleens were collected under sterile conditions. Lymphocytes from the spleen were isolated according to our previously published procedures [33, 35, 39]. Briefly, lymphoid organs were gently passed over a sterile 60

μm-mesh steel screen and the cells were washed in RPMI-1640 media devoid of estrogenic phenol red (Cell Gro, Mediatech, Herndon, VA). Spleen cells were treated with Tris-ACK-NH_4Cl lysis buffer (pH 7.2) and washed two times (250 x g, 7°C, 8 min). Cells were then suspended in RPMI-1640 media supplemented with 10% charcoal-stripped fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine (ICN, Costa Mesa, CA), 50 IU/ml penicillin (Mediatech, Herndon, VA), 50 μg/ml streptomycin (Mediatech, Herndon, VA), and non-essential amino acids (Fisher, Pittsburgh, PA). Cell concentrations were enumerated by a Beckman Coulter Multisizer™ 3 Cell Counter (Miami, FL) and adjusted to 5 x 10^6 cells/ml prior to culture.

Flow cytometry

Thymocytes and splenocytes were stained and subjected to dual color flow cytometric analysis per our previously reported work [37, 40, 41]. Briefly, 100 μl of freshly enriched thymocytes or splenocytes at a concentration of 5 x 10^6 cells per ml (5 x 10^5 cells per well) were plated in Corning 96-well round-bottom tissue-culture plates (Corning, NY). Thymocytes were dual stained with FITC-anti-CD4 and PE-anti-CD8 antibodies or with FITC-anti-CD4 and PE-anti-CD25 antibodies. Splenic lymphocytes were stained with PE-anti-CD45R (B220, a pan B cell marker), PE-anti-CD40 (a B lymphocyte marker), FITC-anti-CD11b (a macrophage and dendritic cell marker), PE-anti-F4/80 (a macrophage marker), or PE-anti-NK1.1 (a natural killer cell marker) in addition to FITC-anti-CD4 and PE-anti-CD8 antibodies (all from eBioscience, San Diego, CA). Single staining was performed with anti-CD45R (B220), anti-CD40, and anti-NK1.1 antibodies, while dual staining was performed with anti-F4/80 and anti-CD11b, as well as anti-CD4 and anti-CD8 antibodies (eBioscience, San Diego, CA). Cells were incubated with the antibodies on an orbital shaker at 4°C for 30 min. Phosphate buffered saline (PBS) (100 μl, pH 7.4, Fisher Scientific, Pittsburgh, PA) was then added to each well and the plates were centrifuged at 250 x g for 10 min. Supernatants were aspirated and 200 μl of 2% paraformaldehyde were added to each well to fix the cells. Cells were analyzed the following day on a Coulter Epics XL/MXL flow cytometer (Hialeah, FL).

Cell culture

Splenic lymphocytes (2.5 x 10^6 cells) were cultured in 24-well plates for 24 and 48 h with either an optimal concentration of Concanavalin A (Con-A) (5 μg/ml, Sigma, St. Louis, MO), or left unstimulated in media only. At the end of the culture period supernatants were collected and frozen at –80^o C until analysis of IFN-γ and nitric oxide levels.

Statistical analysis

Dr. Stephen Werre conducted all statistical procedures at the Laboratory for Study Design and Statistical Services, Virginia-Maryland Regional College of Veterinary Medicine. The mixed procedure of the SAS system (version 9.1.3, Service Pack 4, SAS Institute Inc., Cary, NC 27513) was used to perform an analysis of variance to test for treatment effects. The analysis focused on 2 null hypotheses: 1) Treatment means (for different doses of EE or E2) did not differ from the mean response of the control condition and 2) Mean response for dose-matched treatment groups (e.g., 4.0 μg/25 g BW EE versus 4.0 μg/25 g BW E2) were not significantly different. The first hypothesis was tested using Dunnett's *t*-test at the 0.05 significance level, and the second set of hypotheses were tested using contrasts with a Bonferroni correction for multiple comparisons. Violations of model assumptions were assessed using residual plots. Each response variable was analyzed separately. The experiment was analyzed as a randomized complete block design, where blocks were experiment days to correct for day-to-day variation. It is important to note that comparisons were not performed across the two different routes of exposure. Instead, these studies were performed and analyzed as two distinct experiments. For each exposure situation (subcutaneous or oral), comparisons were performed directly between one treatment (i.e. 4.0 μg/25 g BW EE treatment) to the gender-matched control. Comparing EE to the dose-matched E2 group (i.e. 4.0 μg/25 g BW EE versus 4.0 μg/25 g BW E2) also enabled us to ascertain at which variables the two compounds elicited different responses.

Results

1. Body Weights

Effects of subcutaneous EE or E2 treatment

Neither subcutaneous E2 nor EE treatment of female or male mice significantly affected terminal body weights (data not shown). At termination, all mice had gained body weight during the treatment period. However, both female mice that received 4.0 μg/25 g BW EE exhibited a reduced rate of weight gain, reaching significance only in the females. For example, in female mice, the amount of weight gained during treatment period for controls was 5.43 ± 0.56 g and for the 4.0 μg/25 g BW EE treatment group it was 3.27 ± 0.23 g with $p < 0.01$. In male mice, the amount of weight gained during the treatment period for controls was 6.31 ± 0.75 g and for the 4.0 μg/25 g BW EE treatment group weight gain was 3.22 ± 0.69 g (though this observation was non-significant).

Effects of oral EE or E2 treatment

Neither oral treatment of E2 nor EE significantly affected terminal body weights (data not shown). However, weight gain was significantly diminished in male mice receiving 4.0 μg/25 g BW EE treatment with the male amount of weight gained during the treatment period for controls being 3.64 ± 0.48 g and for the 4.0 μg/25 g BW EE treatment group being 1.69 ± 0.45 ($p < 0.01$). Female weight gain was not significantly altered by oral exposure (data not shown).

2. EE and E2 differentially affected reproductive organ weights

Increased female uterine weights were greater in subcutaneously administered EE-treated mice than E2-treated mice

As predicted, subcutaneous exposure to both EE and E2 affected uterine weights in a dose-dependent fashion. Interestingly, direct comparison of EE with E2 suggested that EE had stronger effects compared to E2 (Figure 1). For example, increased uterine weights were evident in mice given the lowest dose of EE, 0.04 μg/25 g BW, an effect not evident at a comparable dose of E2. EE at 0.04 μg caused a significant increase in uterine weight relative to 0.04 μg/25 g BW E2 but was not significantly increased compared to the female control uterine weight. EE significantly increased uterine weights compared to control ($p < 0.01$) or dose-matched E2 ($p < 0.05$). In addition, both 4.0 EE and E2 caused a significant increase in uterine weight relative to

the control ($p < 0.0001$). It is noteworthy that the increase in uterine weights by EE was evident at a dose that is 10-fold less than E2. For example, to achieve the level of uterine weight increase induced by 0.4 μg/25 g BW EE, it was necessary to utilize a 10-fold higher dose of E2 (4.0 μg/25 g BW). Histopathological analysis of the uterus revealed that EE caused the endometrial glands to become more dilated and cystic as the dose increased. E2 caused the uterine endometrium to become edematous, with cystic distension of glands that became more apparent as the dose increased. Both EE and E2 caused some stimulation of the glandular portion of the uterus, with the highest dose causing cystic hyperplasia. However, neither EE nor E2 altered ovarian weight or ovarian to BW ratio (data not shown).

Female uterine weights were less dramatically affected by oral exposure

Uterine weight was noticeably increased relative to the control by oral exposure only in the highest EE-treated group (4.0 μg/25 g BW, $p < 0.01$, Figure 2A). When adjusted for body weight differences (uterine to BW ratio), none of the treatment groups demonstrated a statistically significant increase in ratio compared to the control group (Figure 2B). Histopathological analysis of the uterine endometrium indicated that the 4.0 μg/25 g BW EE dose caused cystic distension, while the lower doses caused little change. E2 treatment did not reveal striking alterations, though there was a tendency for the endometrium to become more edematous with increasing dose of EE. The uterine glands demonstrated some stimulation at lower doses, while cystic hyperplasia was observed in both 4.0 μg/25 g BW EE and 4.0 μg/25 g BW E2 uterine glands. Neither EE nor E2 altered ovarian weight or ovarian to BW ratio (data not shown) and histopathological analysis revealed no apparent alterations to the ovarian structure.

Seminal vesicle weights were reduced more potently by subcutaneous EE than E2

Both EE and E2 decreased seminal vesicle weights in males (Figure 3). Remarkably, EE decreased seminal vesicle weights at all doses, including the lowest dose used, 0.04 μg/25 g BW EE ($p < 0.0001$). In contrast, comparable profound reduction in seminal vesicle weights by E2 were only evident at the highest dose used, 4.0 μg/25 g BW E2 ($p < 0.0001$). When seminal

vesicle weights were normalized to body weights, EE significantly decreased seminal vesicle to BW ratio at all doses administered ($p < 0.0001$). E2 decreased seminal vesicle to BW ratios at 0.4 μg/25 g BW ($p < 0.05$) and profoundly at 4.0 μg/25 g BW E2 ($p < 0.0001$). Histopathologic examination revealed a trend towards a less cystic seminal vesicle and more active, thicker epithelium in E2-treated mice, but this trend was not significant. The glandular portion of the vesicle became less packed with cells, suggestive of decreased activity, at 4.0 μg/25 g BW E2, but this observation was not tested or verified. In EE-treated males, all doses revealed a less active vesicular portion, with very little activity. In addition, overt squamous metaplasia was observed at 4.0 μg/25 g BW EE. The glandular portion of the seminal vesicle became less active, with less robust cells as the dose increased. At the highest dose, 4.0 μg/25 g BW EE, squamous metaplasia was observed. In contrast to EE and E2-induced seminal vesicle weight changes, these hormones had no profound effects on testicular weight, with the exception of 4.0 μg/25 g BW EE, which significantly decreased testicular weight compared to both 4.0 μg/25 g BW E2 ($p < 0.05$) and male controls ($p < 0.05$) (data not shown). When testicular weights were normalized to mouse body weights, EE at all doses failed to alter the testicular to body weight ratio (data not shown) and histopathological analysis did not reveal significant alterations in the testes of any treatment group.

Seminal vesicle weights were reduced more potently by oral EE than E2

The highest dose of both EE and E2 significantly reduced seminal vesicle weight. Interestingly, when comparing mice treated with 4.0 μg/25 g BW EE to 4.0 μg/25 g BW E2, EE treatment caused a greater than 60% reduction in seminal vesicle weight than E2 (Figure 4A). The 4.0 μg/25 g BW EE dose resulted in seminal vesicle weights that were significantly different from both controls ($p < 0.0001$) and dose-matched 4.0 μg/25 g BW E2 ($p < 0.0001$). Furthermore, 0.4 μg/25 g BW EE modestly but significantly reduced seminal vesicle weights ($p < 0.05$), whereas 0.4 μg/25 g BW E2 did not. Normalization of seminal vesicle weight to body weight revealed that both 4.0 μg/25 g BW EE ($p < 0.0001$) and 4.0 μg/25 g BW E2 ($p < 0.0001$) reduced seminal vesicle to BW ratio (Figure 4B). Histopathology of the vesicular portion of the seminal vesicle did not reveal noticeable changes. At both the 4.0 μg/25 g BW EE and 4.0 μg/25 g BW E2 dose, there was some inhibition of activity, a trend that was indicated upon examination of the glandular portion of the seminal vesicle. At the highest dose of either

treatment, the glandular portion of the seminal vesicle was less packed with cells, implying that it was less active. All doses of EE caused less activity relative to the control, though this was a subtle trend. Neither EE nor E2 induced significant changes in testes weight or testes to BW ratio (data not shown). In addition, histopathology did not reveal remarkable differences among the treatment groups.

3. Thymic effects of treatment

Subcutaneous EE treatment induces more profound thymic atrophy compared to E2 treatment

As expected, female thymic weights were greater than male thymic weights. The mean female control thymus weight was 61% greater than the mean male control weight and the mean female control thymus to BW ratio was 60% greater than the mean male control ratio. Relative to the control, female thymus weight was decreased at 0.4 μg/25 g BW EE ($p < 0.0001$) and 4.0 μg/25 g BW EE ($p < 0.0001$) (Figure 5A), as well as at 0.4 μg/25 g BW E2 ($p < 0.001$) and 4.0 μg/25 g BW E2 ($p < 0.0001$). Comparison of 4.0 μg/25 g BW E2 to 4.0 μg/25 g BW EE yielded a greater decrease in thymus weight by 4.0 μg/25 g BW EE treatment group ($p < 0.05$). An identical trend was found when thymic weights were related to body weights (thymus to BW ratio) at 0.4 μg/25 g BW E2, 4.0 μg/25 g BW E2, 0.4 μg/25 g BW EE and 4.0 μg/25 g BW EE ($p < 0.001$) (Figure 5B).

Male thymus weight was decreased relative to the gender-matched control at the 4.0 μg/25 g BW EE treatment ($p < 0.01$) (Figure 6A). Similar to females, 4.0 μg/25 g BW EE elicited a stronger effect on decreasing thymus weight than 4.0 μg/25 g BW E2 ($p < 0.05$). Subcutaneous E2 doses elicited a non-significant decreasing dose-dependent response with regard to thymus weight (Figure 6A). Body weight-adjusted results (thymus to BW ratio, Figure 6B) demonstrated similar findings, with E2 treatment decreasing the ratio in a dose-dependent but non-significant manner and 4.0 μg/25 g BW EE causing a significant decrease ($p < 0.01$). Subcutaneous 4.0 μg/25 g BW EE was more potent at decreasing thymus to body weight ratio compared to 4.0 μg/25 g BW E2 ($p < 0.05$).

Interestingly, consistent with increased thymic weight of females compared to males, female controls demonstrated a 115% increase in mean thymocyte recovery (i.e. thymocytes yielded per thymus) compared to male control mean thymocyte recovery (Figures 5C and 6C). Similar to thymus weight, EE or E2 treatment of female mice induced a dose-dependent decrease in thymocyte cellularity (Figure 5C). This trend reached significance relative to control at 0.4 µg/25 g BW E2 ($p < 0.01$) and 4.0 µg/25 g BW E2 ($p < 0.0001$), as well as at 0.4 µg/25 g BW EE ($p < 0.0001$) and 4.0 µg/25 g BW EE ($p < 0.0001$). Decreased male thymocyte recovery was observed at 4.0 µg/25 g BW E2 ($p < 0.05$) and at 4.0 µg/25 g BW EE ($p < 0.001$) (Figure 6C).

Neither oral EE nor E2 induced thymic atrophy

Both female and male mouse thymus weight and thymus to body weight ratio were unaffected by oral treatment with EE or E2 (Figures 7 and 8).

Female thymus cellularity following oral dosing was decreased only at 4.0 µg/25 g BW E2 ($p < 0.05$, Figure 8C). A non-significant decrease was evident at 0.4 µg/25 g BW E2, as well as a dose-dependent, non-significant decrease in cellularity with increasing dose of EE. Male thymus cellularity was not decreased at any treatment level of either EE or E2 in the oral model. However, consistent with the subcutaneous results, male controls demonstrated a greater than 100% decrease in thymocyte recovery compared to female controls, a trend that is related to gender rather than treatment.

Effects of subcutaneous treatment on major thymus subsets

The maturation expression markers CD4 and CD8 were measured in the thymus (Table 1). $CD4^+/CD8^+$ cells were decreased only in females at 4.0 µg/25 g BW EE ($p < 0.05$). Single positive $CD4^+$ and $CD8^+$ cells were not markedly altered in either sex or at any subcutaneous dosing level. $CD4^+/CD8^+$ cells were decreased in females at 4.0 µg/25 g BW EE ($p < 0.05$).

Major thymus cellular subsets of orally exposed mice

The maturation expression markers CD4 and CD8 were measured in the thymus (Table 2). Female thymocyte subsets were not altered, though $CD4^-/CD8^-$ thymocytes were noticeably increased at all oral doses, especially 4.0 µg/25 g BW EE and 4.0 µg/25 g BW E2. Similar to females, male thymocyte subsets were not altered, with the exception of $CD4^-/CD8^+$ cells in

0.04 μg/25 g BW EE-treated mice. These mice demonstrated a decrease in thymus $CD4^-/CD8^+$ thymocytes compared to the control mice ($p < 0.01$), though this trend was not significant in any other treatment group. Interestingly, $CD4^-/CD8^-$ cells were also noticeably increased in males, though this was not significant due to wide variation in the data and the resulting high standard error of the mean.

4. Splenic effects of EE or E2 treatment

Spleen weight, spleen to BW ratio, and splenocyte cellularity following subcutaneous dosing

In contrast to EE and E2-induced decrease in thymus weight (a primary lymphoid organ) by subcutaneous treatment, neither spleen weight (a secondary mature lymphoid organ) nor spleen to BW ratio were affected by either estrogenic compound at any subcutaneous dosing level in either sex (data not shown). Female splenocyte recovery was not affected, though at 4.0 E2 and all three EE doses there was a noticeably diminished total splenocyte cell recovery that was non-significant (data not shown). In addition, 0.4 μg/25 g BW EE caused a more potent decrease (E2 = 103.77 ± 9.69 million, EE = 66.75 ± 8.10 million) in splenocyte recovery compared to 0.4 μg/25 g BW E2 ($p < 0.05$), though this was not different from control mice. Male splenocyte recovery was not altered by any subcutaneous treatment relative to the control mice (data not shown).

Spleen weight, spleen to BW ratio, and splenocyte cellularity were unaffected by oral EE or E2 treatment

None of spleen weight, spleen to BW ratio, or splenocyte cellularity was affected by either estrogenic compound at any oral dosing level in either sex (data not shown).

Major spleen cellular subsets following subcutaneous exposure

Splenic $CD4^+$, $CD8^+$, $CD40^+$, $CD45R^+$ (B220), $NK1.1^+$, $F4/80^+$, and $CD11b^+$ cells were all measured by flow cytometry (Table 3). Female $CD4^+$ cells were diminished compared to the control at 0.04 μg/25 g BW EE ($p < 0.01$) and were lower than the dose-matched 0.04 μg/25 g BW E2 ($p < 0.001$). $CD8^+$ splenocytes of female mice were not affected, relative to controls, though 0.04 EE caused a decrease relative to 0.04 μg/25 g BW E2 ($p < 0.001$). Splenic B cells of females, as indicated by CD40 (a mature B cell marker) and CD45R (B220, present on both

early and mature B cells) or natural killer cells were not altered by treatment. However, splenic macrophages, as indicated by F4/80, were increased relative to the oil control at all doses of EE, reaching significance only at 4.0 μg/25 g BW EE ($p < 0.01$). CD11b, commonly expressed on macrophages, NK cells, granulocytes, and activated lymphocytes, was not changed by any subcutaneous treatment (Table 3A).

$CD4^+/CD8^-$ and $CD4^-/CD8^+$ male splenocytes were unaffected by subcutaneous treatment compared to control mice. CD40 expression was unchanged. However, CD45R (B220) was increased at 4.0 μg/25 g BW E2 ($p < 0.05$), 0.04 μg/25 g BW EE ($p < 0.05$) and 0.4 μg/25 g BW EE ($p < 0.01$), but not 4.0 μg/25 g BW EE (Table 3A). $NK1.1^+$ male splenocytes were increased by 0.4 μg/25 g BW E2 treatment ($p < 0.05$), in addition to 0.04 μg/25 g BW EE ($p < 0.01$) and 4.0 μg/25 g BW EE ($p < 0.001$) subcutaneous treatments. Furthermore, 4.0 μg/25 g BW EE was more potent than 4.0 μg/25 g BW E2 in inducing an increase in NK cells ($p < 0.05$). $F4/80^+$ macrophages were increased in a dose-dependent manner, reaching significance at 4.0 μg/25 g BW EE ($p < 0.05$). $CD11b^+$ cells were not affected by treatment.

Major spleen cellular subsets were unaffected by oral EE or E2 treatment in either gender

Splenic $CD4^+$, $CD8^+$, $CD40^+$, $CD45R^+$ (B220), $NK1.1^+$, $F4/80^+$, and $CD11b^+$ cells were all measured by flow cytometry (Table 4) and were observed to be unaffected by oral EE or E2 treatment. The lone exceptions to this occurred in female mice, where 0.4 μg/25 g BW EE caused an increase in CD40 and B220 expression relative to that of 0.4 μg/25 g BW E2, an observation that was not different compared to control mice. In addition, 4.0 μg/25 g BW EE caused an increase in B220 expression compared to that of 4.0 μg/25 g BW E2, again not significant relative to the control group.

5. Functional analysis

Mitogen-induced splenocyte proliferation was not altered by EE or E2 treatment by either subcutaneous or oral administration

Mitogen-induced proliferation of splenocytes with either Con-A (5 μg/ml), lipopolysaccharide (LPS, 10 μg/ml), or phorbol 12-myristate 13-acetate (PMA, 10 ng/ml) and ionomycin (200 pg/ml) was not altered by EE or E2 (data not shown).

Discussion

There are a limited number of studies which have investigated the effects of *in vivo* EE exposure on the immune system, particularly with regard to the thymus. The little data which exist focus on short-term human studies, primarily examining peripheral immune subsets and secondary lymphoid organs of women on oral contraceptives. Data on the effects of EE on primary lymphoid organs, such as the thymus, and across genders and different levels and routes of exposure are extremely limited. This study sought to answer several questions: 1) Does EE exert immunomodulatory properties like other estrogenic compounds? 2) If so, how do those effects compare to the more well-studied E2? 3) What differential effects will a range of doses have on the immune system? 4) Are these effects similar across gender? 5) How do two common routes of exposure to EE (subcutaneous and oral) compare with regard to their influence on the immune system? These companion studies examined the effects of EE and E2 when administed via subcutaneous injection or oral delivery in tocopherol-stripped corn oil via micropipette.

Dosage levels were determined based on multiple factors. First, prior dosing studies in our laboratory demonstrated that 4.0 μg/25 g BW of E2 was a dose suitable for inducing physiological concentrations of estrogen in gonadectomized male mice compared to intact female controls (data not published). Although we used intact females and males in this subcutaneous study, we used this dose as the upper limit and used two lower doses in order to determine dose response patterns to either EE or E2. Second, our dosage ranges were within the upper realm of environmental contamination and the lower range of pharmaceutical (both hormonal contraceptives and estrogen replacement therapy) concentrations, allowing this study to investigate the potential effects across a range of exposure situations.

There were an estimated 16 million women in the United States using hormonal contraceptives as of 2003 [5]. Ortho Evra® is the only skin patch approved for birth control and is the hormonal contraceptive which most closely mimics the subcutaneous route of exposure. This contraceptive is a patch applied to non-mammary tissue of a woman's body for a 21-day

period (1 cycle), with a new patch being applied every 7 days, and contains 750 μg of EE along with 6 mg of norelgestromin (a progestin). It relies on transdermal hormone delivery and it is estimated that 20 μg of EE are delivered daily [43]. Interestingly, though it has been recently noted that the Ortho Evra contraceptive patch exposes women to higher steady-state doses of EE but has diminished EE concentration spikes [44], to our knowledge there have not been any studies performed examining the EE-induced immune alterations or lack thereof. In addition to their use as contraceptives, EE-containing combination pills are also used to alleviate symptoms associated with menopause (Femhrt®). Given that there are an estimated 40 million post-menopausal women in the U.S. today [45] and that this number is expected to grow with increased life expectancy, exposure to synthetic EE is extensive and therefore, further exploration of possible immune effects are warranted.

Intuitively, the reproductive system would be the most sensitive target to reproductive hormones. We assessed male and female reproductive organ weight and preserved organs for histopathological processing as a positive confirmation of successful treatment of mice. The observed estrogen-induced increase in uterine weight is believed to be due to increased uterine fluid retention, increased blood flow to the uterus, and increased epithelial cell proliferation [46-48]. Interestingly, EE elicited a stronger uterine effect at the 0.04 and 0.4 μg/25 g BW levels compared to E2. When adjusted for body weight, only 4.0 μg/25 g BW EE caused an increase in uterine to BW ratio. The uterine effects strongly indicate that the estrogenic compounds exerted similar effects to those that would be elicited in humans.

Reproductive organ histopathological alterations are a strong indicator of EE toxicity in rodents [49]. Seminal vesicle atrophy was dramatic in all EE-treated males (Figures 3 and 4), especially when comparing the atrophy to that induced by E2. The lowest dose of EE (0.04 μg/25 g BW) caused even greater seminal vesicle atrophy than the highest dose of E2 (4.0 μg/25 g BW), indicating that EE has a profound effect on male reproductive health at relatively low levels. Atrophy of seminal vesicles is likely due to decreased levels of male hormones such as testosterone or dihydrotestosterone and/or diminished androgen receptor expression [50] together with increased expression of estrogen receptors on the sex glands [51]. The most intriguing aspect is that EE was more potent than E2 at inducing this atrophy. These reproductive implications are important given that it is conceivable that males could be exposed to low levels of EE via environmental contamination from drinking water, food, or dermal exposure and these

levels may be sufficient to cause reproductive dysfunction. Reports suggest that human sperm counts have decreased over the past 60 years and it has been suggested that estrogenic compounds are contributing to this phenomenon, [52, 53] although the contribution of EE to this effect is not known. Kinomoto *et al*. observed that EE administration in male rats at 0.3 and 3.0 mg/kg for 2 and 4 weeks induced atrophy of Leydig cells, degeneration/necrosis of spermatocytes, vacuolar degeneration of Sertoli cells and retention of spermatids, primarily at the 3.0 mg/kg dose. Our highest dose was approximately 20 times less than that and none of the histopathological changes noted by Kinomoto et al. were evident in the testes of treated mice in our current study. Testes weight was diminished at 4.0 μg/25 g BW EE but spermatogenesis was observed to be normal in all groups.

The gonadal-thymic axis is well-documented [44, 54-56]. Given this connection, it is reasonable to suggest that the immune system is similarly vulnerable to modulation by exogenous estrogens. The thymus is a primary lymphoid organ that is modulated by E2 [57-60]. Similar to our findings, studies in intact female Wistar rats show that increasing doses of EE lead to a dramatic decrease in thymic weights [61]. Doses in those studies ranged from 0.01 μg up to 100 μg but thymic weight did not significantly decrease until 10 μg EE was administered. To our knowledge, there have been no other studies that have reported the effects of EE on thymic weight. Our study expands upon previous findings by examining between sexes in the mouse. Furthermore, we demonstrate effects at a lower dose and in intact animals.

Our data show that female mice were more sensitive to EE-induced thymic atrophy compared to males. EE-induced thymic atrophy and thymocyte cellularity decreases were significant at the 0.4 μg dose in females, but not in males. In males, a higher dose of EE, 4.0 μg/25 g BW, was necessary to induce a similar decrease in thymic weight and thymocyte cellularity. Interestingly, in males, 4.0 μg/25 g BW E2, unlike 4.0 μg/25 g BW EE, did not decrease thymic weights but did decrease thymocyte cellularity. Even though EE decreased thymic cellularity following a relatively short 21-day duration of exposure, in general, there was no decrease in specific thymocyte subsets. At the highest EE dose, 4.0 μg/25 g BW, $CD4^+/CD8^+$ were decreased. There was a biological, but not statistically significant, trend toward increasing $CD^+/CD8^-$ cells in EE and E2-treated mice. It is possible that statistical significance could have been achieved had there been more mice per treatment group. Estrogen-induced thymus involution is characterized primarily by the loss of $CD4^-/CD8^-$ double negative and $CD4^+/CD8^+$

double positive thymocytes, indicating that the signals which attract progenitor cells to the thymus are disrupted by estrogen treatment [62]. Previous studies have addressed the possible mechanism behind E2-induced thymic atrophy. This includes the potential for decreased emigration of prothymocytes from the bone marrow [63], a deleterious alteration in thymocyte movement that is key for survival. It is likely that estrogenic compounds may exert their primary effect on estrogen-receptor-positive thymic epithelial cells [30], which provide vital maturation signals to developing thymocytes. It is also likely that EE, like E2, may induce loss of thymocytes through Fas/FasL-induced or other mechanisms of apoptosis [60, 64]. Furthermore, chemokines such as thymus-expressed chemokine, stromal-derived factor-1α, and secondary lymphoid tissue chemokine, in addition to cytokines such as IL-7 have been demonstrated to be upregulated in the thymus in response to estrogen treatment and concomitant thymic atrophy [65]. It has been suggested that upregulation of these cytokines during thymic atrophy is a mechanism by which the thymus is attempting to regenerate itself and increase thymocyte survival. These cytokines are produced by thymic epithelial cells [66], the primary producers of cytokines in the thymus, and an area that is subject to modulation by estrogen treatment [67]. It is plausible that these signaling mechanisms may also be operative with regard to EE. Given the more extreme thymic involution caused by EE in this study, the above aspects warrant further consideration.

There are multiple possibilities that may explain why EE exerted a more potent response on reproductive organs and the thymus. The half-life of EE is longer than that of E2. In addition, EE has an affinity for the estrogen receptor (ER) almost 2 times greater than that of E2, further explaining how EE can elicit a more potent response [68]. EE may impact expression of the ER, especially given the alteration in uterine weight, which may be indicative of increased exposure to EE or E2 [69].

Unlike pronounced effects of EE on reproductive organs and the thymus, neither EE nor E2 elicited a similar dramatic response in the spleen, a mature lymphoid organ. This was evident by the lack of noticeable effects on splenic weights, splenocyte cellularity, alterations in major splenocyte subsets, mitogen-induced proliferation. These observations are not entirely surprising since the treatment duration was only 21 days, a time period when mature cells would have already emigrated and established residence in the spleen. In general, mature lymphocytes live longer than developing lymphocytes, such as those that would be present in the thymus.

Interestingly, EE (and to a lesser extent, E2) demonstrated a biologically non-significant trend towards increased B cell numbers in males, and increased macrophages in both males and females. It is likely that a longer duration of estrogen-treatment would have yielded greater functional alterations in the spleen. Indeed, studies from our laboratory have previously demonstrated that treatment of mice with E2 (in silastic implants) for 6-8 weeks resulted in marked functional changes in the spleen. These alterations included enhanced release of proinflammatory molecules such as IFN-γ [34], nitric oxide, cyclooxygenase-2 [33], chemokines [38], and autoantibodies [37]. We have also demonstrated that longer duration of E2-treatment induced distinct molecular modifications in splenocytes such as alterations in the transcription factors T-bet, interferon-regulatory factor-1, and NF- κB [70-72]. It is certainly possible that EE may also induce these molecular changes, an aspect to be investigated in future studies. It is noteworthy that EE (and E2) caused marked negative effects on thymocytes, which eventually emigrate as mature T cells to seed mature lymphoid organs such as the spleen, lymph nodes, and Peyer's patches. It is therefore conceivable that eventually these mature T cells may demonstrate estrogen-induced alterations in splenocyte function.

Administration of EE by non-oral routes (i.e. subcutaneous hormone delivery) minimizes metabolic impacts that have been widely reported to be due to estrogens altering hepatic synthesis of proteins such as C-reactive protein and serum amyloid A [73, 74]. In non-oral routes, the hormone does not initially travel through the liver, avoiding the first pass effect and resultant high hormone concentrations within the portal vein and subsequent metabolic alterations [75]. Nonetheless, avoiding hepatic circulation results in greater systemic circulation of EE, as 55-65% of EE is inactivated by the intestinal wall and liver in oral delivery [76]. Furthermore, avoiding hepatic effects will potentially negate the positive alterations that have been linked to estrogen replacement therapy, such as increased hepatic synthesis of high density lipoprotein and other cardiovascular-protective effects [77, 78]. Clearly, the results from this study suggest that non-oral route of exposure may have a greater influence on the immune system, an aspect that must be considered when developing subcutaneous and transdermal means of EE delivery. This study is one of the first to explore the immunological implications of EE across age and gender, and further compare these findings to those of E2.

Future studies need to investigate the influence of EE on the bone marrow. These studies were limited to examination of only one peripheral immune organ, the spleen. The effects of EE

treatment on lymphocyte subsets in other mature lymphoid organs (lymph nodes, mucosal-associated lymphoid tissue, and other secondary sites) should be determined in order to definitively conclude whether or not mature lymphoid organs are unaffected by 21-day, subcutaneous dosing of EE or E2. More studies are needed to elucidate at what levels EE may negatively or positively modulate the immune system, across age and gender, duration of exposure and route of exposure, aspects which have been much more well-studied in E2. Our findings assessing and comparing EE to E2 effects on immune (thymus and spleen) and reproductive tissues are novel and provide a basis for future in-depth studies.

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Table 4.1: Thymus cell subsets following subcutaneous exposure

A: Females

		CD4- CD8- (% Expression)	CD4+ CD8+ (% Expression)	CD4+ CD8- (% Expression)	CD4- CD8+ (% Expression)
Control		4.69 ± 0.94	76.24 ± 2.77	13.75 ± 2.33 [a]	5.33 ± 0.34
	0.04	4.77 ± 0.99	64.85 ± 9.58	14.68 ± 1.24	5.84 ± 0.53
E2 µg/25g	0.4	4.98 ± 0.77	69.60 ± 2.59	18.50 ± 1.84	6.90 ± 0.45
	4	9.95 ± 4.29	64.88 ± 3.08	18.55 ± 2.68	6.62 ± 0.72 [a]
	0.04	5.64 ± 1.42	73.68 ± 1.12	14.03 ± 1.10	6.66 ± 0.41
EE µg/25g	0.4	5.28 ± 0.65	69.99 ± 4.53	17.76 ± 3.57	6.95 ± 0.87
	4	27.03 ± 10.08	51.74 ± 6.88	16.03 ± 2.95 [b]	5.22 ± 0.88 [b]

Significance at $p \leq 0.05$, $n \geq 6$/treatment group.

B: Males

		CD4- CD8- (% Expression)	CD4+ CD8+ (% Expression)	CD4+ CD8- (% Expression)	CD4- CD8+ (% Expression)
Control		5.53 ± 1.60	75.88 ± 2.40	12.78 ± 2.02	5.85 ± 0.47
	0.04	4.16 ± 0.81	78.64 ± 1.62	11.90 ± 2.20	5.32 ± 0.36
E2 µg/25g	0.4	11.24 ± 6.53	70.73 ± 6.03	11.66 ± 1.57	6.39 ± 0.87
	4	19.86 ± 12.97	61.88 ± 10.55	13.35 ± 2.76	4.89 ± 0.89 [a]
	0.04	7.83 ± 2.18	74.05 ± 1.64	13.07 ± 1.17	5.06 ± 0.24
EE µg/25g	0.4	3.75 ± 0.82	77.92 ± 0.79	13.85 ± 1.12	4.50 ± 0.27
	4	7.12 ± 2.83	66.14 ± 5.84	14.84 ± 5.68	8.35 ± 1.73 [b]

Significance at $p \leq 0.05$, $n \geq 6$/treatment group.

Table 4.1: EE and E2 effects on major thymus cellular subsets were assessed by flow cytometry. Female and male 8-week-old mice were given daily subcutaneous doses of EE or E2 at concentrations of either 0.0 µg/25 g BW (Control), 0.04, 0.4, or 4.0 µg/25 g BW in 100 µl of corn oil for 21 d. Control mice were given 100 µl of oil only. Mice were 9 weeks old when euthanized. Thymocytes were dual-stained with FITC-anti-CD4 and PE-anti-CD8 antibodies and analyzed by immunosoftware in a Coulter EPICS XL/MXL flow cytometer. Females (A) and males (B) (n = 6-8). The significance between treatment groups ($p \leq 0.05$) was determined by comparing treatment group to gender-matched control. Furthermore, each treatment group was then compared to the dose-matched counterpart (i.e. 0.04 E2 vs. 0.04 EE, 0.4 E2 vs. 0.4 EE, 4.0 E2 vs. 4.0 EE) to determine if one estrogenic compound elicited a different response than the other.

Within columns, different superscript letters = significantly different from one another (i.e. $a \neq b$).

Table 4.2: Thymus cell subsets following oral exposure

A: Females

		CD4- CD8- (% Expression)	CD4+ CD8+ (% Expression)	CD4+ CD8- (% Expression)	CD4- CD8+ (% Expression)
Control		4.67 ± 1.22	79.27 ± 2.53	11.93 ± 1.31	4.12 ± 0.57
E2 µg/25g	0.04	6.90 ± 2.11	75.52 ± 2.56	13.60 ± 0.70	3.95 ± 0.45
	0.4	9.53 ± 2.45	72.33 ± 2.85	12.96 ± 2.04	5.18 ± 0.73
	4	26.52 ± 10.69	59.40 ± 8.93	11.00 ± 1.54	3.07 ± 0.48
EE µg/25g	0.04	9.16 ± 2.00	75.32 ± 2.77	12.39 ± 1.20	3.13 ± 0.46
	0.4	9.72 ± 2.82	71.75 ± 3.48	14.82 ± 1.25	3.73 ± 0.61
	4	5.92 ± 1.14	76.61 ± 2.14	13.38 ± 0.87	4.03 ± 0.54

Significance at $p \leq 0.05$, $n \geq 6$/treatment group.

B: Males

		CD4- CD8- (% Expression)	CD4+ CD8+ (% Expression)	CD4+ CD8- (% Expression)	CD4- CD8+ (% Expression)
Control		8.28 ± 2.17	73.77 ± 2.56	12.91 ± 2.38	5.07 ± 0.91 [a]
E2 µg/25g	0.04	8.23 ± 1.35	75.13 ± 2.24	12.97 ± 1.60	3.68 ± 0.55
	0.4	9.65 ± 3.94	75.95 ± 4.03	11.25 ± 1.36	3.12 ± 0.37
	4	17.03 ± 4.62	65.48 ± 3.59	14.03 ± 1.76	3.45 ± 0.29
EE µg/25g	0.04	11.86 ± 8.56	72.86 ± 8.00	12.52 ± 0.94	2.79 ± 0.15 [b]
	0.4	17.04 ± 8.89	68.08 ± 8.01	11.81 ± 1.82	3.06 ± 0.64 [b]
	4	11.20 ± 6.08	68.28 ± 5.86	16.78 ± 1.93	3.71 ± 0.40

Significance at $p \leq 0.05$, $n \geq 6$/treatment group.

Table 4.2: EE and E2 effects on major thymus cellular subsets were assessed by flow cytometry. Methods were as detailed in Table 1 legend but with daily oral doses.

Within columns, different superscript letters = significantly different from one another (i.e. $a \neq b$).

Table 4.3: Major splenocyte subset percent expression following subcutaneous exposure

A: Females

		CD4+ CD8- (% Expression)	CD4- CD8+ (% Expression)	CD40 (% Expression)	B220 (% Expression)	NK1.1 (% Expression)	F480+ CD11b- (% Expression)	F480- CD11b+ (% Expression)
Control		21.57 ± 0.97 [a]	15.05 ± 1.60	52.77 ± 1.80	40.72 ± 4.34	9.99 ± 1.61	5.76 ± 1.16 [a]	1.53 ± 0.25
E2 µg/25g	0.04	22.72 ± 0.71 [a]	15.88 ± 0.76 [a]	55.28 ± 1.74	46.45 ± 5.32	10.35 ± 0.25	6.30 ± 0.77	1.27 ± 0.20
	0.4	20.68 ± 1.40	14.28 ± 0.91	51.26 ± 2.91	41.90 ± 4.47	11.50 ± 1.10	6.71 ± 1.13	1.05 ± 0.22
	4	17.85 ± 0.82	16.33 ± 0.77	54.22 ± 2.75	47.20 ± 6.79	11.86 ± 3.94	5.27 ± 1.30 [a]	1.48 ± 0.35
EE µg/25g	0.04	14.38 ± 2.89 [b]	11.19 ± 2.41 [b]	56.47 ± 1.43	47.60 ± 5.12	8.16 ± 2.04	7.83 ± 1.55	1.09 ± 0.11
	0.4	18.22 ± 1.01	15.10 ± 0.92	53.45 ± 2.46	45.84 ± 6.85	6.72 ± ***	8.44 ± 0.50	0.97 ± 0.16
	4	18.83 ± 1.44	14.86 ± 1.17	51.37 ± 2.44	49.43 ± 5.35	9.45 ± 0.45	9.84 ± 1.32 [b]	0.91 ± 0.09

Significance at $p \leq 0.05$, $n \geq 6$/treatment group.

B: Males

		CD4+ CD8- (% Expression)	CD4- CD8+ (% Expression)	CD40 (% Expression)	B220 (% Expression)	NK1.1 (% Expression)	F480+ CD11b- (% Expression)	F480- CD11b+ (% Expression)
Control		19.49 ± 4.20	12.80 ± 0.70	48.36 ± 3.19	36.52 ± 6.09 [a]	10.64 ± 2.16 [a]	7.73 ± 1.53 [a]	1.82 ± 0.23
E2 µg/25g	0.04	20.90 ± 0.90	12.90 ± 0.42	54.80 ± 1.95	43.42 ± 7.60	13.81 ± 2.23 [a]	5.05 ± 1.07	1.56 ± 0.29
	0.4	22.02 ± 1.54	14.42 ± 1.01	53.98 ± 2.52	45.64 ± 3.90	14.96 ± 2.70 [b]	8.64 ± 2.31	1.26 ± 0.09
	4	18.38 ± 0.47	14.22 ± 1.20	48.00 ± 2.42	42.50 ± 5.40 [b]	8.87 ± 0.39 [a]	10.00 ± 1.41	1.27 ± 0.52
EE µg/25g	0.04	19.04 ± 0.92	13.66 ± 0.73	54.64 ± 2.67	48.42 ± 3.50 [b]	11.05 ± 0.45 [c]	7.73 ± 1.44	1.31 ± 0.23
	0.4	20.55 ± 0.61	15.02 ± 1.11	53.32 ± 2.86	47.35 ± 4.19 [b]	8.55 ± 1.15	9.90 ± 1.36	1.09 ± 0.13
	4	17.40 ± 1.09	14.98 ± 1.01	48.48 ± 1.77	42.97 ± 6.79	12.25 ± 1.65 [b]	11.03 ± 1.81 [b]	1.06 ± 0.18

Significance at $p \leq 0.05$, $n \geq 6$/treatment group.

Table 4.3: EE and E2 effects on some major spleen cellular subsets including helper T cells ($CD4^+/CD8^-$), cytotoxic T cells ($CD4^-$ $CD8^+$), B cells (CD40, as well as CD45R/B220), natural killer cells (NK 1.1), and macrophages and dendritic cells (CD11b and F4/80). The significance between treatment groups ($p \leq 0.05$) was determined by comparing treatment group to the gender-matched control. Furthermore, each treatment group was then compared to the dose-matched counterpart (i.e. 0.04 E2 vs. 0.04 EE, 0.4 E2 vs. 0.4 EE, 4.0 E2 vs. 4.0 EE) to determine if one estrogenic compound elicited a different response than the other. *** indicates no SEM to report as there was only one mouse in this group with reportable data for this variable due to poor antibody performance. *Within columns, different superscript letters = significantly different from one another (i.e. a ≠ b).*

Table 4.4: Major splenocyte subset percent expression following oral exposure

A: Females

		CD4+ CD8- (% Expression)	CD4- CD8+ (% Expression)	CD40 (% Expression)	B220 (% Expression)	NK1.1 (% Expression)	F480+ CD11b- (% Expression)	F480- CD11b+ (% Expression)
Control		23.51 ± 1.32	11.14 ± 0.84	55.84 ± 2.73	42.47 ± 1.71	8.16 ± 1.04	5.21 ± 0.61	2.17 ± 0.40
	0.04	24.33 ± 1.11	9.98 ± 1.00	54.68 ± 2.47	44.35 ± 2.75	7.90 ± 1.30	4.83 ± 0.98	2.03 ± 0.45
E2 µg/25g	0.4	25.48 ± 1.35	10.81 ± 0.77	53.13 ± 2.01	40.95 ± 2.79 [a]	7.54 ± 1.18	4.31 ± 0.45	2.72 ± 0.88
	4	26.70 ± 2.03	10.38 ± 0.65	55.98 ± 1.26	36.35 ± 2.10 [a]	7.61 ± 0.94	4.91 ± 0.80	1.96 ± 0.46
	0.04	25.60 ± 1.18	9.13 ± 1.11	57.35 ± 1.91	43.40 ± 2.59	7.47 ± 0.66	4.55 ± 0.72	1.95 ± 0.48
EE µg/25g	0.4	25.58 ± 1.50	8.53 ± 0.45	59.12 ± 1.51	48.13 ± 1.51 [b]	8.61 ± 0.89	5.05 ± 0.73	1.83 ± 0.49
	4	25.30 ± 1.51	8.80 ± 0.70	56.90 ± 0.89	44.26 ± 2.19 [b]	7.09 ± 1.25	5.94 ± 0.71	1.60 ± 0.56

Significance at $p \leq 0.05$, $n \geq 6$/treatment group.

B: Males

		CD4+ CD8- (% Expression)	CD4- CD8+ (% Expression)	CD40 (% Expression)	B220 (% Expression)	NK1.1 (% Expression)	F480+ CD11b- (% Expression)	F480- CD11b+ (% Expression)
Control		22.58 ± 1.99	9.19 ± 1.27	50.00 ± 5.70	44.62 ± 2.84	12.32 ± 3.40	5.62 ± 1.05	4.07 ± 1.72
	0.04	25.82 ± 1.44	10.51 ± 1.28	57.98 ± 1.52	43.03 ± 2.48	9.55 ± 1.02	3.90 ± 0.48	2.17 ± 0.54
E2 µg/25g	0.4	23.80 ± 0.87	10.35 ± 0.84	57.67 ± 1.18	43.93 ± 3.20	9.81 ± 1.06	4.71 ± 0.56	2.15 ± 0.51
	4	27.27 ± 1.55	11.13 ± 1.14	52.17 ± 1.29	39.18 ± 3.58	9.45 ± 0.87	5.07 ± 0.63	2.31 ± 0.41
	0.04	26.25 ± 0.79	9.48 ± 0.87	56.68 ± 0.73	44.03 ± 2.70	8.38 ± 1.15	4.37 ± 1.00	2.47 ± 0.70
EE µg/25g	0.4	27.08 ± 1.76	9.78 ± 0.83	54.70 ± 1.22	45.90 ± 2.84	9.07 ± 0.82	5.11 ± 0.66	2.53 ± 0.58
	4	26.15 ± 0.88	12.45 ± 1.26	54.58 ± 0.70	38.68 ± 4.06	8.65 ± 1.01	5.71 ± 0.71	1.95 ± 0.37

Significance at $p \leq 0.05$, $n \geq 6$/treatment group.

Table 4.4: EE and E2 effects on some major spleen cellular subsets including helper T cells ($CD4^+/CD8^-$), cytotoxic T cells ($CD4^- CD8^+$), B cells (CD40, as well as CD45R/B220), natural killer cells (NK 1.1), and macrophages and dendritic cells (CD11b and F4/80). Methods are as detailed in Table 3 legend.

Within columns, different superscript letters = significantly different from one another (i.e. a ≠ b).

Figure 4.1: Uteri following subcutaneous exposure

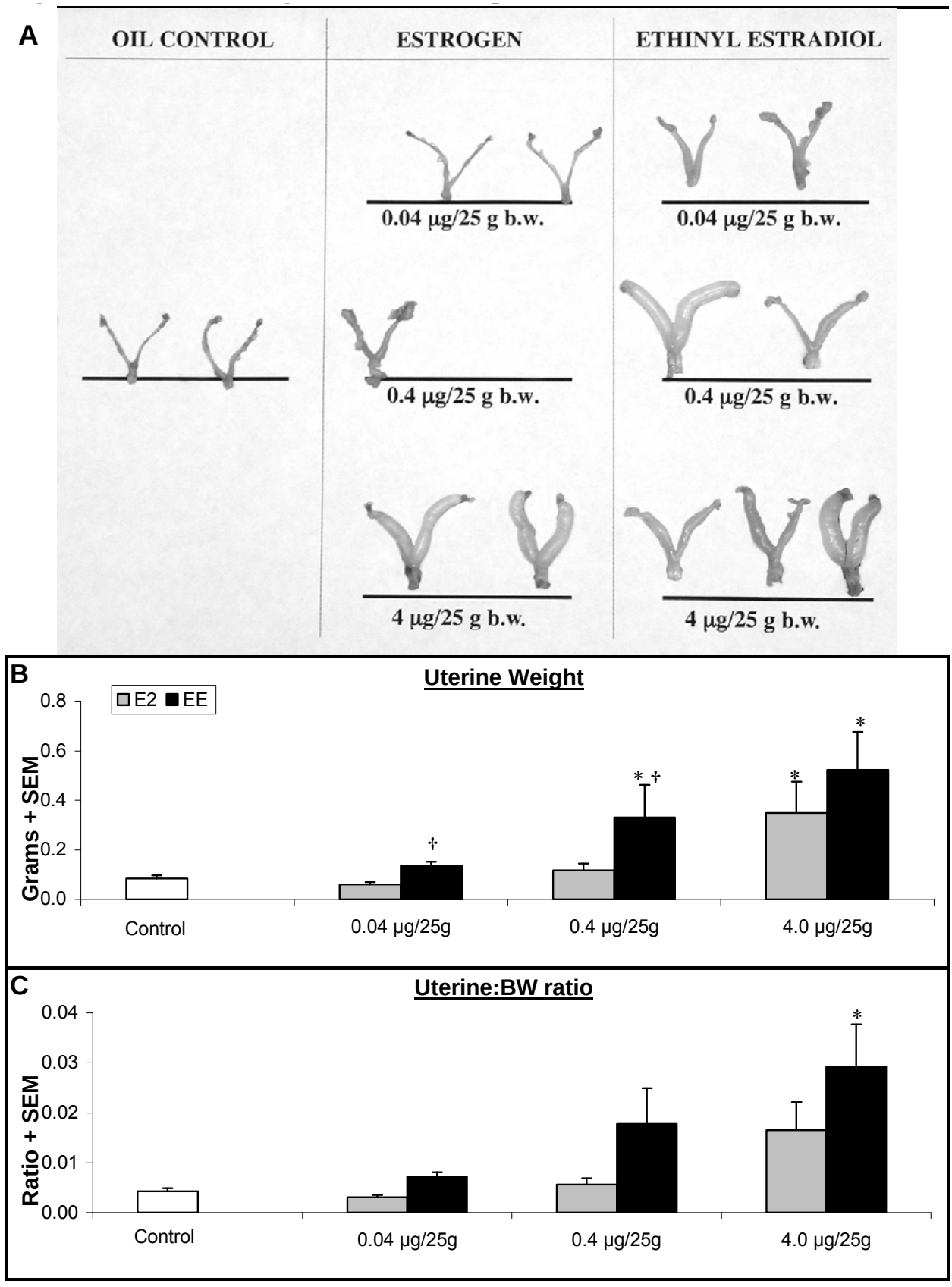
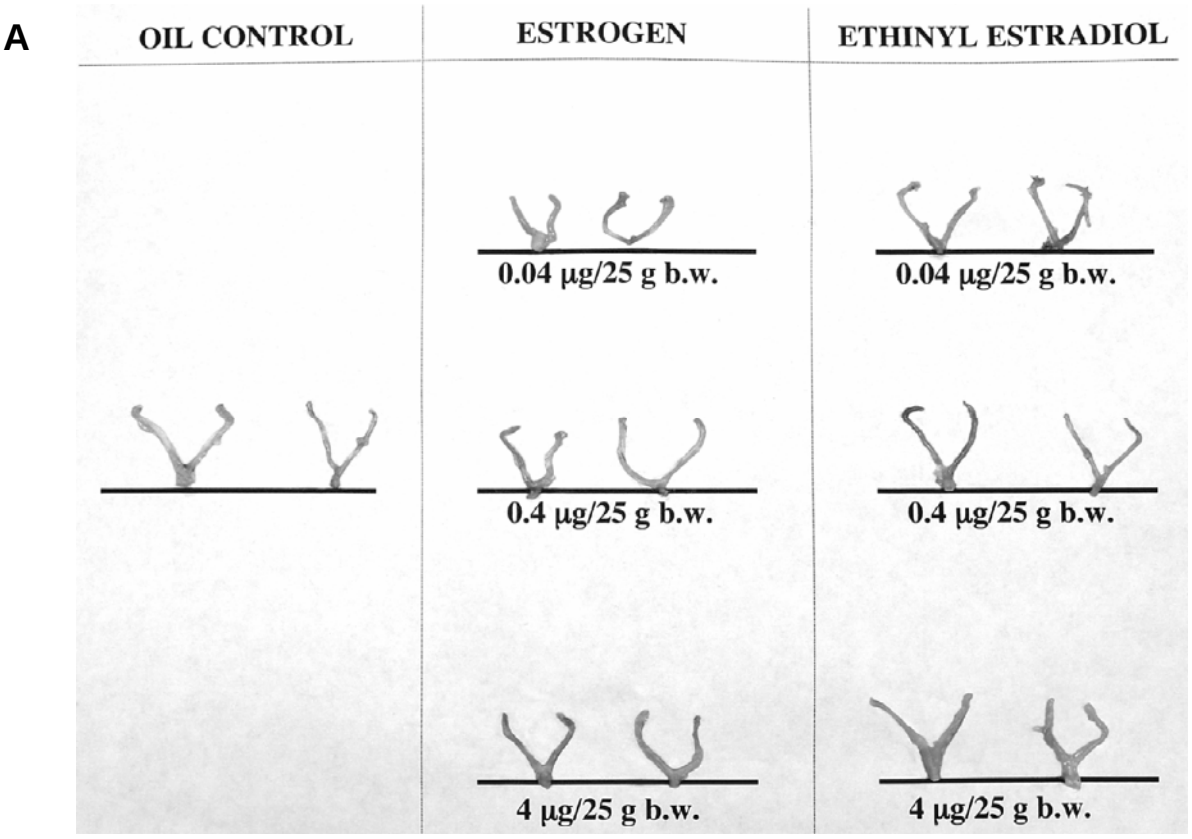


Figure 4.2: Uteri following oral exposure



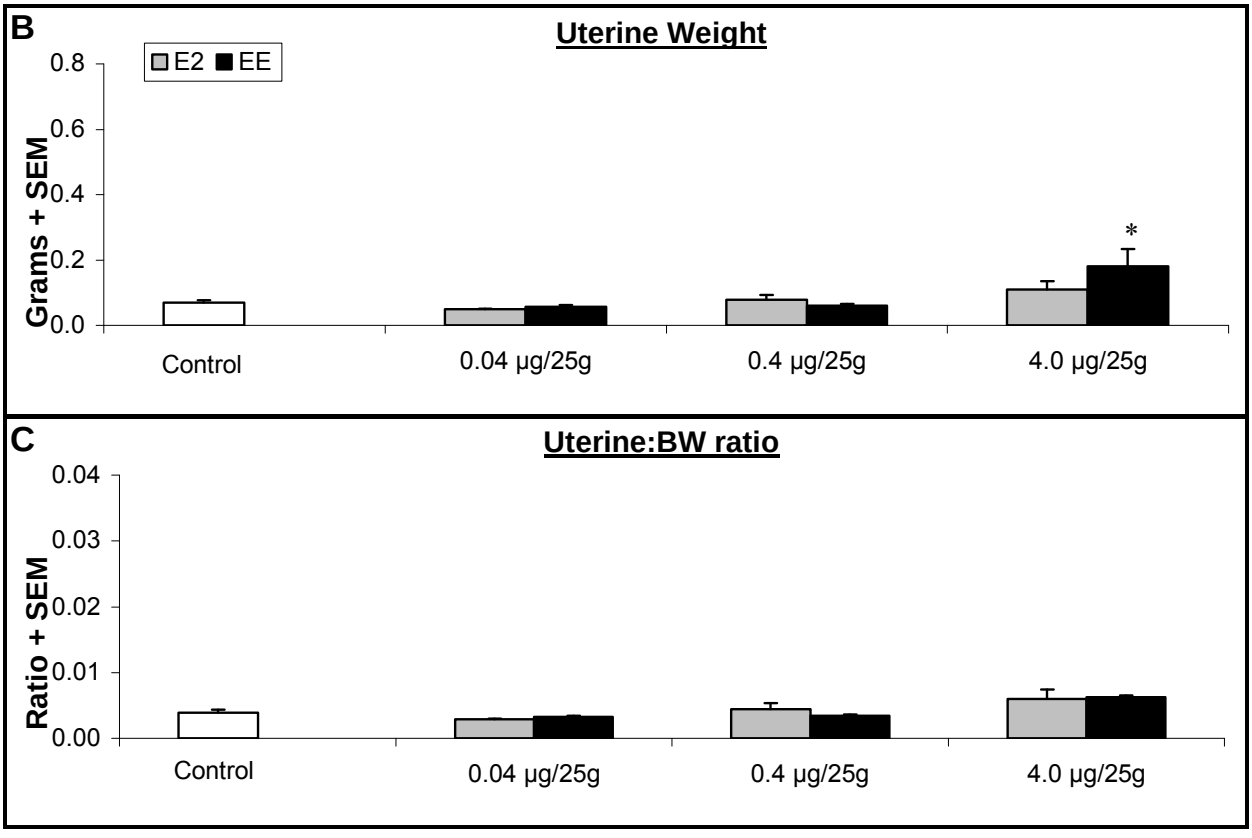


Figure 4.3: Seminal vesicles following subcutaneous exposure

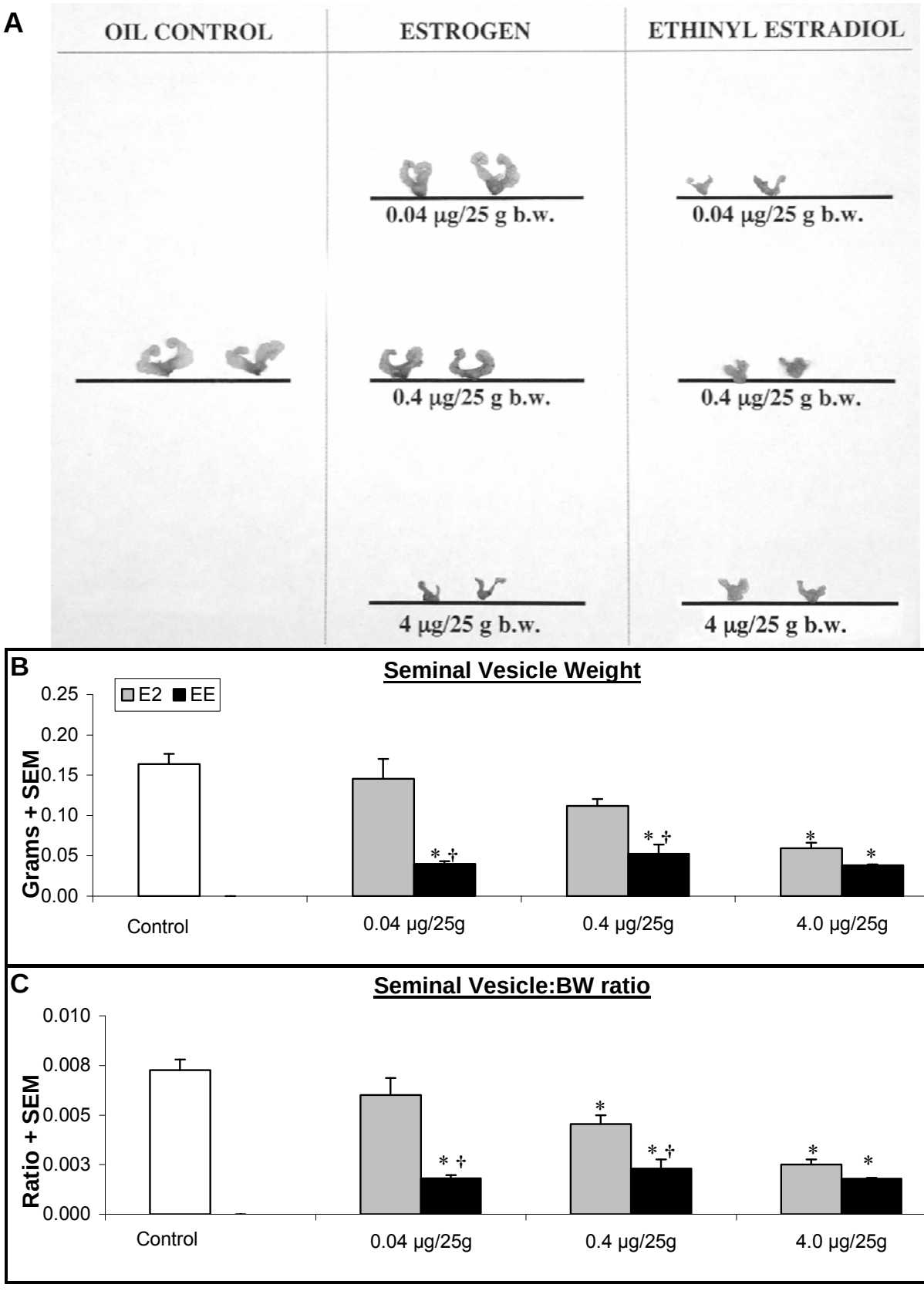


Figure 4.4: Seminal vesicles following oral exposure

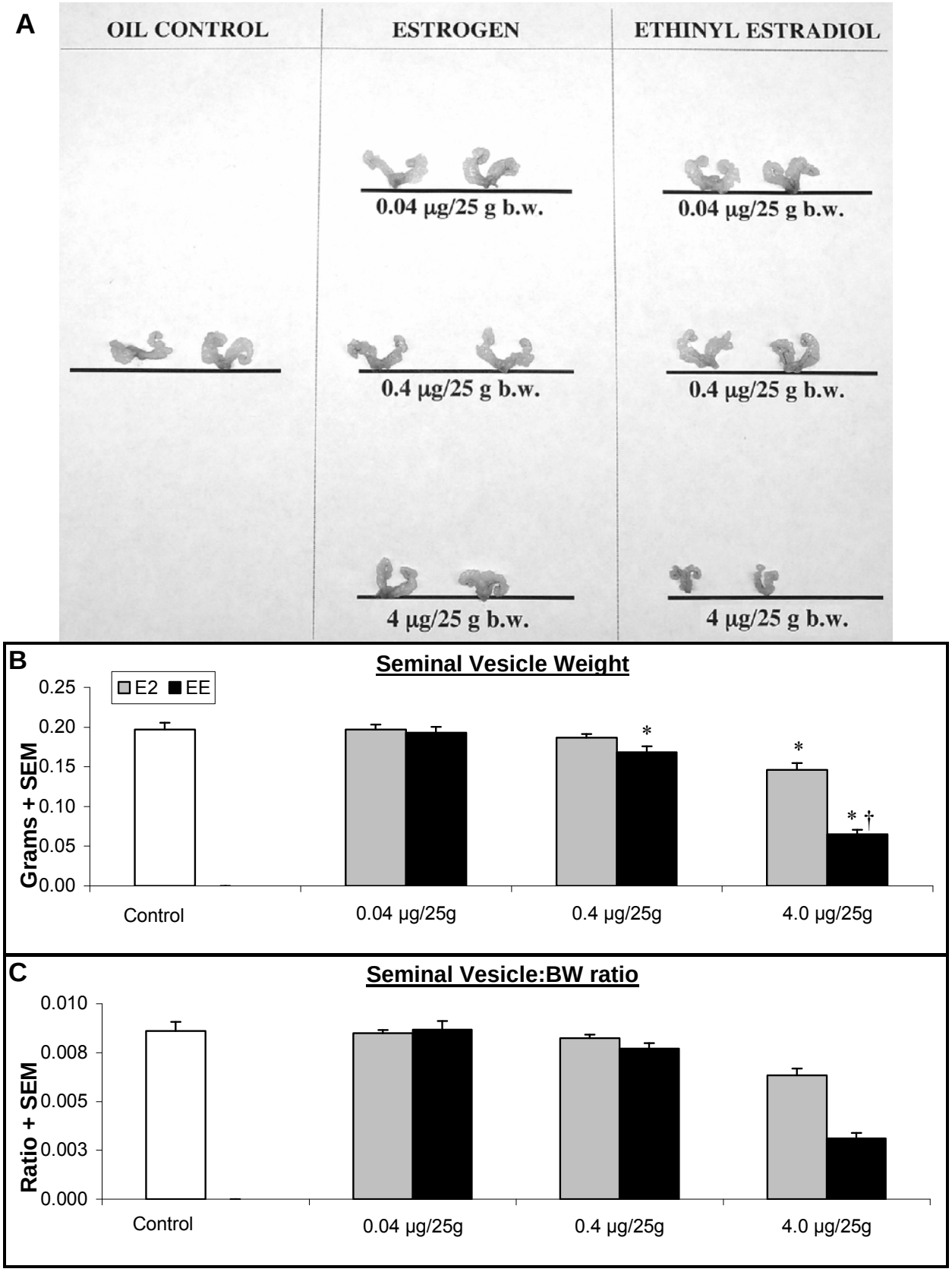


Figure 4.5: Female thymus weight and cellularity following subcutaneous exposure

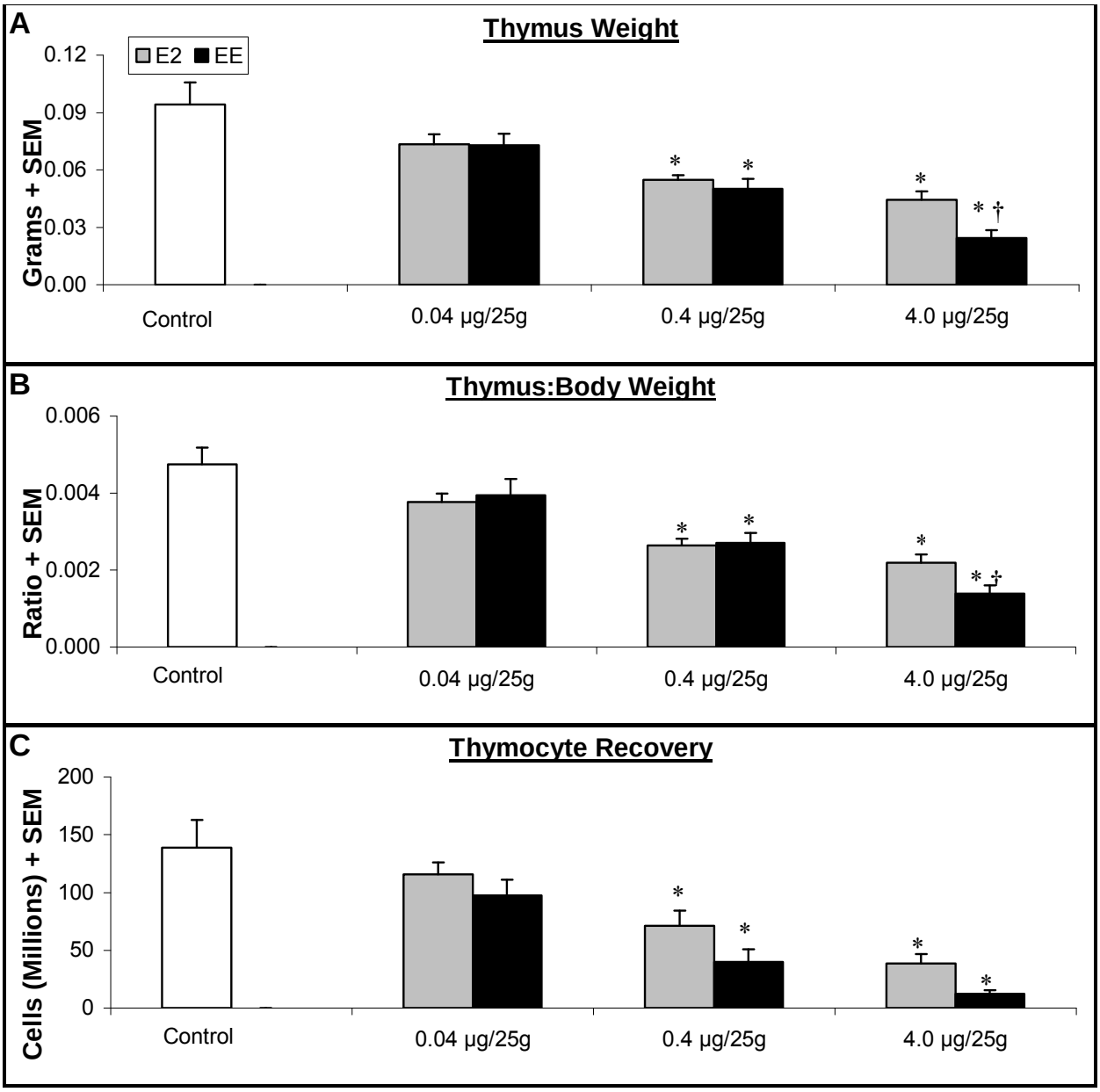


Figure 4.6: Male thymus weight and cellularity following subcutaneous exposure

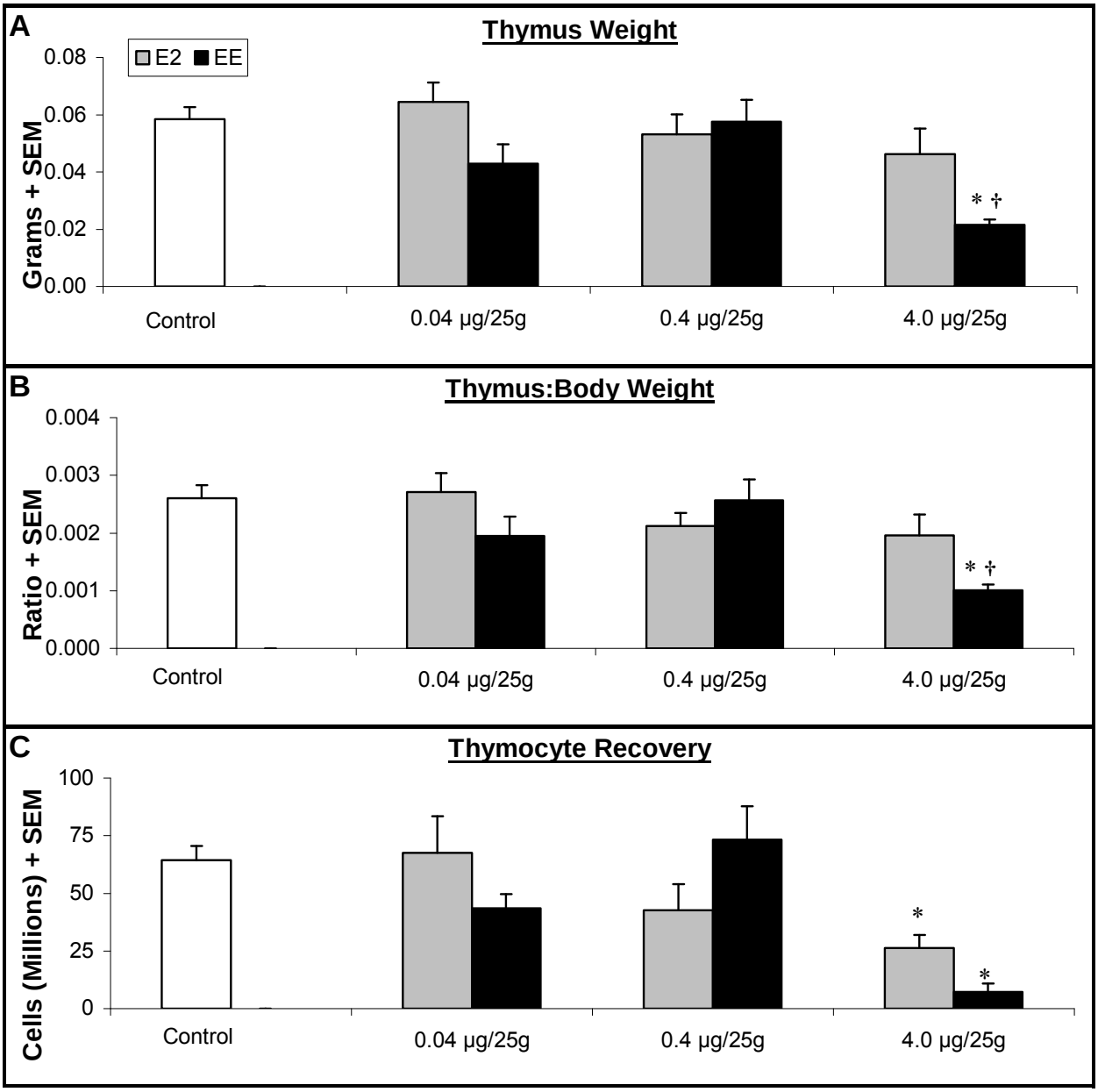


Figure 4.7: Female thymus weight and cellularity following oral exposure

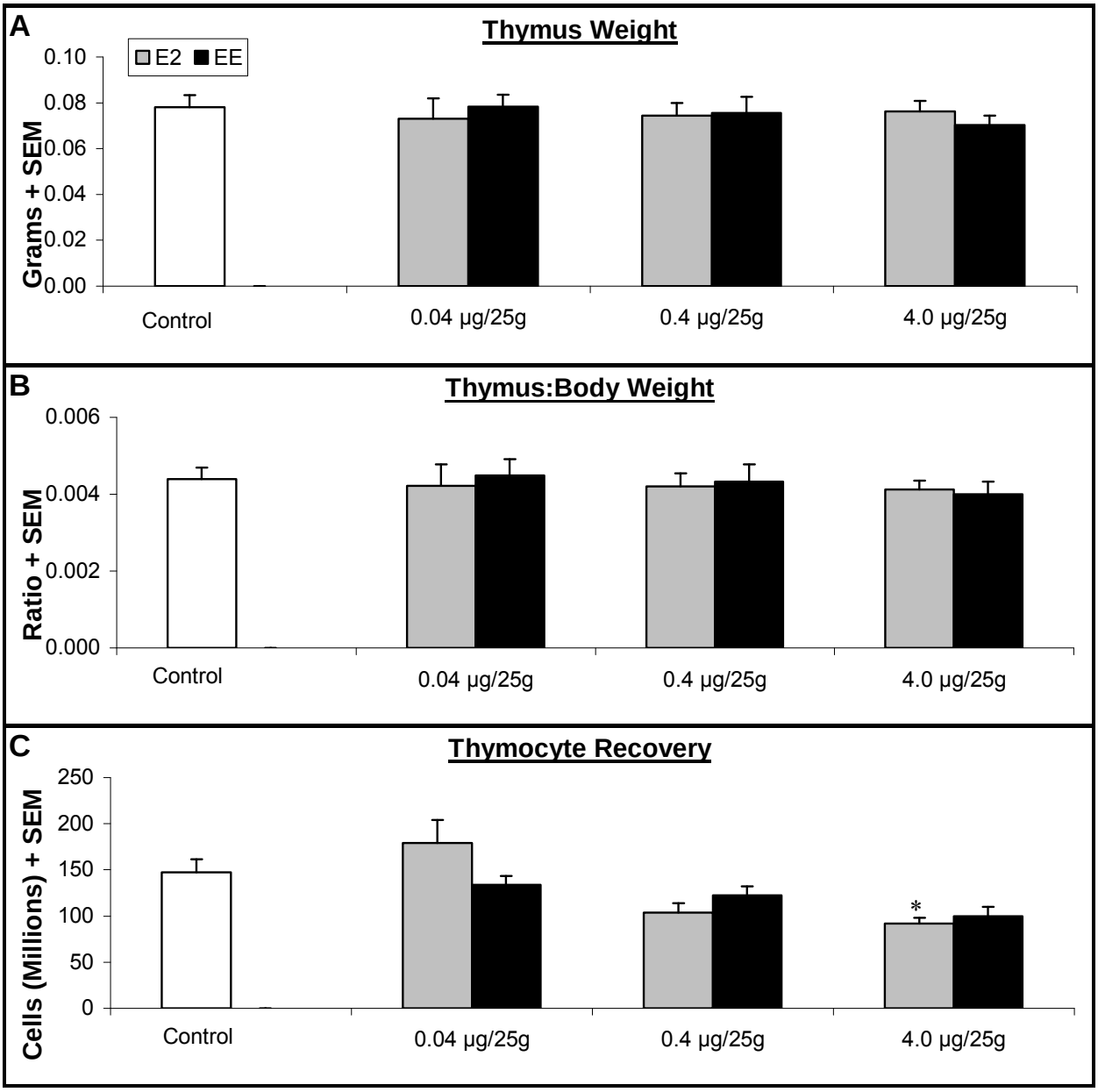


Figure 4.8: Male thymus weight and cellularity following oral exposure

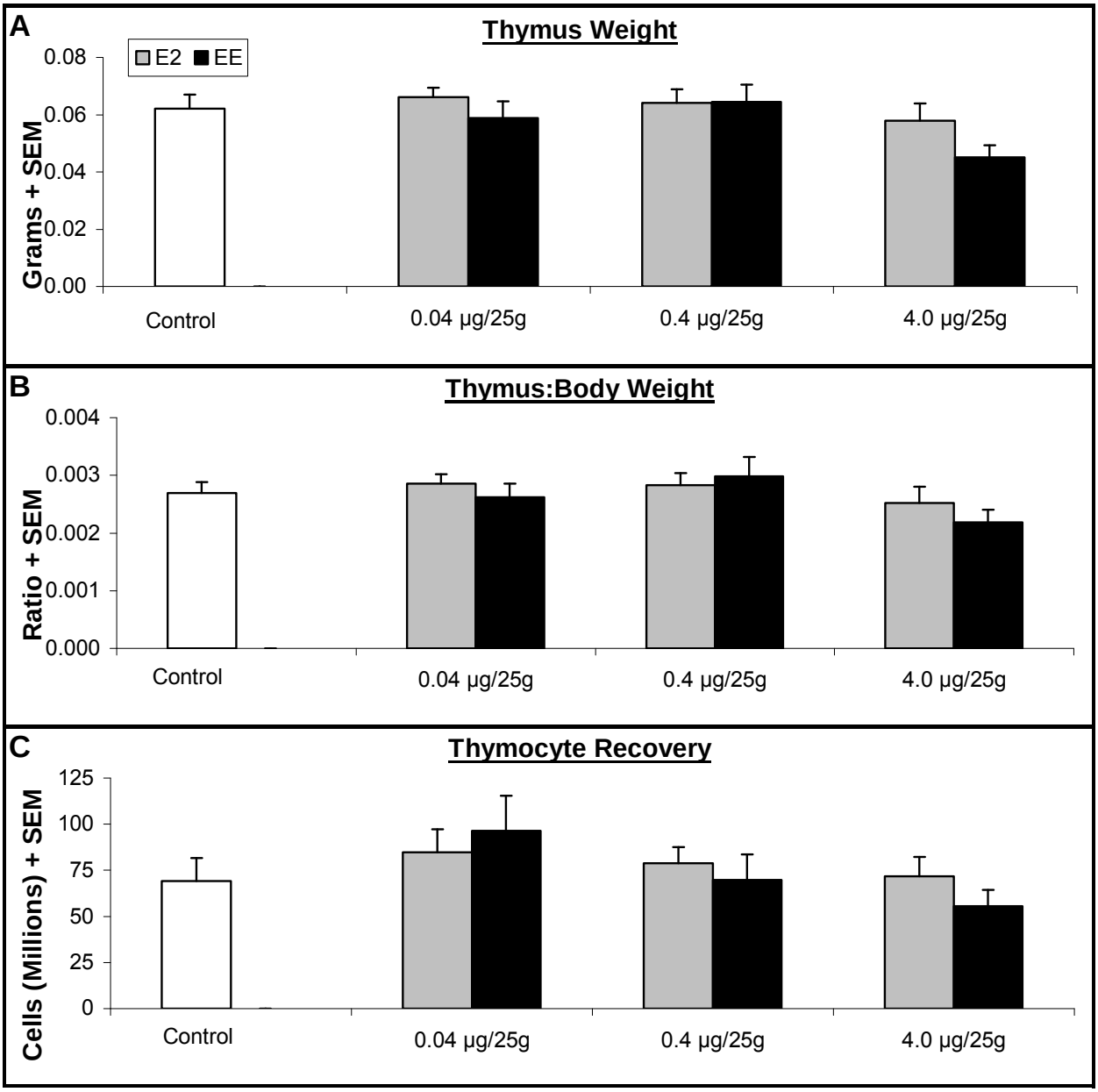


Figure Legends

Figure 4.1. Uterine photograph, weights, and organ to body weight ratios from subcutaneously exposed females. Data are presented as mean + SEM, $n \geq 6$. (**A**) Representative photograph of vehicle-control, E2, and EE-treated female uteri. **(B)** Uterine weight. EE induces a significant increase ($p < 0.05$) in uterine weight at 0.4 and 4.0 µg/25 g BW doses. E2 elicits a significant increase at 4.0 µg/25 g BW. EE induced a significantly more potent response than E2 at 0.04 and 0.4 doses. (**C**) Uterine:BW ratio. Body weight-adjusted results indicate that 4.0 EE induces a significant increase in ratio relative to controls. * denotes significantly different from the gender-matched control and † denotes significantly different from the dose-matched treatment group (i.e. 0.04 E2 vs. 0.04 EE).

Figure 4.2. Uterine photograph, weights, and organ to body weight ratios from orally exposed females. Data are presented as mean + SEM, $n \geq 6$. (**A**) Representative photograph of vehicle-control, E2, and EE-treated female uteri. **(B)** Uterine weight. EE induces significant increase ($p < 0.05$) in uterine weight at 4.0 µg/25 g BW doses compared to controls, while E2 did not cause a significant increase in uterine weight at any dose administered. (**C**) Uterine:BW ratio. None of the treatment groups significantly increased uterine:BW ratio. * denotes significantly different from the gender-matched control and † denotes significantly different from the dose-matched treatment group (i.e. 0.04 E2 vs. 0.04 EE).

Figure 4.3. Reproductive organ photograph, weights, and organ to body weight ratios from subcutaneously exposed males. Data are presented as mean + SEM, $n \geq 6$. (**A**) Representative photograph of vehicle-control, E2, and EE-treated male seminal vesicles. **(B)** Seminal vesicle weight. EE induces a significant decrease ($p < 0.05$) in seminal vesicle weight at all doses and a tremendously more potent response than E2. E2 only elicits a significant decrease at 4.0 µg/25 g BW. EE induced a statistically significant more potent response than E2 at 0.04 EE and 0.4 EE doses. Interestingly, mice treated with 0.04 µg/25 g BW had a similar decrease in seminal vesicle weight to mice exposed to 4.0 µg/25 g BW E2, a dose 100 times greater. (**C**) Seminal vesicle:BW ratio. All doses of EE induced a significant decrease in seminal vesicle:BW ratio compared to the control, as did 0.4 and 4.0 E2. However, EE decreased the ratio significantly

more than E2 at 0.04 and 0.4 dosing levels. * denotes significantly different from the gender-matched control and † denotes significantly different from the dose-matched treatment group (i.e. 0.04 E2 vs. 0.04 EE).

Figure 4.4. Reproductive organ photograph, weights, and organ to body weight ratios from orally exposed males. Data are presented as mean + SEM, n ≥ 6. (**A**) Representative photograph of vehicle-control, E2, and EE-treated male seminal vesicles. **(B)** Seminal vesicle weight. EE induced a significant decrease ($p < 0.05$) in seminal vesicle weight at 0.4 and 4.0 μg/25 g BW doses, while E2 significantly decreased seminal vesicle weight only at 4.0 μg/25 g. EE induced a statistically significant and more potent response than E2 at the 4.0 dose. (**C**) Seminal vesicle:BW ratio. Both 4.0 EE and 4.0 E2 caused a statistically significant decrease in ratio compared to controls. Relative to E2, EE decreased the ratio significantly more at the highest dose used (4.0 μg/25 g BW). * denotes significantly different from the gender-matched control and † denotes significantly different from the dose-matched treatment group (i.e. 0.04 E2 vs. 0.04 EE).

Figure 4.5. Female thymus weight, thymus to body weight ratio, and thymocyte cellularity from subcutaneously exposed mice. Data are presented as mean + SEM, n ≥ 6. (**A**) Thymus weight. EE induces a significant decrease ($p < 0.05$) in thymus weight at 0.4 and 4.0 μg. E2 elicits a significant decrease at 0.4 and 4.0 μg/25 g BW. EE induced a statistically significant response compared to E2 at the 4.0 μg dose. (**B**) Thymus:BW ratio. Relative thymus weight (adjusted for body weight) indicated the same pattern as thymus weight, with both EE and E2 significantly decreasing the ratio at 0.4 and 4.0 doses, while EE induced a more potent decrease in ratio at 4.0 μg. (**C**) Thymocyte Cellularity. A significant decrease in thymocyte recovery (number of thymocytes recovered from each thymus) was observed at 0.4 and 4.0 EE, as well as 0.4 and 4.0 E2. * denotes significantly different from the gender-matched control and † denotes significantly different from the dose-matched treatment group (i.e. 0.04 E2 vs. 0.04 EE).

Figure 4.6. Male thymus weight, thymus to body weight ratio, and thymocyte cellularity from subcutaneously exposed mice. Data are presented as mean + SEM, n ≥ 6. (**A**) Thymus weight. EE induces a significant decrease ($p < 0.05$) in thymus weight at 4.0 μg, a response that is

significantly more potent than 4.0 E2. (**B**) Thymus:BW ratio. Relative thymus weight (adjusted for body weight) indicated the same pattern as thymus weight, with 4.0 EE significantly decreasing the ratio. (**C**) Thymocyte Cellularity. A significant decrease in thymocyte recovery (number of thymocytes recovered from each thymus) was observed at 4.0 EE, as well as 4.0 E2. * denotes significantly different from the gender-matched control and † denotes significantly different from the dose-matched treatment group (i.e. 0.04 E2 vs. 0.04 EE).

Figure 4.7. Female thymus weight, thymus to body weight ratio, and thymocyte cellularity from orally exposed mice. Data are presented as mean + SEM, $n \geq 6$. (**A**) Thymus weight. In stark contrast to subcutaneous exposure, thymus weight was unaffected by oral exposure regardless of treatment group. (**B**) Thymus:BW ratio. Relative thymus weight (adjusted for body weight) indicated the same pattern as for thymus weight, with none of the treatment groups demonstrating a significant change in ratio. (**C**) Thymocyte Cellularity. A significant decrease in thymocyte recovery relative to controls (number of thymocytes recovered from each thymus) was observed at 4.0 E2 only. * denotes significantly different from the gender-matched control and † denotes significantly different from the dose-matched treatment group (i.e. 0.04 E2 vs. 0.04 EE).

Figure 4.8. Male thymus weight, thymus to body weight ratio, and thymocyte cellularity from orally-exposed mice. Data are presented as mean + SEM, $n \geq 6$. (**A**) Thymus weight. (**B**) Thymus:BW ratio. (**C**) Thymocyte Cellularity. There were no significant alterations in thymus weight, thymus:BW ratio, or thymocyte cellularity in any of the male treatment groups.

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Chapter 5: General Discussion and Conclusions

The synthetic estrogen, 17α-ethinyl estradiol (EE), is one of the most widely prescribed and environmentally detected environmental compounds. Due to the wide range of typical exposure conditions (pharmaceutical or environmental contamination) exposure is likely across a wide cross-section of the population. Past reports of contamination of drinking water, food, and soil would indicate that there is a potential for regular exposure of all ages from infancy (including *in utero* development) to the elderly, and between sexes. This poses a threat not only to humans but to cattle and possibly other animals as well. Polycystic ovarian syndrome has been observed in cattle and linked to disruptions in endocrine balance and the hypothalamic-hypophysial-axis due to environmental sources, including estrogen [1-3]. Overall, our studies have demonstrated that EE is both a reproductive and immunomodulatory compound, particularly when administered subcutaneously. Route of exposure of the estrogens appears to have a greater influence on the degree of immunological alterations, particularly in this 21-day exposure model. It is likely that acute exposure of higher doses and chronic exposure of lower doses would produce different results. Further, our studies indicate that the thymus is a sensitive target of EE-modulation. The most important observation in this experimental system is that EE is significantly more potent than E2 with regard to induction of reproductive and thymic effects. It is therefore conceivable that even lower exposure levels of EE relative to E2, a known immunomodulatory agent, may have health implications.

These preliminary studies reveal that EE induces striking alterations in the gross anatomy of the rodent reproductive organs, as well as the thymus. However, alterations in IFN-γ and nitric oxide production by Con-A-stimulated splenocytes *in vitro* were not observed. This suggest that treatment duration was insufficient to induce functional alterations in splenocyte cytokine production, or possibly the lack of an antigenic challenge *in vivo* may not have stimulated immune alterations. Further, since these mice were intact, it is possible that the endogenous hormonal milieu may have a protective effect with regard to molecular changes in short-term exposure situations. This possibility may be good in real life human exposure situations but is not ideal in scientific settings if trying to determine biological mechanisms of action of specific estrogenic compounds. However, there are several biomarkers of estrogenic exposure, both molecular and non-molecular, that were not assessed in these studies including *in*

vitro assessment of MCF-7 cell proliferation, estrogen receptor binding and transactivation, and *in vivo* assessment of endometrial thickness [4].

It is important to note that differences likely exist across species with regard to response to exogenous EE [5]. Differences in hormone cycling, sensitivity to estrogen, and hormone metabolism patterns will impact the effects that are observed. Extrapolation of results from rodents to humans should be done conservatively. Synthetic steroids are less effective orally in rodents than in people, so direct extrapolation from animal doses to human doses may lead to human overdosing [6]. However, doses used in the subcutaneous (second) and oral (third) study were comparable to that of what is administered to women in hormonal contraceptives. Modern hormonal contraceptive formulations range from 20-50 μg. More studies are needed to elucidate at what levels EE may have a positive effect and at what levels it may have a negative effect on the immune system, across age and sexes, duration of exposure, and route of exposure. It must also be noted that our studies assessed only the immune-modulating properties of EE, but almost all EE-containing hormonal contraceptives contain a progesterone component (Table 2.1). There is an interrelation between the two compounds when administered for therapeutic or contraceptive purposes, meaning that if they had been administered together in these studies observations would have very likely differed. Whether those differences would be positive in nature (i.e. diminished immunologic and reproductive alterations) or negative in nature (i.e. greater immunologic and reproductive consequences) remains to be determined by future work.

There are many questions and aspects of this work that remain unanswered. It is critical that these preliminary studies be expanded upon to decipher the exact nature and mechanism through which EE may induce immunological alterations. Our findings with regard to the potency of EE compared to E2 and effects on the thymus are unequivocal. However, the lack of effects of EE on other less sensitive immune parameters may be because our studies needed to address the role of age, sex, route of exposure, and comparative potency of E2 to EE with regard to immunological effects. This particular body of work attempted to address several gaps in the literature. The number of treatment groups dictated a rather limited statistical approach, an analysis of variance (ANOVA). This procedure has low statistical power when the numbers of mice per treatment group are as low as six to eight, combined with the fact there were numerous treatment groups [7]. This resulted in several multiple comparisons in the analysis, further reducing statistical power. In short, the paucity of data with regard to immunological effects of

EE across age, gender, and route of exposure necessitated somewhat limited study design with several treatment groups. These data form the foundation for which future studies should be designed to address remaining questions.

Future studies need to investigate the influence of EE on the bone marrow, as all thymic T cells are derived from hematopoietic stem cells that originate from there [8]. Specifically, how does EE affect prothymocyte trafficking from the bone marrow to the thymus? Are the thymic effects of EE mediated directly on the thymus or are they mediated via bone marrow stem cells targeted for distribution to the thymus, as in the case with DES [9]? Future studies would certainly need to concentrate on the thymus, attempting to determine the precise mechanism by which EE causes thymic involution and decreased thymocyte recovery. Since there is so little information available on the effects of EE, these studies have merit, although preliminary in nature. Currently lacking is a deeper investigation into the mechanistic pathways of thymus cytokine production, apoptosis, and negative selection, or thymocyte migration into and out of the thymus. Histopathological analysis of the thymus would answer structural questions regarding the effect of EE treatment on the cortex and medullary region of the thymus, regions which are affected by other estrogenic compounds.

Furthermore, these studies were limited to examination of only one peripheral immune organ, the spleen. It would be prudent to examine the effect of estrogen treatment on lymphocyte subset expression in lymph nodes, mucosal-associated lymphoid tissue, and other secondary sites in order to definitively determine whether or not mature lymphoid organs are unaffected by subacute, subcutaneous dosing of EE or E2. The measurement of IgG and IgM levels would provide a useful measurement of the differences in effect of EE compared to E2 on humoral immunity, a common immune component known to be altered by E2. In addition, levels of E2, EE, and testosterone should be assessed prior to and following treatment to have a better understanding of how EE or E2 treatment is affecting the endocrine system and primary hormone levels. Effects on the liver should be observed, as short term doses tend to exert effects on the liver and hematological system. Increased liver weight and hepatocyte hypertrophy and hyperplasia have been noted in rats treated with EE at 1-10 mg/kg for several days [10, 11]. Assessment and comparison on the effects of EE versus EW2 on the liver in oral administration would be useful for determining any metabolic endpoints that may be altered [12].

Vascular lesions are commonly observed in women using oral contraceptives (OC) [13], while anti-EE antibodies are also commonly observed in women taking OCs. Anti-EE antibodies have been observed in both healthy OC users and OC users with SLE [14, 15]. The observations that anti-EE antibodies play a role in the development of vascular thrombosis [16, 17] suggests that EE may promote an inflammatory state in particular systems of the body. Inflammation is mediated by the immune system, further suggesting that EE is an immunomodulatory compound.

There are many conflicting reports regarding steroid-based oral contraceptives on plasma immunoglobulin levels [18], as well as numerous other immunological endpoints that have previously been assessed. Several studies from the 1970s and 1980s have produced conflicting results. As technology has advanced and the number of uses/target population for EE-containing drugs has increased, it is imperative that immunological alterations be investigated more vigorously. More advanced immunological and molecular assays now exist which can more accurately assess precisely what the effects of hormonal therapies are on the immune system. Formulations of hormonal contraceptives and routes of delivery have varied greatly in the ensuing years [19] and many of the immunological studies from the 1960s, 1970s, and 1980s may not be applicable to modern formulations. Immunological studies have investigated only oral formulations of hormones and even those studies do not adequately address immunological effects and function. There are no studies which examine the immunological effects of transdermal, subcutaneous, or intramuscular injection of hormonal contraceptives or hormone replacement therapy, as aspect which warrants intense investigation.

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Appendix A

Additional Figures and Tables for Chapter 3: Subacute oral administration of low dose 17β-estradiol or 17α-ethinyl estradiol does not markedly alter the immune system of young adult and aged C57BL/6 mice

Table A1: Body Weights

A: Females

		Initial BW (g)	Terminal BW (g)	BW Change (g)
Young	Control	13.33 ± 0.11	19.16 ± 0.42	5.84 ± 0.34
	E2	13.39 ± 0.29	19.31 ± 0.46	5.92 ± 0.38
	EE	12.72 ± 0.43	18.21 ± 0.47	5.49 ± 0.60
Aged	Control	29.21 ± 1.81	28.84 ± 1.51	-0.37 ± 0.81
	E2	27.58 ± 1.92	26.01 ± 1.43	-1.58 ± 1.12
	EE	28.28 ± 1.49	27.45 ± 1.39	-0.83 ± 0.45

B: Males

		Initial BW (g)	Terminal BW (g)	BW Change (g)
Young	Control	16.67 ± 0.81	24.11 ± 0.46	7.44 ± 0.88
	E2	16.49 ± 0.47	23.96 ± 0.60	7.46 ± 0.48
	EE	15.66 ± 0.56	23.81 ± 0.53	8.15 ± 0.35
Aged	Control	34.52 ± 3.65	32.84 ± 3.00	-1.68 ± 1.11
	E2	41.42 ± 1.35	38.33 ± 3.00	-3.09 ± 2.67
	EE	38.02 ± 4.93	37.18 ± 4.72	-0.84 ± 0.73

Table A2: Spleen Weight and Cellularity

A: Females

		Splenic Weight (Grams)	Spleen:Body Weight (Ratio)	Splenic Cellularity Cells (Million)
Young	Control	0.09 ± 0.01	0.005 ± 0.0003	63.37 ± 10.08
	E2	0.09 ± 0.01	0.005 ± 0.0003	59.01 ± 10.97
	EE	0.09 ± 0.00	0.005 ± 0.0003	63.50 ± 6.17
Aged	Control	0.12 ± 0.01	0.004 ± 0.0005	42.39 ± 13.62
	E2	0.19 ± 0.05	0.008 ± 0.0021	73.53 ± 11.92
	EE	0.10 ± 0.02	0.004 ± 0.0005	48.62 ± 6.69

B: Males

		Splenic Weight (Grams)	Spleen:Body Weight (Ratio)	Splenic Cellularity Cells (Million)
Young	Control	0.08 ± 0.01	0.003 ± 0.0003	48.91 ± 6.43
	E2	0.09 ± 0.01	0.004 ± 0.0005	47.82 ± 6.64
	EE	0.08 ± 0.00	0.003 ± 0.0001	53.38 ± 8.66
Aged	Control	0.09 ± 0.01	0.003 ± 0.0005	50.05 ± 3.74
	E2	0.10 ± 0.01	0.003 ± 0.0004	67.00 ± 18.22
	EE	0.08 ± 0.01	0.002 ± 0.0002	43.57 ± 9.51

Figure A1: Female reproductive tract weight

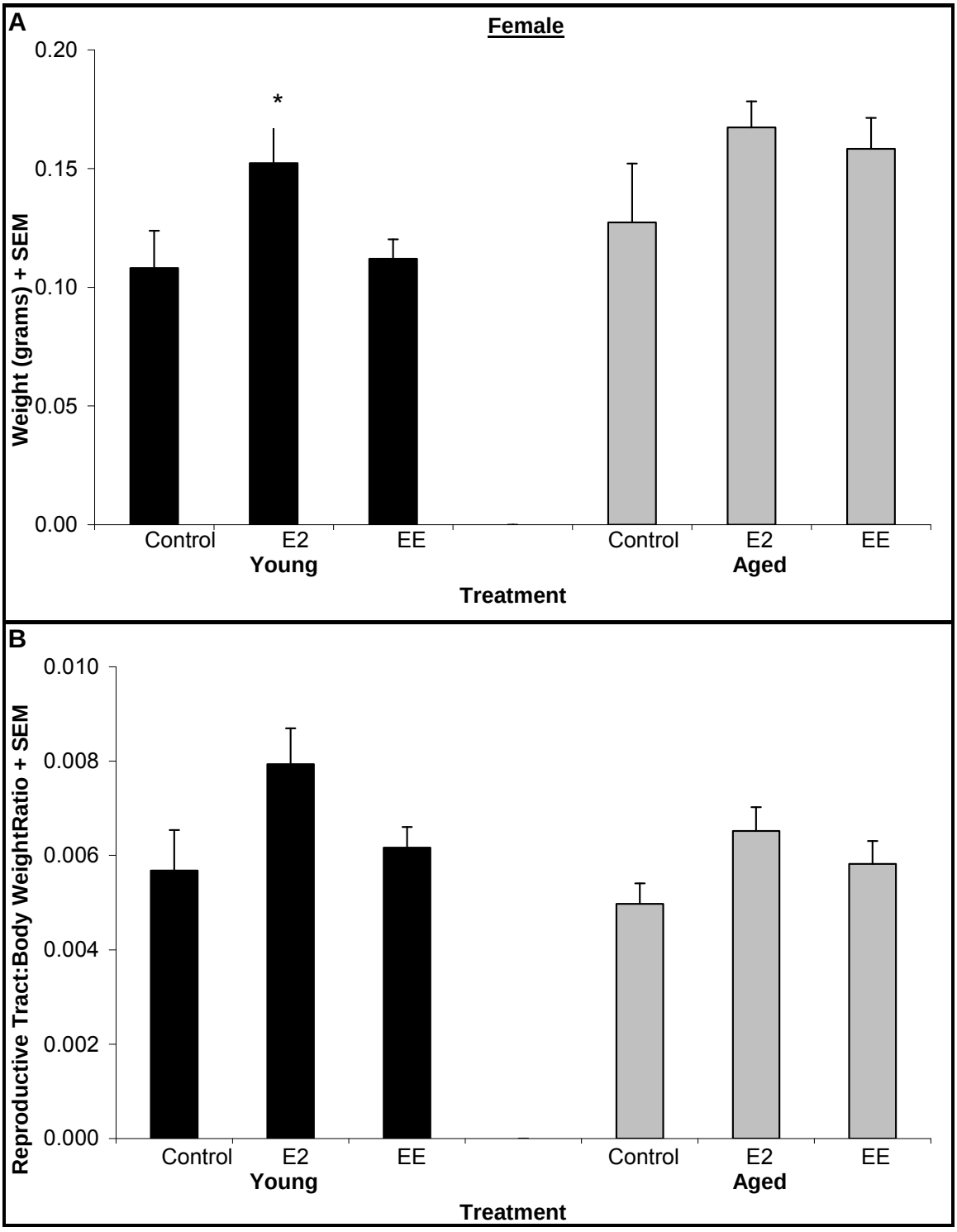


Figure A2: Male reproductive organ weight

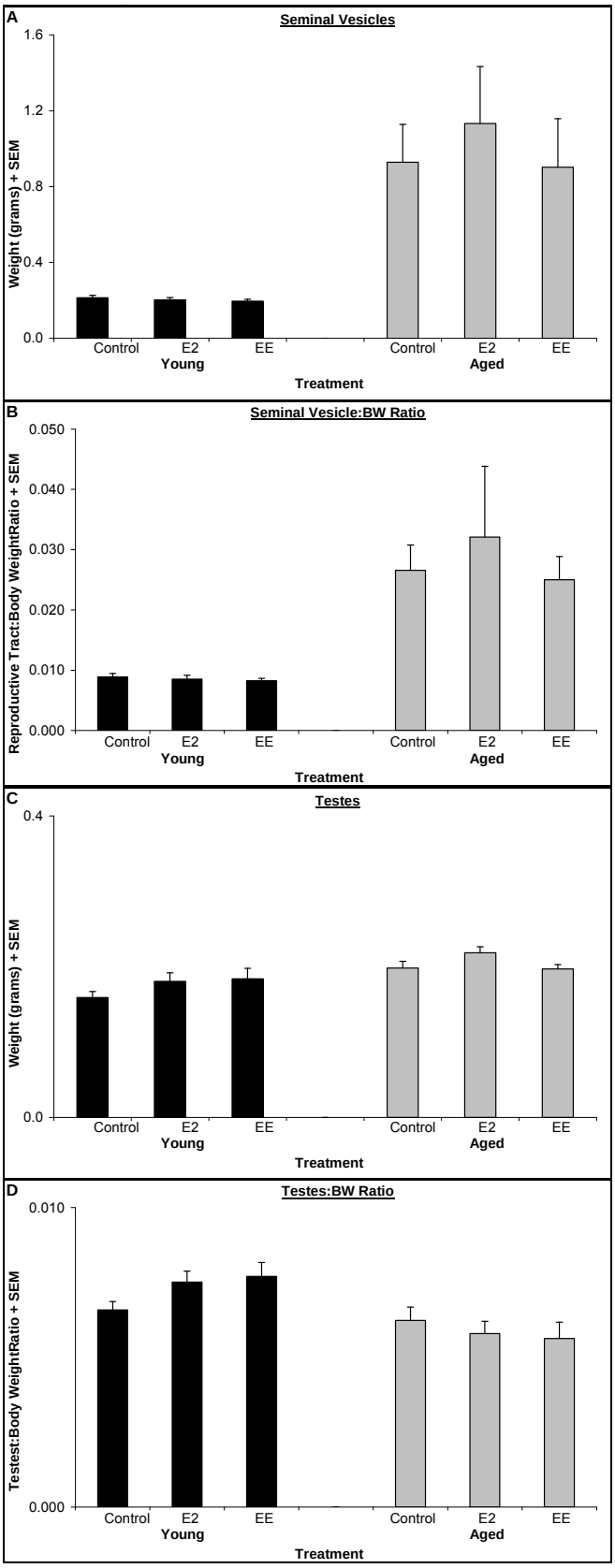
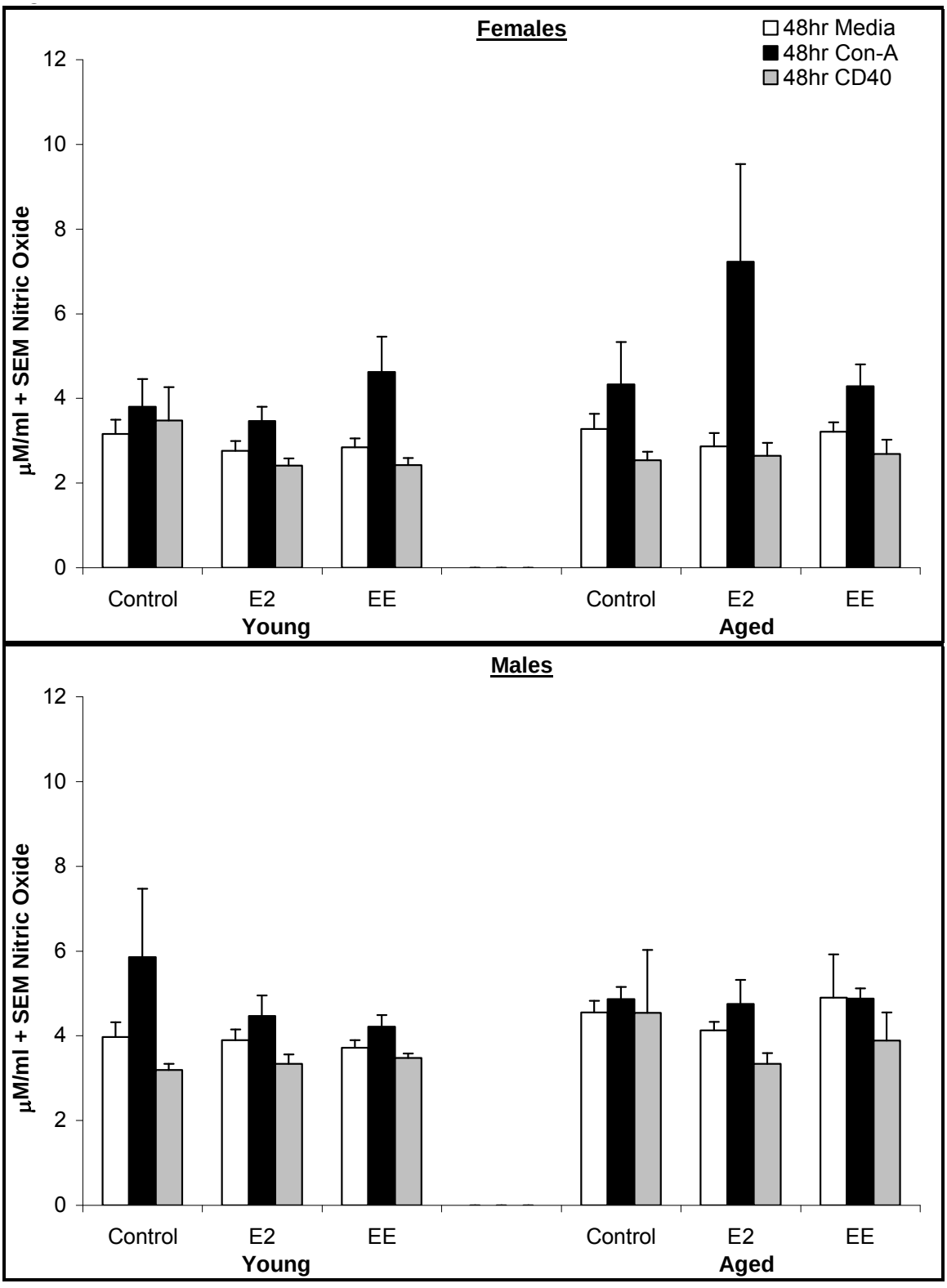


Figure A3: Nitric oxide at 48 hours



Appendix B

Additional Figures and Tables for Chapter 4: Subcutaneous, but not oral, administration of 17α-ethinyl estradiol markedly alters the thymus and reproductive organs of juvenile C57BL/6 mice relative to 17β-estradiol

Table B1: Body weights following subcutaneous exposure

A: Females

		Initial BW (g)	Terminal BW (g)	BW Gain (g)
Control		14.18 ± 0.59	19.61 ± 0.59	5.43 ± 0.56 [a]
E2 µg/25g	0.04	13.99 ± 0.35	19.43 ± 0.28	5.44 ± 0.46
	0.4	15.28 ± 0.32	20.83 ± 0.41	5.40 ± 0.36
	4	15.14 ± 0.70	20.53 ± 0.60	5.39 ± 0.48 [a]
EE µg/25g	0.04	14.31 ± 0.28	18.71 ± 0.56	4.40 ± 0.54
	0.4	13.49 ± 0.66	18.49 ± 0.32	5.00 ± 0.58
	4	14.23 ± 0.52	17.50 ± 0.50	3.27 ± 0.23 [b]

B: Males

		Initial BW (g)	Terminal BW (g)	BW Gain (g)
Control		16.24 ± 0.88	22.55 ± 0.53	6.31 ± 0.75
E2 µg/25g	0.04	16.13 ± 0.81	23.84 ± 0.84	7.52 ± 0.49
	0.4	17.55 ± 0.30	24.84 ± 0.86	7.29 ± 0.95
	4	16.30 ± 0.44	21.99 ± 1.65	5.68 ± 1.62
EE µg/25g	0.04	16.56 ± 0.39	22.17 ± 0.37	5.61 ± 0.31
	0.4	16.64 ± 0.35	22.46 ± 0.81	5.82 ± 0.57
	4	18.16 ± 0.44	21.38 ± 0.50	3.22 ± 0.69

Significance at $p \leq 0.05$, $n \geq 6$/treatment group.

Table B2: Body weights following oral exposure

A: Females

		Initial BW (g)	Terminal BW (g)	BW Gain (g)
Control		15.55 ± 0.26	17.84 ± 0.43	2.29 ± 0.36
E2 µg/25g	0.04	15.91 ± 0.30	17.44 ± 0.36	1.53 ± 0.19
	0.4	16.12 ± 0.19	17.83 ± 0.45	1.71 ± 0.31
	4	16.65 ± 0.31	18.44 ± 0.40	1.79 ± 0.44
EE µg/25g	0.04	15.30 ± 0.31	17.64 ± 0.64	2.34 ± 0.47
	0.4	15.65 ± 0.40	17.56 ± 0.25	1.91 ± 0.30
	4	15.54 ± 0.22	17.69 ± 0.18	2.02 ± 0.16

B: Males

		Initial BW (g)	Terminal BW (g)	BW Gain (g)
Control		19.30 ± 0.32	23.06 ± 0.67	3.64 ± 0.48 [a]
E2 µg/25g	0.04	19.47 ± 0.31	23.21 ± 0.47	3.73 ± 0.22
	0.4	18.83 ± 0.32	22.65 ± 0.17	3.83 ± 0.38
	4	19.49 ± 0.30	23.08 ± 0.43	3.59 ± 0.38 [a]
EE µg/25g	0.04	19.16 ± 0.36	22.32 ± 0.44	3.16 ± 0.25
	0.4	18.97 ± 0.30	21.88 ± 0.62	2.91 ± 0.37
	4	19.10 ± 0.22	20.79 ± 0.33	1.69 ± 0.45 [b]

Significance at $p \leq 0.05$, $n \geq 6$/treatment group.

Table B3: Spleen weight and cellularity following subcutaneous exposure

A: Females

		Spleen Weight (Grams)	Spleen:Body Weight (Ratio)	Splenic Cellularity Cells (Million)
Control		0.099 ± 0.009	0.0050 ± 0.0004	88.64 ± 7.67
E2 µg/25g	0.04	0.103 ± 0.007	0.0053 ± 0.0003	93.66 ± 9.01
	0.4	0.131 ± 0.006	0.0063 ± 0.0002	103.77 ± 9.69
	4	0.122 ± 0.009	0.0059 ± 0.0004	66.64 ± 7.95
EE µg/25g	0.04	0.105 ± 0.009	0.0056 ± 0.0004	82.52 ± 10.47
	0.4	0.116 ± 0.008	0.0062 ± 0.0004	66.75 ± 8.10
	4	0.107 ± 0.012	0.0061 ± 0.0006	72.02 ± 11.89

B: Males

		Spleen Weight (Grams)	Spleen:Body Weight (Ratio)	Splenic Cellularity Cells (Million)
Control		0.129 ± 0.024	0.0057 ± 0.0011	77.83 ± 8.60
E2 µg/25g	0.04	0.114 ± 0.006	0.0048 ± 0.0002	92.85 ± 8.80
	0.4	0.103 ± 0.007	0.0042 ± 0.0003	67.83 ± 11.05
	4	0.126 ± 0.008	0.0053 ± 0.0003	67.11 ± 2.86
EE µg/25g	0.04	0.109 ± 0.012	0.0049 ± 0.0005	75.94 ± 10.35
	0.4	0.102 ± 0.007	0.0046 ± 0.0003	83.42 ± 7.06
	4	0.143 ± 0.008	0.0067 ± 0.0004	70.34 ± 8.01

Table B4: Spleen weight and cellularity following oral exposure

A: Females

		Spleen Weight (Grams)	Spleen:Body Weight (Ratio)	Splenic Cellularity (Cells (Million))
Control		0.089 ± 0.0075	0.0049 ± 0.0004	95.25 ± 9.72
E2 µg/25g	0.04	0.093 ± 0.0092	0.0053 ± 0.0005	114.06 ± 11.78
	0.4	0.086 ± 0.0026	0.0048 ± 0.0002	98.69 ± 9.35
	4	0.093 ± 0.0037	0.0050 ± 0.0001	98.43 ± 6.88
EE µg/25g	0.04	0.087 ± 0.0053	0.0049 ± 0.0003	104.62 ± 7.05
	0.4	0.085 ± 0.0071	0.0048 ± 0.0004	90.89 ± 5.04
	4	0.076 ± 0.0048	0.0041 ± 0.0004	83.96 ± 5.20

B: Males

		Spleen Weight (Grams)	Spleen:Body Weight (Ratio)	Splenic Cellularity (Cells (Million))
Control		0.097 ± 0.0084	0.0042 ± 0.0003	82.45 ± 14.82
E2 µg/25g	0.04	0.100 ± 0.0145	0.0044 ± 0.0007	83.18 ± 6.50
	0.4	0.079 ± 0.0039	0.0035 ± 0.0002	85.49 ± 5.84
	4	0.099 ± 0.0103	0.0043 ± 0.0004	87.82 ± 6.13
EE µg/25g	0.04	0.082 ± 0.0042	0.0037 ± 0.0002	78.74 ± 7.94
	0.4	0.084 ± 0.0103	0.0038 ± 0.0004	79.19 ± 6.78
	4	0.080 ± 0.0031	0.0039 ± 0.0001	80.51 ± 3.90

Figure B1: Ovarian weight following subcutaneous exposure

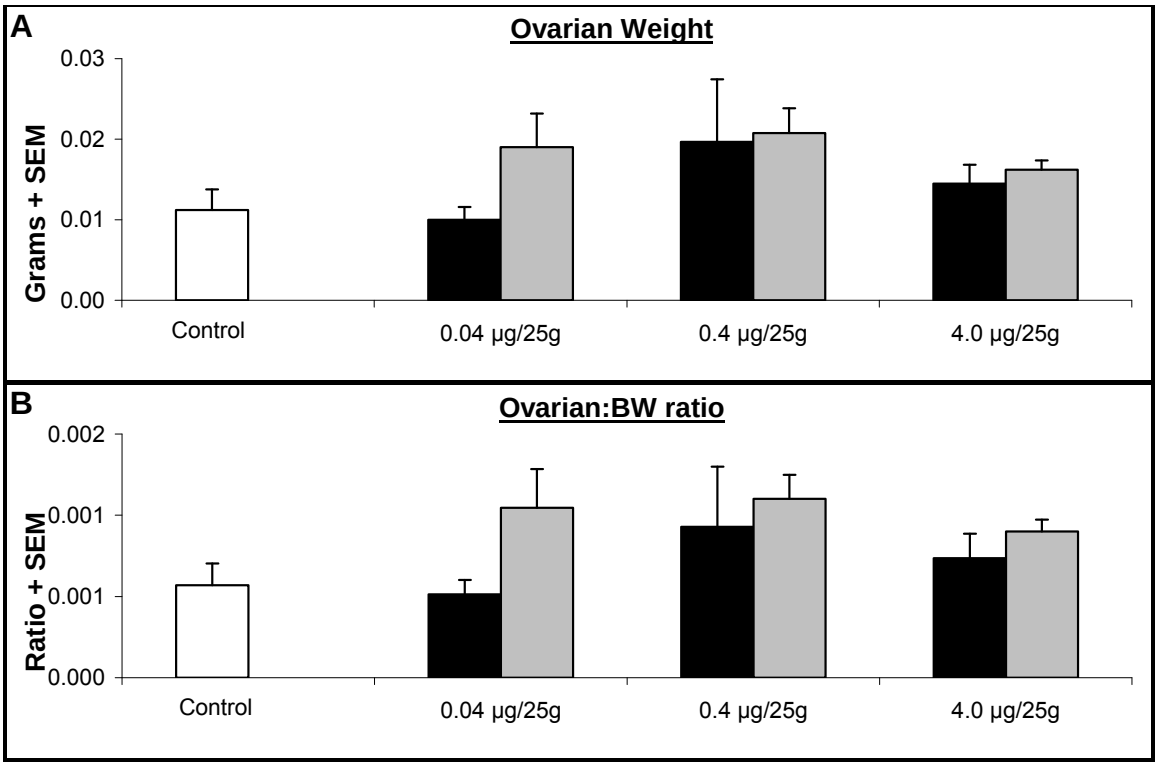


Figure B2: Ovarian weight following oral exposure

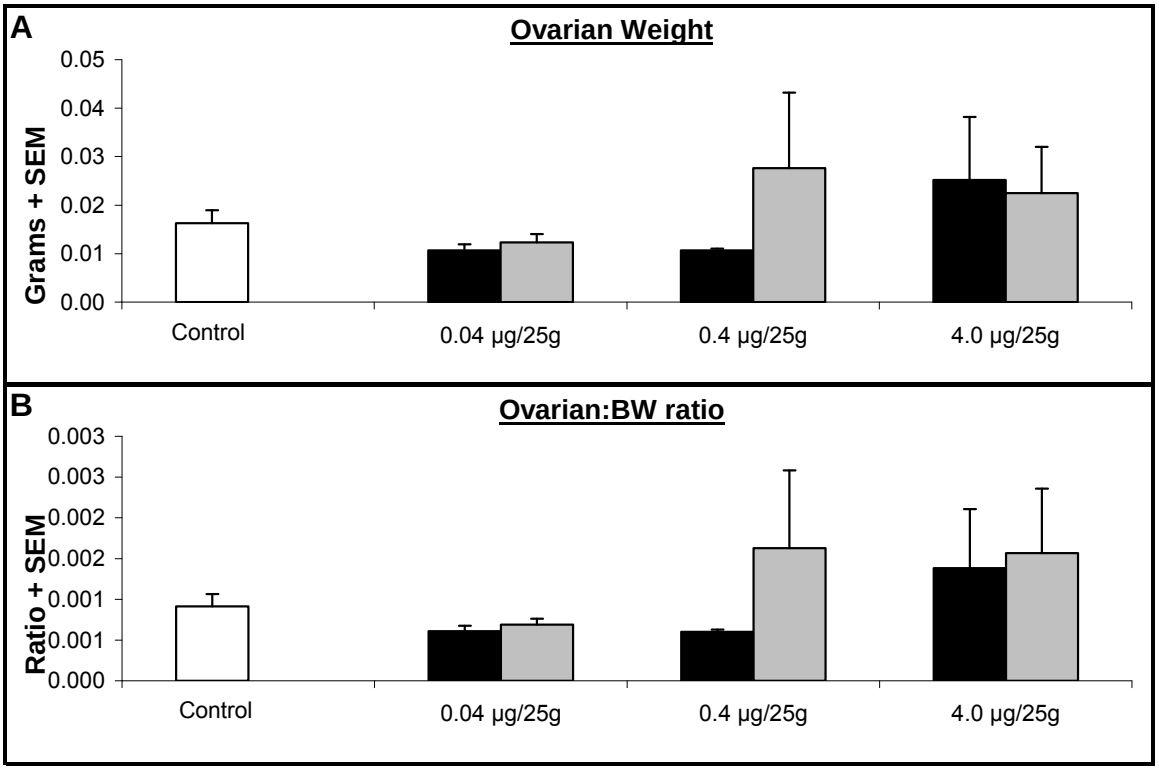


Figure B3: Testes weight following subcutaneous exposure

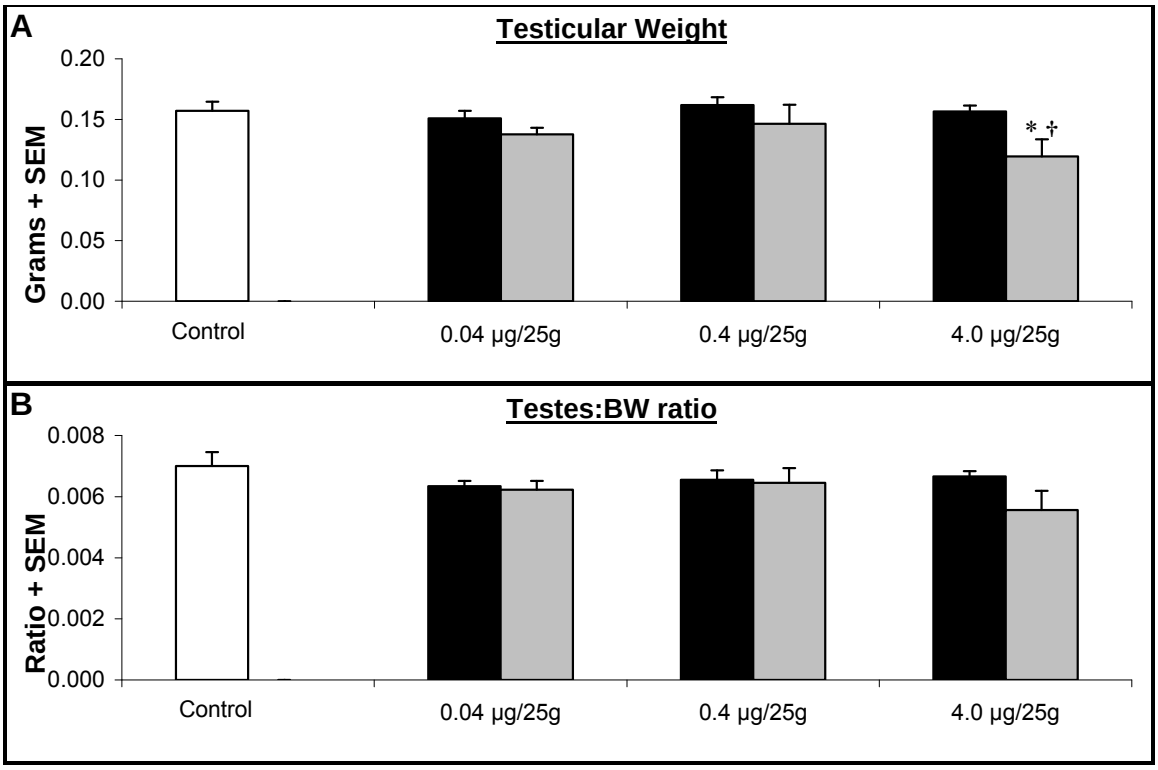


Figure B4: Testes weight following oral exposure

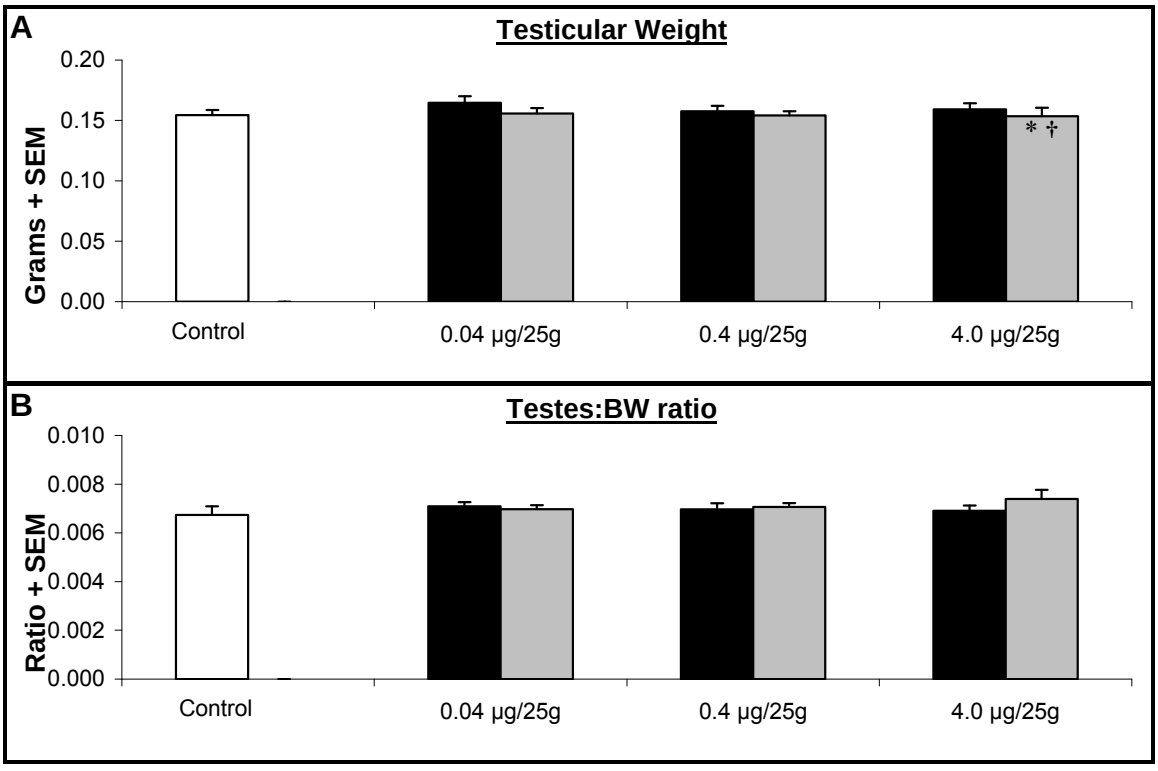


Figure B5: Female and male interferon-γ at 24 hours following subcutaneous exposure

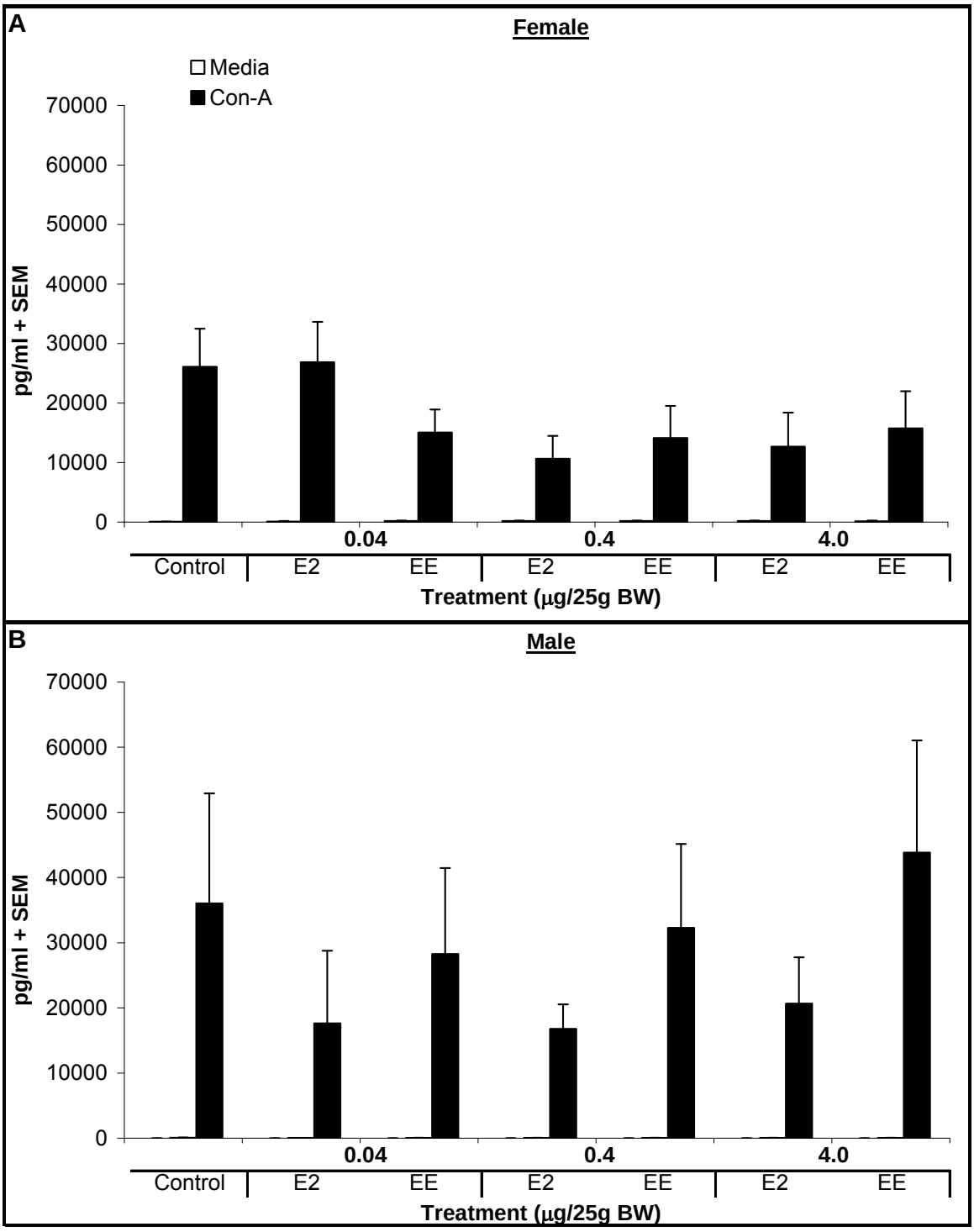


Figure B6: Female and male interferon-γ at 24 hours following oral exposure

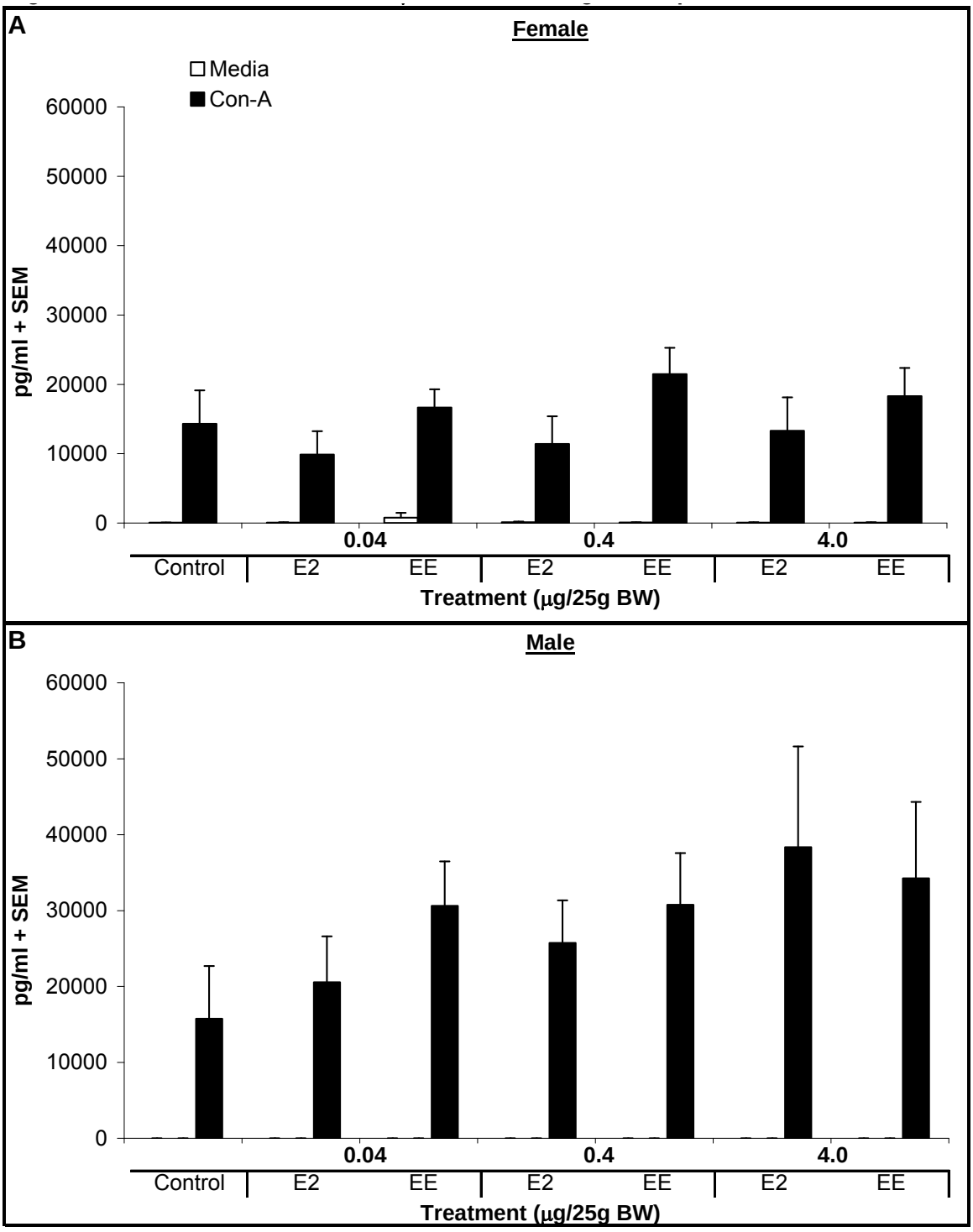


Figure B7: Female and male nitric oxide at 48 hours following subcutaneous exposure

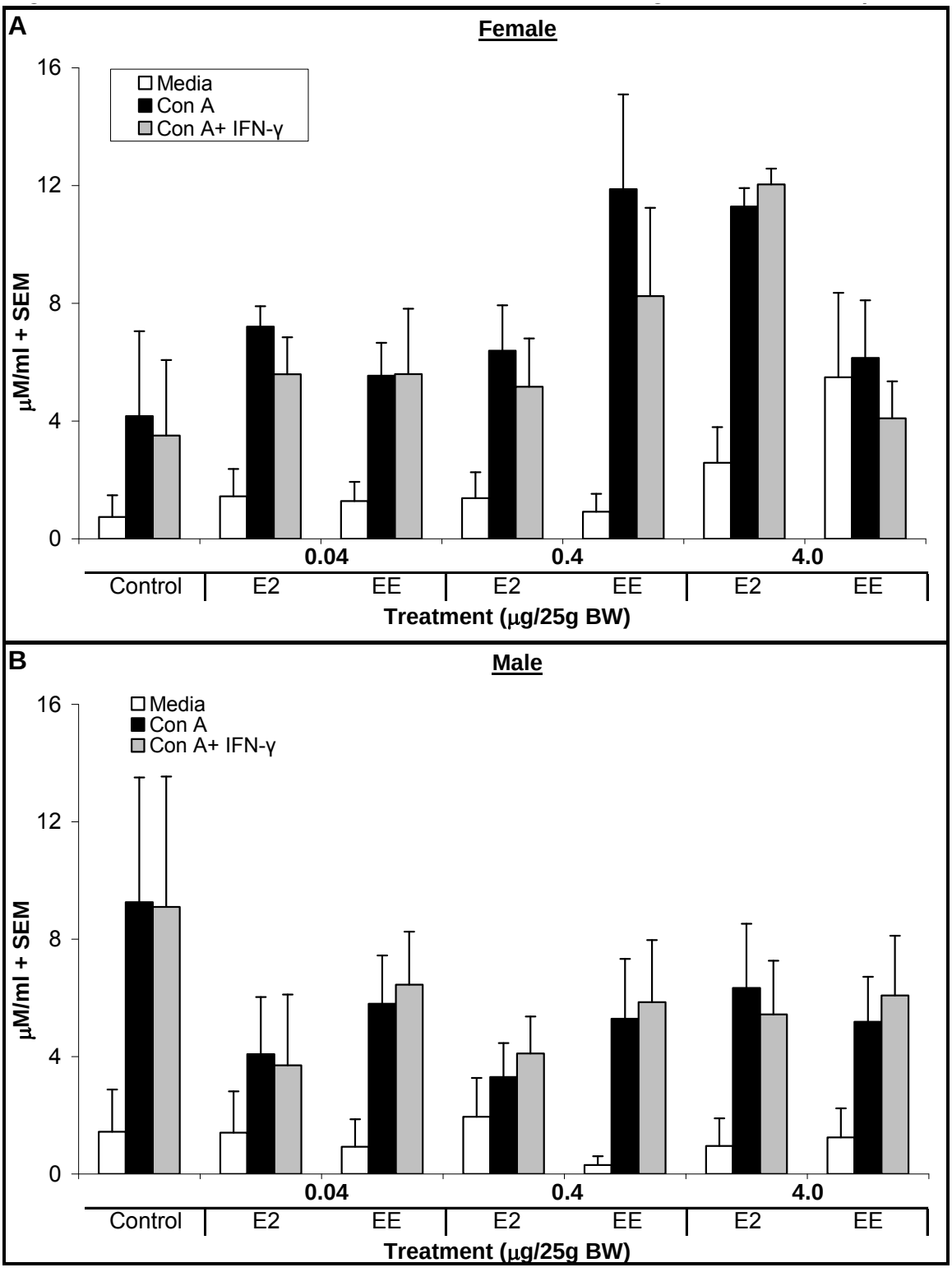
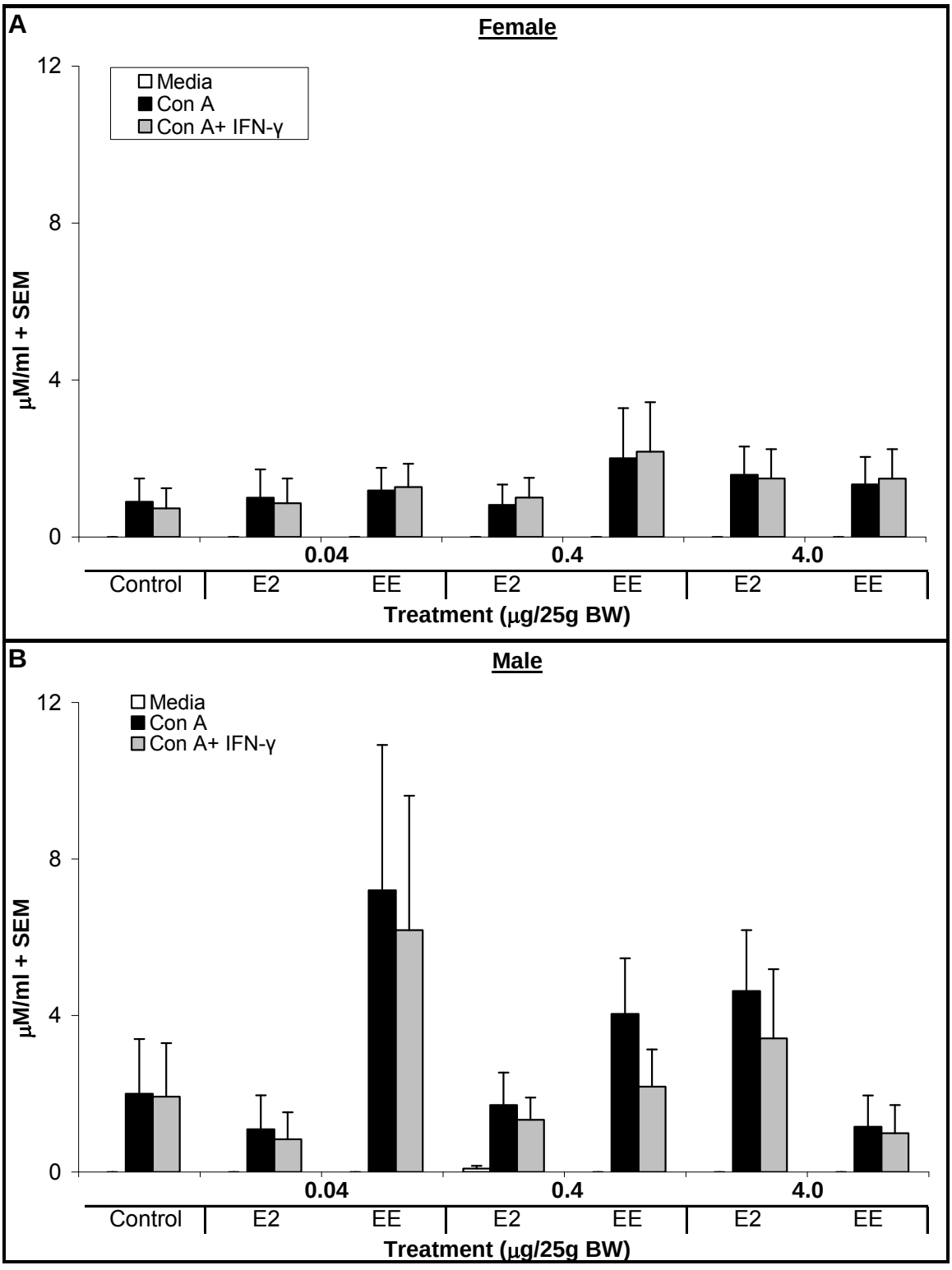


Figure B8: Female and male nitric oxide at 48 hours following oral exposure



Vita

Tyson Peter Thomas Brummer was born on January 4, 1982, in Toronto, Ontario, Canada. He moved to Christiansburg, Virginia in 1999 and graduated from Christiansburg High School in 2000. He attended Virginia Tech in Blacksburg, VA and graduated with a Bachelor's of Science Degree in Biology in May, 2004. In August of 2004 he enrolled in the Master of Science program at the VA-MD Regional College of Veterinary Medicine, Department of Biomedical Sciences and Pathobiology and defended his thesis in the Summer 2007 session. He studied under the supervision of Dr. S. Ansar Ahmed. Following graduation, he will defer his acceptance to the Virginia-Maryland Regional College of Veterinary Medicine DVM Class of 2011 and enter the DVM Class of 2012 in 2008.