# The Effect of Estrogen, Coumestrol, Triclosan, and ICI 182,780 on GnRH Secretion in GT1-7 Cells

Rachael Dooley

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Rachael R. Dooley

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To the College of Graduate Studies:

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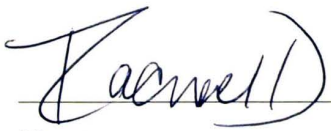

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## ABSTRACT

The Effect of Estrogen, Coumestrol, Triclosan, and ICI 182,780 on GnRH Secretion in GT1-7 Cells (under the direction of Dr. Gilbert Pitts).

Endocrine disrupting chemicals (EDC)s are exogenous agents that can alter the normal functioning of the endocrine system by mimicking or blocking natural hormones found in the body. They are found in many of the foods we eat, in food container linings, in fabrics we wear, and in plastics we use every day. EDCs that mimic estrogen are called xenoestrogens. Estrogen is a hormone that plays a large role in the maintenance of reproductive cycles by participating in positive and negative feedback loops with gonadotropin-releasing hormone (GnRH) neurons, which are part of the hypothalamic-pituitary-gonadol (HPG) axis. GnRH is a neurohormone that is critical for proper reproductive function. Numerous studies have determined the effects of estrogen, coumestrol, and triclosan, on various parts of the HPG axis but none have looked at their effects on GnRH secretion under the conditions in this study. We hypothesize estrogen, coumestrol, and triclosan will have an effect on GnRH secretion. Because primary GnRH cultures are difficult to obtain, an immortalized GnRH secreting cell line namely, GT1-7, was utilized. In this study, GT1-7 cells were incubated for 24-hours with varying concentrations and combinations of estrogen, the xenoestrogens: coumestrol and triclosan, and the ER-$\alpha$ and $\beta$ antagonist/GPR30 agonist, ICI 182,780 (which was added as a possible control) to determine their effect on GnRH secretion. GnRH peptide levels in the media were quantified using enzyme-linked immunoassays (ELISA)s. It was found that neither estrogen, coumestrol, nor triclosan significantly altered GnRH secretion. However, it was shown that ICI 182,780 significantly increased GnRH in GT1-7 cells. These results are surprising however, recent literature has shown that ICI 182,780 can deviate from its traditional antagonistic roles to take on new agonistic ones, as shown in this study. These results give new insight to the effect of ICI 182,780 to GT1-7 cells.

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## Acknowledgements

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## Chapter 1: Introduction

People and other animals encounter endocrine disrupting chemicals (EDCs) daily. EDCs are defined by the U.S. Environmental Protection Agency (EPA) as "exogenous agents that interfere with the synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process (EPA, 2017)." Any disruption in the endocrine system can cause disease or even be fatal, which is why EDCs are so important to study. This thesis describes the effects of estrogen, the xenoestrogens: triclosan and coumestrol, and the ER-α and β antagonist/GPR30 agonist, ICI 182,780 on reproduction.

Synthetic or plant-derived EDCs that mimic estrogens are called xenoestrogens. Coumestrol is a plant-derived xenoestrogen (phytoestrogen) that is found in foods such as, clover, soybeans, brussel sprouts, spinach, legumes, chick peas, and alfalfa sprouts. Some studies have suggested that phytoestrogens may reduce menopausal symptoms (Sunita and Pattanayak, 2011) and prevent breast cancer (Hedelin *et al.*, 2007; Trock *et al.*, 2006). Hedlin *et al.* (2007) reported a 50% decrease in the incidence of estrogen receptor (ER) negative and progesterone receptor (PR) negative breast tumors in women who ate a diet containing coumestrol compared to women that did not eat coumestrol. Eating a diet high in phytoestrogens has also been shown cause negative effects such as premature estrous cycles, disruption of ovarian cycles, and suppressed lordosis behavior (Al-Anazi *et al.*, 2011; Barrett, 1996); thus, further investigation into the effects of phytoestrogens on reproductive health is warranted.

The regulation of estrogen during a normal reproductive cycle starts with gonadotropin-releasing hormone (GnRH) neurons. GnRH neurons integrate many types of information and control reproduction by modulating luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion from the anterior pituitary (reviewed in Conn and Crowley, 1994). In turn, LH and FSH control ovarian secretion of estrogen. Finally, estrogen participates in feedback loops with the cells that secrete GnRH, LH, and FSH. Coumestrol reduced the frequency of multiunit electrical volleys within the hypothalamus (McGarvey *et al.*, 2001) where GnRH neurons are located. The same study also showed that coumestrol inhibited pulsatile LH secretion. This suggests that coumestrol may reduce GnRH secretion from the hypothalamus. Bowe *et al.* (2001) showed that GnRH mRNA levels were significantly reduced when coumestrol was applied to immortalized mouse GnRH (GT1-7) cells. GT1-7 cells are an invaluable model to study GnRH secretion because primary GnRH cultures are difficult to obtain.

GnRH and estrogen may also be impacted by triclosan, a synthetic xenoestrogen. Heath *et al.*, (1999) reported triclosan bound and subsequently inhibited enoyl-acyl carrier protein reductase (FabI); thus, preventing fatty acid synthesis in bacteria. This information led to the use of triclosan as a bacteriostatic and antiseptic agent. It was not expected to exert effects on mammalian cells because the bacterial FabI enzyme is structurally different from its mammalian counterpart (Ling *et al.*, 2004). Nevertheless, some work has been done to examine triclosan's potential endocrine disrupting (Veldhoena *et al.*, 2006; Ajaoa *et al.*, 2015) and carcinogenic (Dinwiddie *et al.*, 2014) properties. These studies have not been conclusive. Interestingly, on September 2, 2016 the U.S. Food and Drug Administration (FDA) ruled that over-the-counter (OTC)

consumer antiseptic wash products containing triclosan, could no longer be sold (FDA, 2016). This ban was issued because the manufacturers could not demonstrate that soaps containing triclosan were better at reducing the number of bacteria than non-triclosan containing soaps (FDA, 2016). However, triclosan is still found in many of the products that we use every day such as makeup, toothpaste, fabrics, and plastics, so its effect on reproductive health must be investigated.

The goal of my thesis was to determine the effects of estrogen, the EDCs coumestrol and triclosan, and the ER-α and β antagonist/GPR30 agonist, ICI 182,780 (which was initially used for a control) on GnRH secretion from GT1-7 cells.

## Introduction

There are hundreds of different EDCs that we encounter daily, and some of them can adversely affect people and wildlife. Many of EDCs are found in everyday products such as toothpastes, make-up, plastics, and even in the U.S. drinking water and bottled water purchased in China, France, Italy, and Germany (Benotti *et al.*, 2009; Li *et. al.*, 2009; Wagner *et al.*, 2013). Although EDCs can affect all aspects of human and wildlife health, this review focuses on their effects on reproductive health. Estrogen-like EDCs (xenoestrogens) are of interest as estrogen has a large role in maintaining reproductive health. Therefore, my goal has been to examine the effects of two xenoestrogens commonly found in many of the foods and products we consume and use; namely, coumestrol and triclosan.

To clearly lay out the scope of issues related to xenoestrogens, this review will describe EDCs, xenoestrogens, and their mechanism of action. Next, the hypothalamic-pituitary-gonadal (HPG) axis will be examined leading up to a description of potential endocrine disrupting events. Ultimately, mammalian reproduction is governed by GnRH, which is a neurohormone that indirectly causes the release of sex hormones from the gonads (Marshall *et al.*, 1992). Proper maintenance of the HPG axis crucial for healthy reproductive and sexual development and any disturbances in its functioning can cause deleterious effects. Therefore, it is important to investigate the effects of xenoestrogens on the HPG axis, specifically GnRH secreting neurons. Unfortunately, *in vivo* studies of GnRH neurons are extremely difficult to perform because they are diffusely scattered throughout the hypothalamus (Okubo, 2006). To sidestep this issue, Mellon and

colleagues, developed immortalized GnRH secreting neurons from immature mice called GT1-7 cells. These cells express mouse GnRH mRNA and secrete GnRH when depolarized (Mellon *et al.*, 1990). GT1-7 cells provide an invaluable model to study the effects of estrogen, the xenoestrogens coumestrol and triclosan, and the ER-α and β antagonist/GPR30 agonist, ICI 182,780 on GnRH secretion and ultimately reproductive health.

**Introduction to EDCs**

There are over 85,000 manufactured or synthetic chemicals that are used in many products today; some of which may be EDCs (Gore, 2007). Table 1 shows a list of some EDCs found in common products. These products are nearly ubiquitous and impact food production from seed to table and personal care from the crib to the tomb.

| Category/Use | Example EDCs |
|---|---|
| Pesticides | DDT, chlorpyrifos, atrazine, 2,4-D, glyphosate |
| Children's products | Lead, phthalates, cadmium |
| Food contact materials | BPA, phthalates, phenol |
| Electronics and Building materials | Brominated flame retardants, PCBs |
| Personal care products, medical tubing | Phthalates |
| Antibacterials | Triclosan |
| Textiles, clothing | Perfluorochemicals |

Abbreviations: BPA: bisphenol A; 2,4-D: 2,4-dichlorophenoxyacetic acid; DDT: dichlorodiphenyltrichloroethane; PCBs: polychlorinated biphenyls

**Table 1.** A list of some known EDCs and the products that contain them (Gore, 2007).

The amount of research concerning EDCs has greatly increased over the past 30 years. In the mid 1990's major changes were made in the methodologies for testing endocrine-mediated toxicity (Marty et al., 2011). Perhaps most significantly, these changes include the suggestion that some EDCs be tested at lower concentrations than previously examined (Gore, 2007; Marty et al., 2011). These changes were due to the development of the "low-dose hypothesis" which suggests that the relationship between dose and response is non-monotonic, or non-linear. In such situations, it is possible that low EDC doses may have greater physiological effects than higher doses (Marty et al., 2011). However, the U.S. EPA is not on board with this hypothesis stating "until there is an improved scientific understanding of the low-dose hypothesis, EPA believes that it would be premature to require routine testing of substances for low-does effects in the Endocrine Disruptor Screening Program. EPA recognizes that in the future, relevant information may become available on specific chemicals. Such information may support testing for low-dose effects on a case-by-case basis" (Marty et al., 2011). On the contrary, the Endocrine Society issued its support for the hypothesis in a scientific statement published in 2009 (Diamanti-Kandarakis et al., 2009) and then again in 2015 (Gore et al., 2015). Although there may not be an agreement among the entire scientific community about whether the low-dose hypothesis is correct, there is an agreement that EDC's can possibly be harmful to the reproductive health of humans and the wildlife around us.

EDC's can exert their affects because they are chemically similar to endogenous hormones. Each hormone has a certain chemical composition and structure which allows it to bind to specific receptors. For example, the steroid hormone, estrogen (Figure 1), has

a specific order and arrangement of elements which allow it to bind specifically to estrogen receptors.

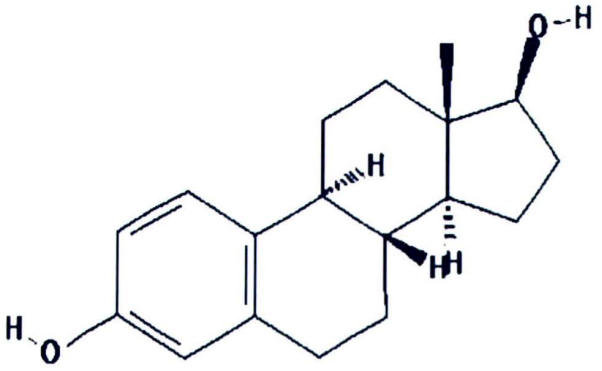



**Figure 1.** The chemical structure of the steroid hormone estrogen (PubChem, 2017).

**Estrogenic Signaling**

There are many diverse steroidal and non-steroidal, natural plant-derived and synthetic compounds which can mimic estrogen (Singleton and Kahn, 2003). Some have a similar chemical structure to estrogen, while others do not. EDCs that mimic estrogen are known as xenoestrogens. Like estrogen, some xenoestrogens can have stimulatory and inhibitory effects, while others can be pleiotropic in nature (Paech *et al.*, 1997; Mueller *et al.*, 2004). Xenoestrogens exert their effects by binding to estrogen receptors (ER)s, thus mimicking estrogen.

There are two main types of nuclear ERs: ER-α and ER-β. Both are expressed in diverse tissues with ER-α populating the hypothalamus, pituitary, uterus, and gonads (Jacob et al., 2001; Lubahn *et al.*, 1993; Couse *et al.*, 1999; Schomberg *et al.*, 1999; Dupont *et al.*, 2000) and ER-β in the hypothalamus, cerebellum, olfactory lobe, prostate, uterus, ovary

and lung (Jacob *et al.*, 2001; Couse *et al.*, 1997). Research has shown that ERs may be found in equal numbers in various cellular structures, moving between the plasma membrane, cytoplasm, and nucleus (Parikh *et al.*, 1987). Figure 2 shows the four different signaling mechanisms used by ERs to cause their diverse physiological effects. The first mechanism is direct which involves the ER directly binding to the DNA. This occurs when one ER-ligand complex dimerizes to another ER-ligand complex. The ER-ligand complex dimer then binds directly to the DNA altering transcription (Kushner, *et al.*, 2000; reviewed in Mueller, 2004; reviewed in Safe and Kim, 2008). It is also noteworthy that ER has been reported to exist as a dimer without a ligand (Tamrazi, *et al.*, 2002). The second mechanism is tethered and it occurs the same way as the direct mechanism however the ER-ligand complex undergoes indirect binding via transcription factors (TF)s to the DNA (Kushner, et al., 2000; reviewed in Mueller, 2004; reviewed in Safe and Kim, 2008). The third mechanism is non-genomic. It is not well understood but involves interaction between a classical ER or a membrane bound receptor such as GPR30, ligands, and second messengers (SM)s however there is no binding onto the DNA, and ultimately no gene regulation (Revankar *et al.*, 2005). Unlike the previous two mechanisms, the non-genomic exerts its effects rapidly through signaling cascades that either alter ion channels or increase nitric oxide (NO) levels within the cytoplasm (reviewed in Mueller, 2004); this is also known as membrane-initiated steroid signaling (MISS) (Nemere, *et al.*, 2003). The fourth mechanism utilizes growth factor (GF) induced signaling which can be activated by steroidal and non-steroidal (Schiff, *et al.*, 2005) stimuli. This involves phosphorylation of the ER and activation via peptide growth factors (Nicholson *et al.*, 1999) such as IGF-I (Aronica and Katzenellenbogen, 1993),

EGF and TGFα (Bunone *et al.*, 1996) and heregulin (Pietras *et al.*, 1995). Once activated, the ER can alter transcription like the direct and tethered methods.

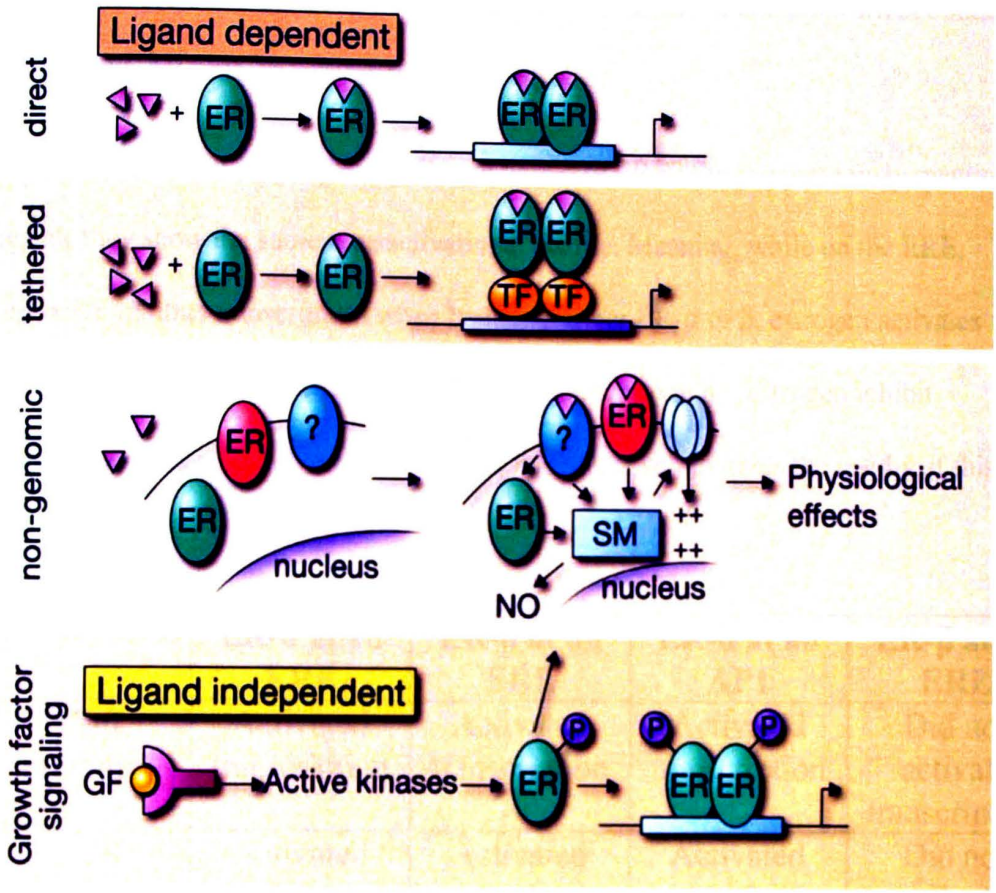



**Figure 2.** The four known mechanisms through which ERs exert their effects (Heldring *et al.*, 2007).

The signaling actions of each nuclear or membrane bound ER is dependent on the ligand or growth factor present and where applicable, which response element it binds to. Paech *et al.*, (1997) demonstrated this in a study that showed what happens to transcription when differing combinations of ERs and ligands are bound to either an estrogen response element (ERE) or activator protein (AP1) binding site; the ERE and AP1 sites are sequences of DNA within a promoter region of a gene to which certain TFs bind to initiating transcription of estrogen related genes. They showed that when bound to ER-α on an AP1 site, the ligands 17β-estradiol ($E_2$), diethylstilbestrol (DES), and the

antiestrogen ICI 164,384, tamoxifen, and raloxifene activate transcription. Transcription was inhibited when $E_2$ and DES combined, and $E_2$ alone were bound with ER-β on the AP1 site. Tamoxifen, ICI 164,384, and raloxifene initiated transcription when bound to ER-β on the AP1 site.

Alternatively, when both types of ERs are bound to ERE with the previously mentioned ligands they show the same transactivational profile. Meaning, while on the ERE, tamoxifen inhibits transcription when bound to either ER-α or β, estrogen activates transcription when bound to either ER-α or β, and tamoxifen + estrogen inhibit transcription when bound to either ER-α or β. Table 2 summarizes the results of this study.

| | **ER-α at an ERE** | **ER-β at an ERE** | **ER-α at an AP1** | **ER-β at an ERE** |
|---|---|---|---|---|
| **17-β estradiol** | Activated transcription | Activated transcription | Activated transcription | Did not activate transcription |
| **DES** | Activated transcription | Activated transcription | Activated transcription | Did not activate transcription |
| **ICI 164,384** | Did not activate transcription | Did not activate transcription | Activated transcription | Activated transcription |
| **tamoxifen** | Did not activate transcription | Did not activate transcription | Activated transcription | Activated transcription |
| **raloxifene** | Did not activate transcription | Did not activate transcription | Activated transcription | Activated transcription |

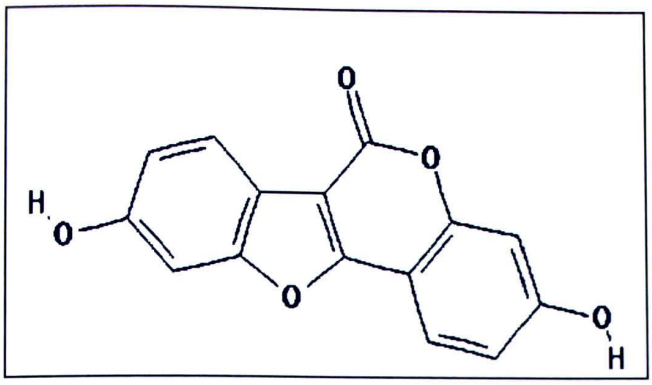
**Table 2.** The transactivational profiles of ER-α and β and the ligand activation profiles of 17-β estradiol, DES, ICI 164,384, tamoxifen, and raloxifene at ERE and AP1 sites (Paech *et al.*, 1997).

Recently, the roles of a novel seven-transmembrane-spanning (7TM) G-protein coupled receptor (GPR)-ER, GPR30, were investigated (Noel *et al.*, 2009). This ER acts independently of the more well-studied nuclear ER-α and ER-β, and activates adenyl cyclase and matrix metalloproteinase (MMP), which in turn generates cAMP and on some occasions, the cleavage and release of heparin-binding epidermal growth factor in response to estrogen (HB-EGF; Filardo and Thomas, 2005). This is quite interesting as recently, HB-EGF expression was found to be significantly elevated in numerous human cancers at levels much higher than other epidermal growth factor receptor (EGFR) ligands (Miyamoto, 2006). Also, the ER antagonists ICI 182,780 and tamoxifen act as agonists when they bind to GPR30 (Filardo and Thomas, 2005).

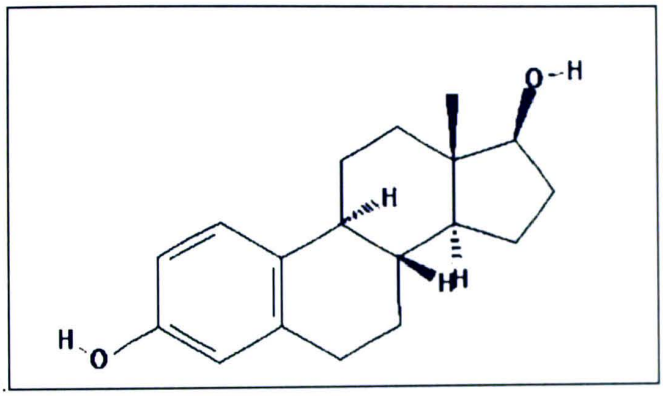
These studies indicate that the effects of hormones are largely dependent on the ligand, the type of receptor and in some cases, which response element the ER-ligand complex is bound to. This shows that one cannot generalize the effects of hormones and EDCs; they must be examined under a variety of conditions/environments. Two EDCs, coumestrol and triclosan, have been investigated for effects on reproductive physiology.

**Coumestrol as an EDC**

Coumestrol is a plant-derived xenoestrogen that is also known as a phytoestrogen (Jacob *et al.*, 2001). It is found in high concentrations in clover and alfalfa sprouts and in lower concentrations in sunflower seeds, lima beans, pinto beans, soybeans, vanilla soymilk, and round split peas (Frank *et al.*, 1995; Bhagwat *et al.*, 2008). It was first discovered in 1957 when it was isolated from ladino clover, alfalfa, and strawberry clover (Bickoff *et al.*, 1957). Coumestrol's chemical structure is like that of estrogen as seen in Figure 3.




A.




B.

**Figure 3.** The chemical structures of: (A) coumestrol and (B) estrogen (PubChem, 2017).

The estrogenic and antiestrogenic effects of coumestrol have been shown in numerous studies. A report by Nelson *et al.*, (1984) showed that coumestrol caused cytoplasmic estrogen receptors to translocate to the nucleus (Nelson *et al.*, 1984). This same study also showed that coumestrol induced progesterone receptor (PR) expression in the mouse uterus. Estrogen is also capable of inducing transcription of the progesterone receptor gene (Lee and Gorski, 1996). A different report by Ashby *et al.*, (1999), showed in the rat, both coumestrol and estrogen increased uterine fluid and the weights of the uterus, cervix and vagina when compared to the non-treated groups. ICI 182,780 blocked this

effect when combined with coumestrol. The combination of ICI 182,780 and estrogen was not tested. Coumestrol administration to neonatal female rats suppressed lordosis behavior in adulthood (Louki *et al.*, 2005). Interestingly, histological examinations revealed that the corpus luteum (CL) was not present in these rats. This latter study suggests that coumestrol altered the level of LH secreted. It was also reported that GnRH mRNA levels were significantly reduced when GT1-7 cells were incubated for 6 or 24 hours with varying concentrations of coumestrol (Bowe, *et al.*, 2003). Estrogen exerted a similar effect (Bowe, *et al.*, 2003). It should be pointed out that GnRH hormone levels were not measured.

Interestingly, coumestrol can also have anti-estrogenic effects on the brain. Jacob *et al.*, (2000), used ER-α knock out (ERαKO) mice to demonstrate this. Female mice were ovariectomized (OVX) and half of them received an estrogen containing implant that yielded a plasma concentration equivalent to that found during proestrus (OVX+estradiol), the other mice received a vehicle-filled implant. Then, for ten days, half of each group ate diets containing coumestrol while the other half ate a coumestrol-free diet. Coumestrol significantly reduced the number progesterone receptor immunoreactive cells in both the medial preoptic area POA and the ventral medial nucleus of the hypothalamus in OVX+estradiol treated mice. However, this effect was not observed in OVX+estradiol treated mice that were not fed coumestrol. This showed that coumestrol not only has estrogenic but antiestrogenic properties as well.

**Triclosan as an EDC**

Triclosan is a synthetic compound which functions as abroad spectrum antimicrobial and antifungal agent that is used in many hygiene products such as toothpaste, hand soaps, body washes, and dish detergents; it is also found in items such as fabrics and plastics (reviewed in Gore, 2007). Triclosan has been marketed under many other names, including: Microban®, Irgasan, Ultra-fresh®, Sanitized®, Bactonix®, and Amicor® (Adolfsson-Erici, 2002.) Li *et al.*, (2010) found triclosan in bottled water purchased from Chinese grocery stores and consumed every day. Triclosan was even detected in human urine, plasma, and breast milk (Calafat *et al.*, 2008; Xiaoyun *et al.*, 2008; Hovander *et al.*, 2002). The chemical structure of triclosan is very similar to that of the widely known xenoestrogen, bisphenol A (BPA, Figure 4; PubChem, 2017.)

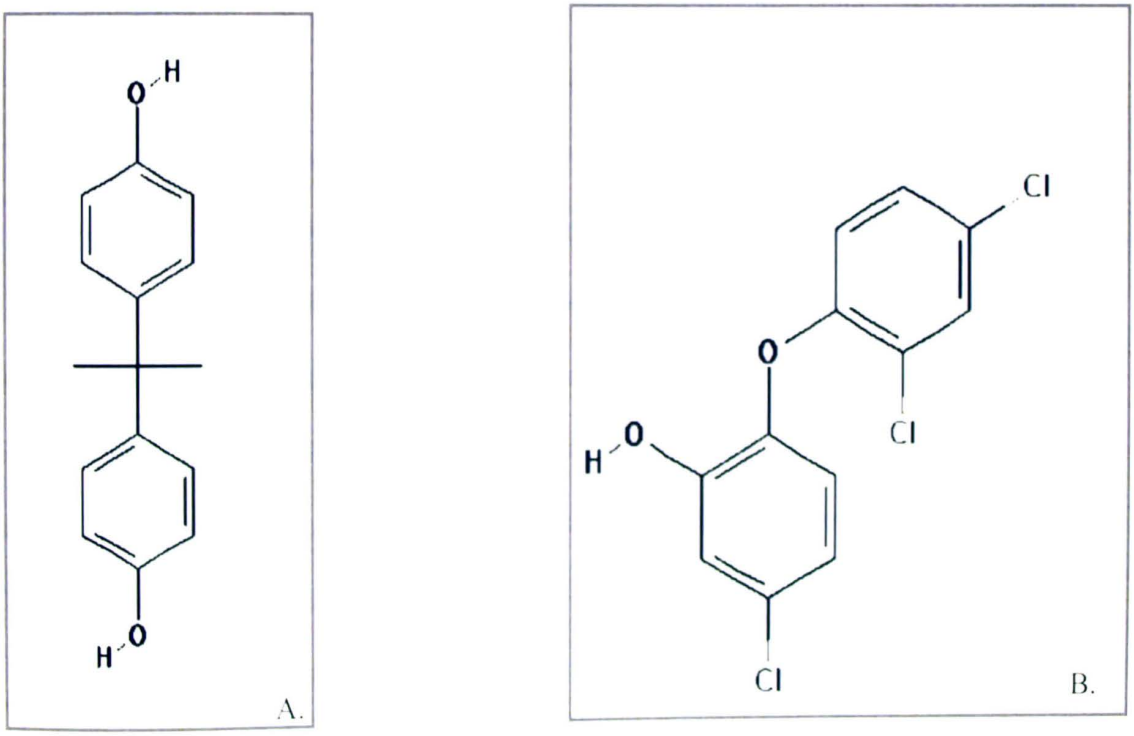



**Figure 4** The chemical structures of (A) BPA and (B) triclosan (PubChem, 2017).

Triclosan has been shown to alter reproduction and thyroid function. For example, triclosan exposure suppressed thyroxine concentrations of juvenile male rats in a dose-dependent manner (Zorilla *et al.*, 2009). Regarding the reproductive system, triclosan significantly reduced testosterone secretion from rat Leydig cells (Kumar *et al.*, 2008). Furthermore, Stoker *et al.*, (2010) found that triclosan significantly advanced the age of vaginal opening in rats. Triclosan appears to function as a xenoestrogen as it was also able to inhibit the binding of estradiol to both human recombinant ER-α and ER-β and cytosolic ER from MCF-7 cells (Gee *et al.*, 2009). Lee *et al.*, (2014) also showed triclosan's estrogenicity using MCF-7 cells. In this study, MCF-7 cells were incubated with varying concentrations triclosan ranging from $10^{-7}$ M to $10^{-5}$ M or with a triclosan/ ICI 182,780 combination for 4 hours. An MTT assay, which is a cell viability assay, was performed after the 4-hour incubation. It was shown that triclosan significantly increased MCF-7 cell proliferation when compared to the control, also that ICI 182,780 reversed this effect. After they discovered this effect, they examined the effect of triclosan on cyclin-D1 (a cyclin-dependent kinase regulator) and p21 (a cyclin-dependent kinase inhibitor) gene expression levels. They incubated the cells with $10^{-6}$M triclosan for 0, 6, and 24 hours. At each increment, they performed the reverse-transcription polymerase chain reaction (RT-PCR) and found that cyclin-D1 expression was significantly increased after 24 hours and p21 expression was significantly decreased after both the 6 and 24 hour incubations. ICI 182,780 and siRNA-ER-α treatment inhibited both these effects. This report suggested that triclosan may work via an ER-mediated signaling cascade.

Clearly, EDCs such as triclosan and coumestrol have endocrine disrupting effects on the reproductive system, and possibly by interfering with ERs. To understand the influences of these EDCs, a review of hormonal regulation of reproduction is required.

## The Hypothalamic-Pituitary-Gonadal (HPG) Axis

Estrogen is a steroid hormone and its involvement with the HPG axis is vital to the maintenance of reproductive health. Essentially, it works by participating in positive and negative feedback loops with the hypothalamus and anterior pituitary gland causing them to secrete GnRH, or the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), respectively (reviewed in Conn and Crowley, 1994). The female and male HPG axis are depicted in Figures 5 and 6, respectively.

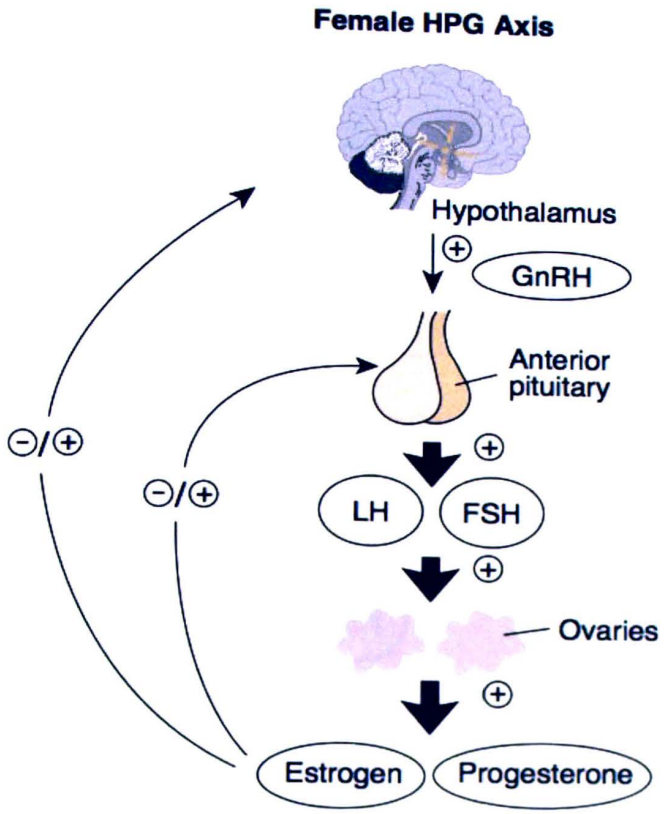



**Figure 5.** A schematic representation of the HPG axis in females (Sturmhöfel and Bartke, 1998).

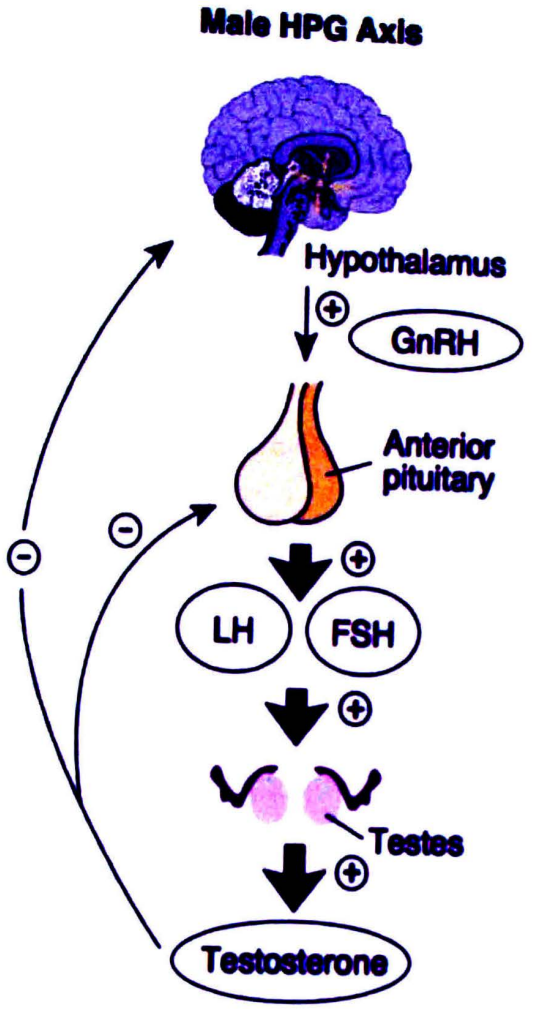



**Figure 6.** A schematic representation of the HPG axis in males (Sturmhöfel and Bartke, 1998).

GnRH is a decapeptide that is secreted into the hypophyseal portal system from neurons in the preoptic area of the hypothalamus (reviewed in Conn and Crowley, 1994). GnRH binds to specific high-affinity receptors within the anterior pituitary where it regulates biosynthesis and secretion of gonadotropins such as LH and FSH (Kaiser *et al.*, 1997). The secretion of LH and FSH have been shown to be dependent on the pulsatile nature of

GnRH release (Vazquez-Martinez *et al.* 2001); whereas, continuous exposure to GnRH reduces LH and FSH secretion (Jayes *et al.*, 2001). High frequency GnRH pulses stimulate the secretion of LH, while low frequency pulses stimulate FSH secretion (Jayes *et al.*, 2001). The gonadotropins induce growth of the gonads and release of sex steroids, estrogen and progesterone, which act on diverse targets, including the brain to mediate reproductive function and behaviors (Moore *et al.*, 2006). The next few paragraphs describe the reproductive cycles in females and males followed by a summary table (Table 2) of the hormones: estrogen, FSH, and LH and their effects on males and females.

**Hormonal Regulation of Female Reproduction**

*Ovarian cycle*

The ovarian cycle consists of two phases: the follicular and luteal phases. During the follicular phase in a normal 28-day ovarian cycle in the female, GnRH causes the release of FSH and LH (Kaiser *et al.*, 1997). FSH causes the recruitment of follicles as well as the secretion of estrogen from the granulosa cells of the vesicular follicles; as estrogen levels in the plasma rise, it causes a negative feedback loop with the hypothalamus and anterior pituitary, thus inhibiting the release of GnRH, FSH, and LH This lack of FSH causes all but the dominant follicle to deteriorate (reviewed in Baerwald *et al.*, 2012). This dominant follicle in turn starts secreting high levels of estrogens which forms a positive feedback with the hypothalamus and anterior pituitary. Around midcycle, these high levels of estrogen cause a burst of LH to be secreted (Hall *et al.*, 1992). This LH surge triggers many events leading up to ovulation in which the oocyte is released from the ovary. After ovulation, the ovary enters the luteal phase. LH causes the corpus luteum

(CL) to form and secrete large amounts of progesterone and some estrogen thus causing the inhibition of FSH, and LH (Edwards and Steptoe, 1975). This decline in gonadotropins prevents follicle maturation and ovulation of additional oocytes (Reame *et al.*, 1984). If fertilization does not occur, the CL deteriorates as well as the levels of estrogen and progesterone and the cycle starts again.

*Uterine cycle*

The uterine cycle happens simultaneously with the ovarian cycle. It consists of three different phases: 1) menstrual phase, 2) proliferative phase, and 3) secretory phase. During the menstrual phase (when the ovarian hormones are at their lowest and the gonadotropins are rising) menstruation occurs in which the outer layers of the endometrium is shed (reviewed in Curry and Osteen, 2003). The proliferative phase consists of proliferation and increased progesterone receptors in the endometrium caused by rising estrogen levels from the ovaries (reviewed in Fazleabas and Strakova, 2004). The secretory phase includes progesterone stimulating many events to prepare the endometrium for implantation (reviewed in Fazleabas and Strakova, 2004).

**Hormonal Regulation of Male Reproduction**

GnRH stimulates the release of FSH and LH from the anterior pituitary. LH causes the Leydig cells to release testosterone (T); which plays a large role in the maintenance of spermatogenesis (McLachlan *et al.*, 2002). FSH aids in the function of Sertoli cells which secrete various proteins that are essential for testis formation and spermatogenesis (Griswold, 1998). Estrogen plays a role as well by influencing the differentiation and

maintenance of reproductive tissues (Krege *et al.*, 1998) as well as regulating reabsorption of luminal fluid (Hess *et al.*, 1997).

| | Estrogen | FSH | LH |
|---|---|---|---|
| **Female** | Helps develop endometrium<br><br>Stimulates LH secretion (follicular phase)<br><br>Inhibits LH and FSH (luteal phase) | Recruits follicles<br><br>Stimulates follicular growth<br><br>Stimulates follicles to secrete estrogen | Triggers ovulation<br><br>Aides in CL development<br><br>Stimulates progesterone secretion |
| **Male** | Differentiation/maintenance of reproductive tissues<br><br>Reabsorption of luminal fluid | Acts on Sertoli cells to aid in spermatogenesis | Acts on Leydig cells to stimulates T secretion |

**Table 2**. Summary table of the roles of estrogen, FSH, and LH in males and females.

**Evidence for Indirect and Direct Effects of Estrogen on GnRH Neurons**

While the role of estrogen in reproduction is clear, there is much controversy concerning how estrogen mediates GnRH secretion. On the one hand, some studies that indicate estrogen exerts its effects indirectly on GnRH neurons. This is supported by many immunocytochemical studies that found few, if any, GnRH neurons that were immunoreactive to estrogen (Herbison and Theosis 1992; Watson *et al.*, 1992; Leman and Karsch, 1993; Sullivan *et al.*, 1995). In addition, Shivers *et al.* showed that the nuclei of GnRH secreting neurons rarely contained estrogen, suggesting that estrogen's nuclear retention-dependent effects were not found within GnRH neurons. On the other hand, GnRH mRNA was significantly reduced when immortalized GnRH cells (GT1-7) were treated with estrogen (Roy *et al.*, 1999); however, it cannot be certain that reduced GnRH

mRNA levels equivalates to reduced GnRH levels. Roy *et al.*, (1999) may have gotten these results because GT1-7 cells were derived from immature mice (Mellon *et al.*, 1990) and this may have affected which receptors are present. Another study showed that exposure to estrogen caused rapid hyperpolarization of isolated cultured primate GnRH neurons as well as a 250% increase in action potential firing frequency, and an increase in number and duration per burst (Abe and Terasawa, 2005). However, it is possible that these results were due to a possible contamination with other types of neurons that contain ERs which reacted when the estrogen was applied. Taken together, these last studies suggest the possibility that estrogen may have a direct effect on GnRH neurons.

**GT1-7 Cells**

There is also much debate about which types of ERs are found in the hypothalamus *in vivo*, specifically, GnRH neurons. This is due to the difficulty in obtaining primary cultures. To avoid this issue, Mellon *et al.*, (1990) created an immortalized GnRH cell line using genetically-targeted tumorigenesis. The resultant GT1 cells have provided an invaluable model system for the study of hypothalamic neurons that regulate mammalian reproduction. This process consisted of creating a DNA construct containing the murine GnRH promoter coupled to the coding region for the Simian virus 40 (SV40) T-antigen (Tag) oncogene. SV40 replication requires only one viral protein, the large T-antigen (Tag), which acts as both an initiator of replication and as a replicative helicase (Vanloock *et al.*, 2002). The construct was then injected into a single cell of a mouse embryo where it was then randomly integrated into the mouse genome. Consequently, any cell capable of producing GnRH would also express the SV40 oncogene. The oncogene would then trigger unregulated cell division. Essentially, this created tumors

which were harvested from the mice and subsequently subcultured (Mellon *et al.*, 1990). The resultant GT1-7 cells express GnRH mRNA Angelini and Belsham, (1999), nuclear ERs-α and β; Noel *et al.*, (2009) the membrane bound GPR30 Filardo and Thomas, (2005), and release GnRH in response to depolarization (Mellon *et al.*, 1999). GT1-7 cells were chosen as a model system determine the effects of coumestrol and triclosan's on GnRH secretion because GT1-7 cells are completely isolated without contamination of other types of cells, so observations of direct effects can be made. GT1-7 cells have been shown to express both classical ERs and the novel membrane-associated estrogen receptor, GPR30. Both coumestrol and triclosan have been shown to bind to the classical ERs however there has been no report on whether they can bind to GPR30; both EDCs warrant further investigation on their effect on GnRH secretion. I hypothesize that coumestrol and triclosan will influence GnRH secretion.

**Summary**

EDCs are exogenous agents that interfere with the normal functioning of the endocrine system by either mimicking or blocking natural hormones found in the body. EDCs that mimic estrogen are called xenoestrogens. Estrogen helps maintain normal reproductive cycles by participating in positive and negative feedback loops with GnRH neurons. GnRH is crucial for the proper functioning of the reproductive system. Estrogenic signaling is extremely complex and can vary depending on the ligand, type of receptor, and where at on the DNA the ER-ligand complex is bound to. Whether estrogen acts indirectly or directly on GnRH neurons remains controversial as there is evidence for both sides. GnRH neurons are difficult to study because they are so scare which makes primary cultures difficult to obtain. To sidestep this issue, this study utilized

immortalized mouse GnRH secreting neurons, namely GT1-7 cells. GT1-7 cells express the estrogen receptors ER-α and β, and GPR30. The endocrine disrupting properties of coumestrol and triclosan have been studied however none have looked at their effects on GnRH secretion. In the present study, GT1-7 cells were incubated for 24-hours with coumestrol, triclosan, 17-β estradiol and the ER-α and β antagonist and GPR30 agonist, ICI 182,780 to determine their effects on GnRH secretion.

## GT1-7 Cell Culture

GT1-7 cells (Dr. Pamela Mellon, University of California, Berkeley) were cultured in 100 mm poly-D lysine-coated tissue culture dishes (BD Falcon, Franklin Lakes, NJ; Catalog # P35GC-1.0-14-C) in Dulbecco's Modified Eagle Medium (DMEM/F12; Invitrogen, Grand Island, NY; Catalog # 12634-010) and supplemented with heat-inactivated fetal bovine serum (10%), penicillin (100 µg/ml), and streptomycin (100 µg/ml) (Invitrogen, Catalog # 10378016, respectively) until experimentation. The cells were incubated at 37°C in 5% $CO_2$, the media was replaced 2-3 times weekly and the cells were passed once they reached 80-90% confluence.

## Cell Preparation and Experimentation

The following is the method for each run, a total of five runs were conducted. Once the plates reached 80-90% confluency, the cells were removed from the carrying plates and counted using a hemocytometer. Approximately 50,000-70,000 cells were plated in each well of four tissue-culture treated polystyrene 12-well plates (BD Falcon; Catalog #352043). Once the plates reached approximately 80% confluency, the media was aspirated and replaced with 1.0 ml phenol red-free DMEM (Invitrogen, Catalog #21041025) supplemented with 10% charcoal stripped heat inactivated fetal bovine serum, penicillin (100µg/ml), and streptomycin (100 µg/ml). The charcoal stripped FBS was heat inactivated by heating it at 56±2°C for 30 minutes, gently swirling every 10 minutes. The cells were incubated for 24 hours with the following solutions:

- 0.1, 1.0, or 10.0 μM of coumestrol (Sigma-Aldrich, SKU# 27885)
- 0.00001, 0.0001, 0.001, 0.01, or 0.1 μM of 17-β estradiol (Sigma-Aldrich, SKU# E8875)
- 0.1, 1.0, or 10.0 μM of triclosan (Sigma-Aldrich, SKU# 1682206)
- 10.0 μM ICI 182,780 (Sigma-Aldrich, SKU# 14409)
- 10.0 μM ICI 182,780 with 10.0 μM coumestrol
- 10.0 μM ICI 182,780 with 0.1 μM 17-β estradiol
- 10.0 μM triclosan with 0.1 μM 17-β estradiol.

Some wells contained controls having with less than 0.2% of the appropriate vehicle of either DMSO, EtOH, or DMSO and EtOH, and lastly phenol red-free DMEM supplemented with 10% charcoal stripped heat inactivated fetal bovine serum and 10% pen/strep. The concentrations of triclosan were chosen because they were reported in the serum of pregnant women (Bhargava *et al.*, 1996). The plates were then placed in the incubator for 24 hours at 37°C in 5% $CO_2$. After which, the media was aspirated and stored at -20°C until needed for GnRH quantification.

**Enzyme-linked Immunoassay**

An enzyme linked immunoassay (EIA) was performed to quantify the amount of GnRH. Each well of a 96-well plate (Pierce, #15042) was coated with 100 μl of donkey anti-rabbit IgG antibody (Jackson Immunoresearch, #711-005-152) at a concentration of 0.55 μg/ml in coating buffer (0.1 M $NaHCO_3$, 0.1 M $Na_2CO_3$, pH9.5). The plate was then

wrapped in foil and left to sit at room temperature for approximately 24 hours. The plate was then rinsed twice with assay buffer (0.1 M sodium phosphate buffer/pH 7.8, 0.15 M NaCl, 0.1% Tween 20) and washed three times with assay buffer. Anti-GnRH primary antibody (100 µl per well, courtesy of Dr. Nett, Colorado State University, R1245) was diluted 1:50,000 in assay buffer and added to the plate which was incubated for 24 hours at 4° C on a shaker set at 60 RPM. The plate was then rinsed twice with assay buffer and then washed three times with assay buffer. The GnRH standards of: 100 pg/µl, 33.33 pg/µl, 11.11 pg/µl, 3.70 pg/µl, 1.23 pg/µl, 0.41 pg/µl, 0.14 pg/µl (BaChem, Torrence, CA, H-4005) and samples were added in 100 µl quantities each to the wells, and the plate was incubated for 24 hours at 4° C on shaker setting 60 rpm. The plates were then allowed to come to room temperature completely and 50 µl of biotinylated GnRH (BaChem, Torrence, CA, H-4792) was added (diluted 1: 2,500 in assay buffer) to each well and the plate was incubated at room temperature for one hour. Next, the plate was washed three times in assay buffer which then 100 µl of Avidin D-HRP (Vector Laboratories, A2004; diluted 1:5,000 in assay buffer) was added to each well then plate was incubated at room temperature for one hour. The plate was then washed 8 times with assay buffer and 100 µl of supersignal substrate (Pierce, #37075,240 µl diluted into in 12 ml $H_2O$) was added to each well. The chemiluminescence of the samples was then read by a BioTek Synergy HT Multidetection Microplate Reader. A standard curve was constructed from the GnRH standards, and the GnRH in the samples was quantified using the equation from the standard curve. Minimal detectable concentrations ranged from 0.0125 - 1.786 pg/µl, any value below these was discarded.

## Statistical Analysis

Each treatment sample was duplicated in each of the experimental runs. Each sample was then assayed in triplicate. The experiment was run 5 times, resulting in sample sizes ranging from n=4-5.

A one-way Kruskal-Wallis test was done to compare GnRH levels in all the experiments besides the ICI 182,780 experiment, in which a Wilcoxin was utilized; significance set at $p<0.05$. The statistical software used was JMP Pro 10.0, manufactured by SAS.

*17-β Estradiol ($E_2$)*

GT1-7 cells were incubated with varying concentrations of 17-β estradiol for 24 hours; after which, GnRH levels were quantified and the results are shown in Figure 1. The culture medium collected from control treatments contained 205.9±60.6 pg GnRH/ml; while the 17-β estradiol (0.1, 1.0, 10.0, and 100 nM) resulted in levels of 103.7±41.6, 211.2±100.9, 202.5±63.8 and 365.3±141.3 pg GnRH/ml, respectively. 17-β estradiol was unable to significantly alter GnRH secretion ($p>0.05$). In this experiment, 17-β estradiol was dissolved in 100% ethanol; therefore, the control contained the same amount of ethanol.

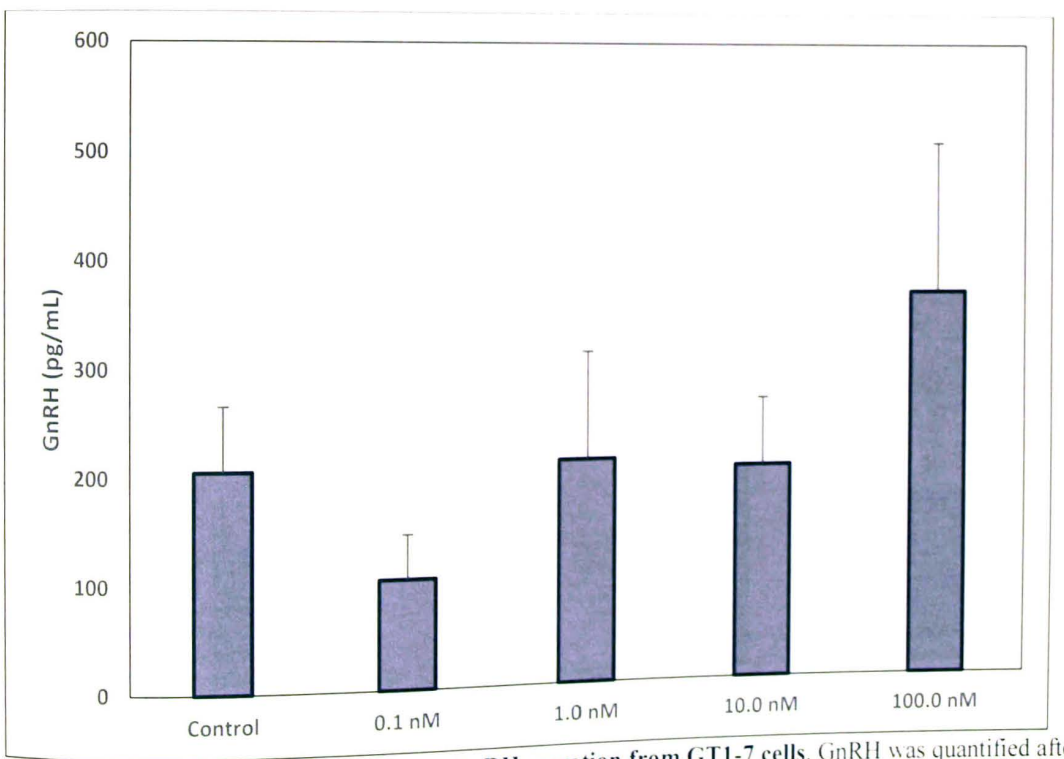



**Figure 1. The effect of 17-β Estradiol on GnRH secretion from GT1-7 cells.** GnRH was quantified after static 24-hour incubations with varying concentrations of 17-β Estradiol and EtOH control. Data is presented as mean ± SEM, $p>0.05$, n=4 for the control, 10.0 nM and 0.1 nM treatment groups and n=5 for the rest of the treatment groups.

*17-β estradiol and ICI 182,780*

17-β estradiol was incubated with ICI 182,780 to determine if ICI 182,780 could block or modulate the effects of estrogen. GnRH levels were quantified and the results are shown in Figure 2. The culture medium collected from control treatments contained 205.9±60.6 pg GnRH/ml; the 100 nM $E_2$ resulted in levels of 365.3±141.3 pg GnRH/ml, the 10.0 μM ICI 182,780 (ICI), 596.3±195.7 pg GnRH/ml, and the combination of $E_2$ and ICI ($E_2$/ICI), 231.9±87.2 pg GnRH/ml. In this experiment, $E_2$ was dissolved in 100% EtOH; therefore, the control contained the same amount of EtOH. None of the treatments significantly altered GnRH secretion ($p>0.05$).

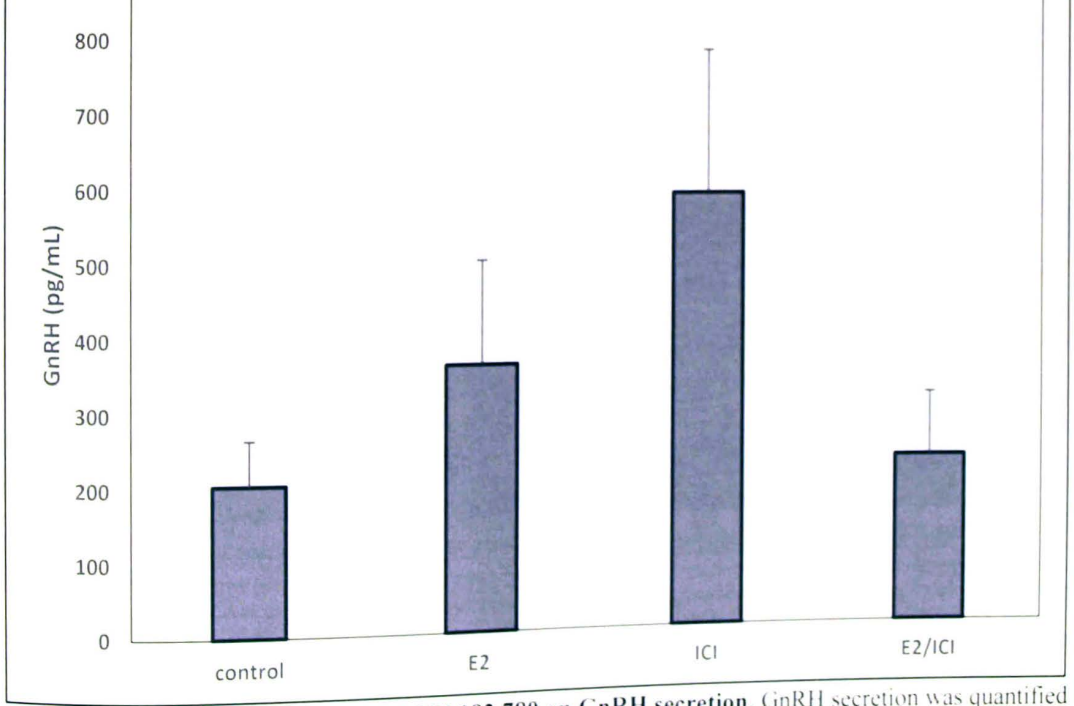



**Figure 2 Effect of 17-β Estradiol and ICI 182,780 on GnRH secretion.** GnRH secretion was quantified after static 24-hour incubations with 0.1 μM 17-β estradiol, 10.0 μM ICI 182,780, and 0.1 μM 17-β estradiol in combination with 10.0 μM ICI 182,780. Data is presented as mean ± SEM. $p>0.05$, n=5 for all treatment groups.

*Coumestrol*

GT1-7 cells were incubated with varying concentrations of coumestrol for 24 hours; after which, GnRH levels were quantified and the results are shown in Figure 3. The culture medium collected from control treatments contained 465.5±162.7 pg GnRH/ml; while the coumestrol (0.1, 1.0, and 10.0 nM) resulted in levels of 769.1±443.4, 888.8±515.1, and 614.7±181.4 pg GnRH/ml, respectively. Coumestol was unable to significantly alter GnRH secretion ($p>0.05$). In this experiment, coumestrol was dissolved in 100% DMSO; therefore, the control contained the same amount of DMSO.

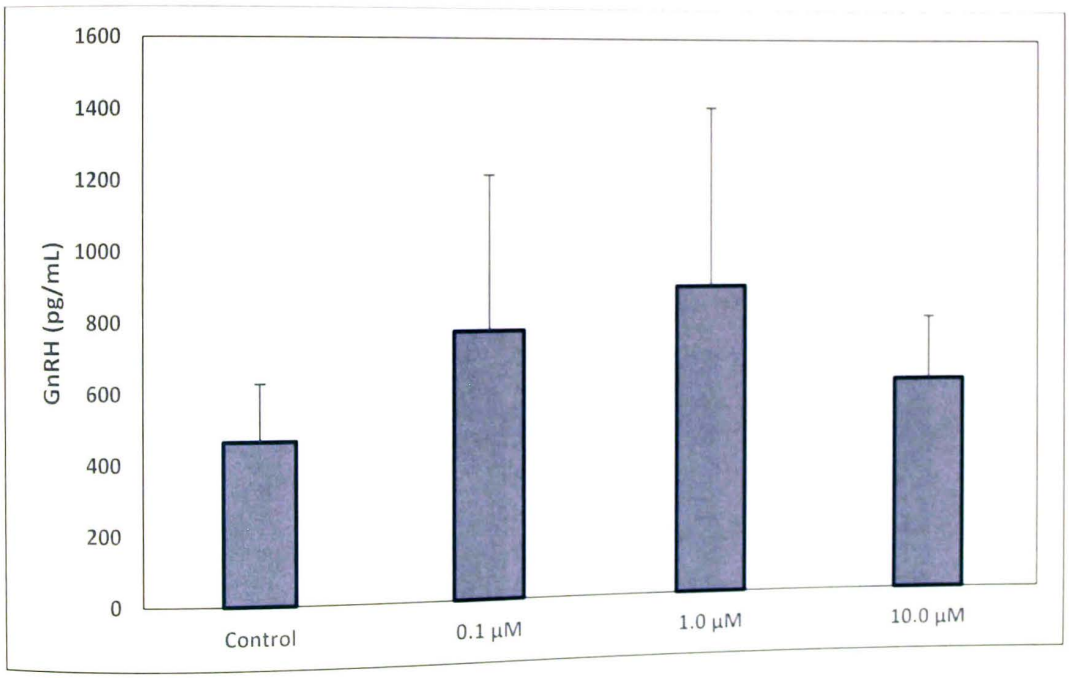



**Figure 3. Effect of coumestrol on GnRH secretion.** GnRH was quantified after static 24-hour incubations with several concentrations of coumestrol and the DMSO control. Data is presented as mean ± SEM, $p>0.05$, n=4 for the 10.0 μM treatment and n=5 for the rest of the treatment groups.

*Coumestrol and ICI 182,780*

Coumestrol was incubated with ICI 182,780 to determine if ICI 182,780 could block or modulate the effects of coumestrol. GnRH levels were quantified and the results are shown in Figure 4. The culture medium collected from control treatments contained 465.5±162.7 pg GnRH/ml, the 10.0 nM Coumestrol (COU) resulted in levels of 614.7±181.4 pg GnRH/ml, the 10.0 μM ICI 182,780 (ICI) resulted in 596.3±195.7 pg GnRH/ml, and the combination of COU and ICI (COU/ICI) resulted in 462.3±105.7 pg GnRH/ml. In this experiment, coumestrol was dissolved in 100% DMSO; therefore, the control contained the same amount of DMSO. The COU/ICI was unable to significantly alter GnRH secretion. ($p>0.05$).

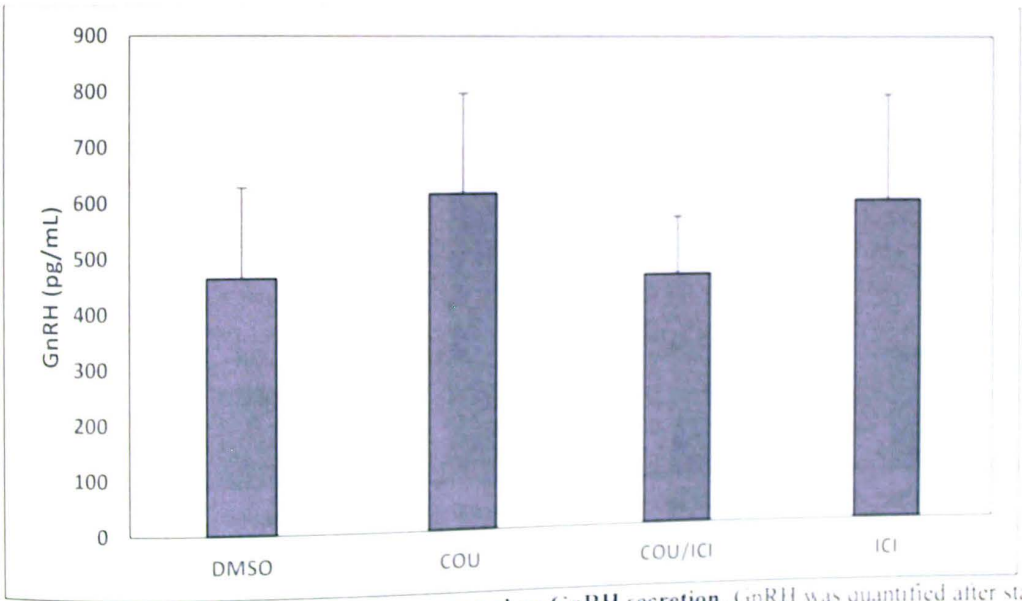



**Figure 4. Effect of ICI 182,780 and coumestrol on GnRH secretion.** GnRH was quantified after static 24-hour incubations with 10.0 μM coumestrol, 10.0 μM coumestrol in combination with 10.0 μM ICI 182,780, and 10.0 μM ICI 182,780, and. Data is presented as mean ± SEM, $p>0.05$, n=4 for the COU treatment and n=5 for the COU/ICI and ICI groups.

*Triclosan*

GT1-7 cells were incubated with varying concentrations of triclosan for 24 hours; after which, GnRH levels were quantified and the results are shown in Figure 5. The culture medium collected from control treatments contained 205.9±60.6 pg GnRH/ml; while the triclosan (0.1, 1.0, and 10.0 nM) resulted in levels of 715.0±312.3, 178.3±60.1, and 254.9±68.8 pg GnRH/ml, respectively. Triclosan was unable to significantly alter GnRH secretion ($p>0.05$). In this experiment, triclosan was dissolved in 100% EtOH; therefore, the control contained the same amount of EtOH.

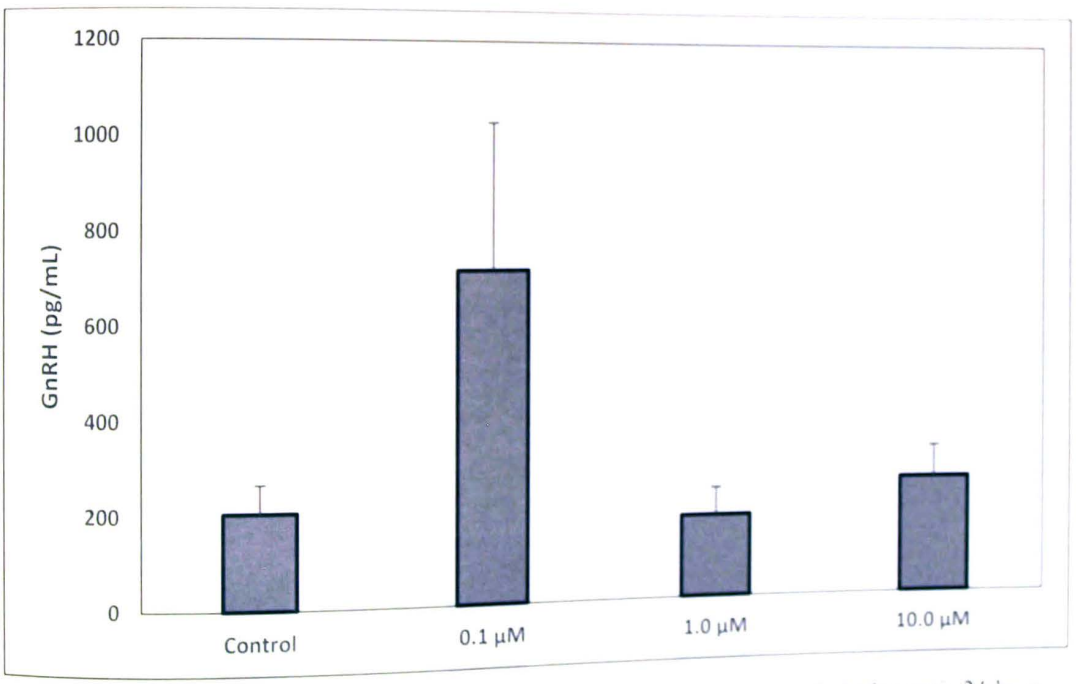



**Figure 5. Effect of triclosan on GnRH secretion.** GnRH secretion was quantified after static 24-hour incubations with the varying concentrations of triclosan and the vehicle EtOH. Data is presented as mean ± SEM. $p>0.05$, n=4 for the control and 0.1 µM treatment groups and n=5 for the rest of the treatment groups.

*Triclosan and ICI 182,780*

Triclosan was incubated with ICI 182,780 to determine if ICI 182,780 could block the effects of triclosan. GnRH levels were quantified and the results are shown in Figure 6. The culture medium collected from the control treatments contained 205.9±60.6 pg GnRH/ml. The 10.0 nM triclosan (TCS) resulted in levels of 254.9±68.8 pg GnRH/ml, the 10.0 μM ICI 182,780 (ICI) in 596.3±195.7 pg GnRH/ml, and the combination of TCS and ICI (TCS/ICI) contained 340.2±94.7 pg GnRH/ml. In this experiment, triclosan was dissolved in 100% EtOH; therefore, the control contained the same amount of EtOH. The TCS/ICI was unable to significantly alter GnRH secretion. ($p$>0.05).

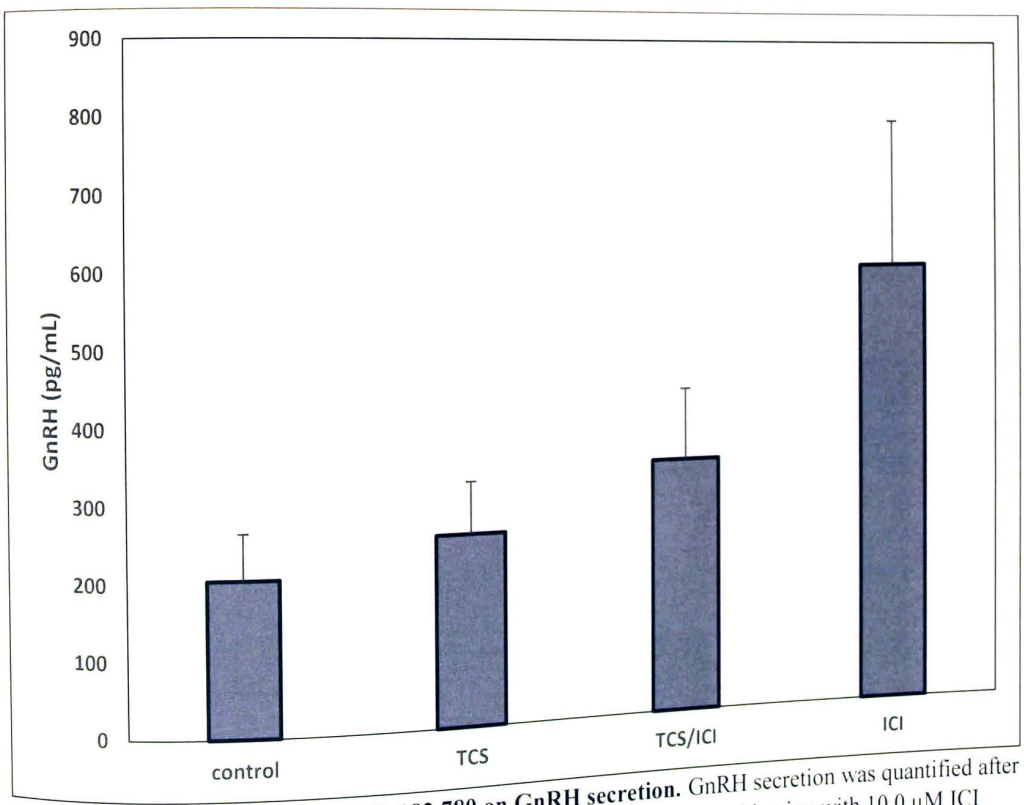



**Figure 6. Effect of triclosan and ICI 182,780 on GnRH secretion.** GnRH secretion was quantified after static 24-hour incubation with 10.0 μM triclosan, 10.0 μM triclosan in combination with 10.0 μM ICI 182,780, and 10.0 μM ICI 182,780. Data is presented as mean ± SEM, $p$>0.05, n=5 for all treatment groups.

*Triclosan and 17-β estradiol*

To determine if triclosan possibly inhibited or modulated the effects of estrogen, cells were incubated for 24 hours with triclosan and estrogen alone and a combination of the two (Figure 7). The culture medium collected from the from control treatments contained 205.9±60.6 pg GnRH/ml, the 100 nM 17-β estradiol (E2) contained 365.3±141.3 pg GnRH/ml, the 10.0 μM triclosan (TCS) resulted in 254.9±68.8 pg GnRH/ml, and the combination of E2 and TCS (E2/TCS) resulted in 121.2±44.1 pg GnRH/ml. In this experiment, estrogen was dissolved in 100% EtOH; therefore, the control contained the same amount of EtOH. The E2/ICI was unable to significantly alter GnRH secretion. ($p$>0.05).

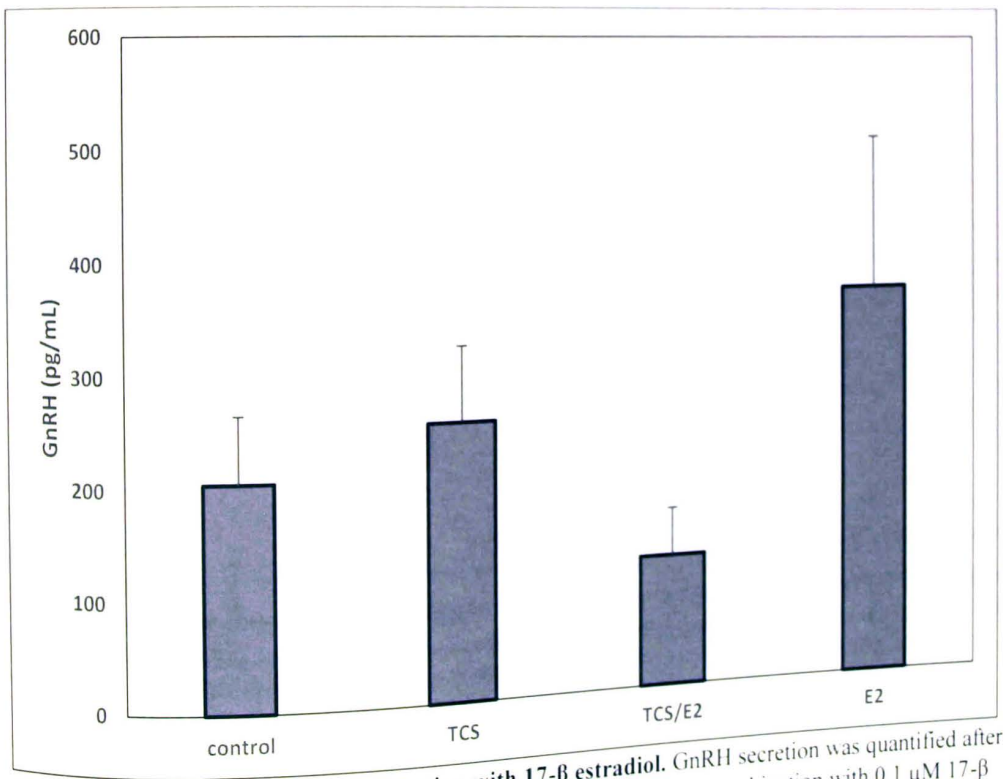



**Figure 7. Effect of triclosan in combination with 17-β estradiol.** GnRH secretion was quantified after static 24-hour incubations with 10.0 μM triclosan, 10.0 μM triclosan in combination with 0.1 μM 17-β estradiol and 0.1 μM 17-β estradiol alone. Data is presented as mean ± SEM, $p$>0.05, n=4 for the TCS/E2 group and n=5 for the TCS and E2 groups.

*ICI 182,780*

To determine the effects of ICI 182,780 on GnRH secretion (Figure 8), GT1-7 cells were incubated with 10.0 μM ICI 182,780 and the EtOH control for 24 hours. The culture medium collected from the control resulted in 153.0±73.3 pg GnRH/ml and 10.0 μM ICI 182,780 (ICI) resulted in 596.3±195.7 pg GnRH/ml. In this experiment, triclosan was dissolved in 100% EtOH; therefore, the control contained the same amount of EtOH. The results show that 10.0 μM concentration of ICI 182,780 significantly increased GnRH secretion when compared with the EtOH control.

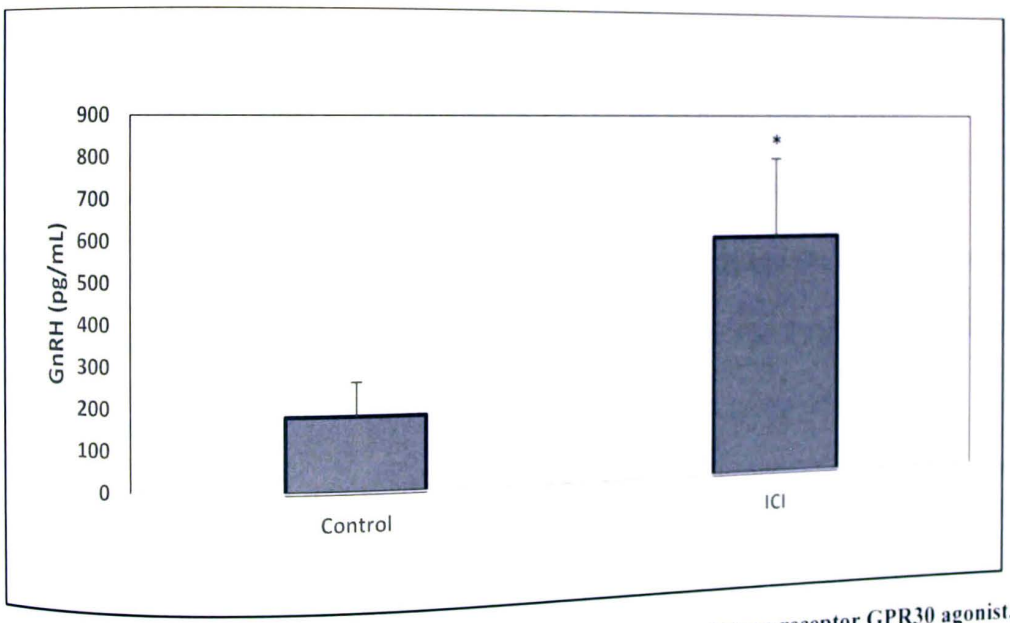



**Figure 8. The effect of the ER-α and β antagonist and membrane estrogen receptor GPR30 agonist, ICI 182 780, on GnRH secretion in GT1-7 cells**. GT1-7 cells with incubated for 24 hours with 10.0 μM ICI 182,780 and EtOH.Data is presented as mean ± SEM, $p<0.05$, n=5 for both treatment groups.

## Chapter 5. Discussion

The goal of this research was to determine the effects of the EDCs coumestrol and triclosan on GnRH secretion and compare them to the effect of estrogen in GT1-7 cells. The anti-estrogen ICI 182,728 was also investigated to determine if it blocked or modulated these effects. The results showed that neither estrogen, coumestrol, nor triclosan significantly altered GnRH secretion nor did ICI 182,780 inhibit or modulate the effects. It was found, however, that ICI 182,780 significantly increased GnRH secretion when compared with the control.

Recently, reports have shown the estrogenic and antiestrogenic effects of coumestrol (Ashby *et al.*, 1999; Bowe, *et al.*, 2003; Jacob *et al.*, 2000). To demonstrate coumestrol's estrogenicity, GT1-7 cells were incubated for 6, 12, or 24 hours with differing concentrations of coumestrol (Bowe, *et al.*, 2003). This resulted in a significant reduction in GnRH mRNA. While Bowe's *et al.*, (2003) study and the current one were similar in that they examined the effect of coumestrol, Bowe's study examined mRNA levels, but not GnRH peptide levels. Because altered mRNA levels do not directly correspond to altered protein levels (reviewed by Maier *et al.*, 2009), it could be that in Bowe's study the mRNA did not actually go on to produce the GnRH peptide in the timeframe given. In addition, Pitts *et al.*, (2001) demonstrated that inhibition of transcription and translation did not alter GnRH secretion over a period of three hours; suggesting that changes in mRNA levels do not alter GnRH secretion in the short term. Another study by Hughes (1988) reported that coumestrol did not influence GnRH-induced LH secretion in ovariectomized rats but estrogen did have a significant effect on

the GnRH-induced secretion. My results support these findings by demonstrating that coumestrol does not directly influence GnRH secretion.

Triclosan is another EDC which was shown to exert estrogenic effects in a breast cancer cell line (MCF-7) where it was shown to significantly increase cell proliferation and cyclin-D (a cyclin-dependent kinase regulator) expression while significantly decreasing p21 (a cyclin-dependent kinase inhibitor) expression.(Lee *et al.*, 2014). These effects were blocked by ICI 182,780 (Lee *et al.*, 2014). Because this study looked at the effects on MCF-7 cells and not GT1-7 cells it is possible that the GT1-7 cells were not displaying the correct receptors to cause an effect. It is also possible that the current experiment utilized a suboptimal TCS concentration or incubation period, preventing the effects of triclosan from being observed. It has been shown that exposing GT1-7 cells to different estrogen concentrations and incubation periods produces contrasting results. For example, Navarro *et al.*, (2004) demonstrated that administration of picomolar estrogen levels to GT1-7 cells for 5-60 minutes caused a rapid, sustained and dose-dependent inhibition of cAMP; conversely, exposure to nanomolar estrogen concentrations for 60 minutes increased cAMP production. These studies demonstrate how easy it is for hormones to have contrasting effects. This is similar to some new reports on ICI 182,780 in which it takes on new roles other than a classical ER antagonist (Howell *et al.*, 2002).

ICI 182,780 was recently shown to be a GPR30 agonist (Meyer *et al.*, 2010). GPR30 is a G-protein coupled receptor expressed by GT1-7 cells that is activated by estrogen (Filardo *et al.*, 2002). Activation of GPR30 causes intracellular calcium mobilization, cAMP signaling, and synthesis of phosphatidylinositol 3,4,5-trisphosphate in the nucleus resulting in rapid non-genomic signaling events (Revankar, et al., 2005; Kang et al.,

2010). Zhao *et al.* (2006) also showed agonistic effects when they investigated the neuroprotective efficacy of ICI 182,780 against neurodegenerative diseases such as Alzheimer's in rat primary hippocampal neurons. They found that, like17-β estradiol, ICI 182,780 promoted neuron survival when they were exposed to excitotoxic glutamate or β-amyloid-induced neurodegeneration. Furthermore, they found that ICI 182,780 induced rapid intracellular $Ca^{2+}$ oscillations, exerted dual regulation of physiological tolerable and excitotoxic ranged glutamate-induced rises in $Ca^{2+}$ levels, and significantly increased expression of spinophilin (a protein that aids in dendritic morphology and glutamatergic synaptic activity) and Bcl-2 (an antiapoptotic protein involved in estrogen promotion of neuronal survival) all with efficacy comparable with neurons treated with 17-β estradiol. Another study by Long *et al.*, (2017) showed that ICI 182,780, via GPR30, facilitated lordosis in estradiol benzoate primed OVX rats. These studies show that ICI 182,780 can deviate from its traditional role as an ER antagonist and act as an agonist causing stimulatory effects. Our results support these findings as ICI 182,780 acted as a stimulatory agent by significantly increasing GnRH secretion.

Clearly there is dynamic relationship between estrogen, xenoestrogens, anti-estrogens, and their target tissues; much of which remains elusive. However, what is known, is estrogen and estrogen-related signaling actions are largely dependent upon which ligand the ER is bound with and which response element the ER-ligand complex is bound to. These criteria can also be applied to xenoestrogens.

In the current study, it is possible that the incubation time was not adequate to observe the effects of triclosan, coumestrol, and estrogen. Although other studies with similar incubation periods have shown coumestrol and triclosan as having significant effects,

either a different cell line was used or GnRH peptide levels were not analyzed. The differing results could be attributed to which receptors were present on the GT1-7 cells, the classical ERs α and β are expressed on both murine GnRH neurons and in GT1-7 cells and the novel GPR30 is expressed in GT1-7 cells (Jacobi *et al.*, 2007), but perhaps there was is some alteration of the estrogen receptor expression due to over-saturation or incubation of the hormones/xenoestrogens. There is evidence of this occurring in MCF-7 cells. In this case, the cells were exposed to 10 nM estrogen for 24 hrs. (one of the concentrations used in this study and the same incubation time) this caused the down-regulation of hundreds of genes, a lot of which coded for receptors (Frasor, *et al.*, 2003). It could be that in GT1-7 cells, the current concentrations or incubation periods of coumestrol, triclosan, and estrogen triggered a down regulation of estrogen receptors thus causing no effect in GnRH secretion.

In conclusion, our results demonstrate that GnRH secretion is not stimulated by coumestrol, triclosan, or estrogen under the current concentrations and incubation periods; GnRH *is* however, stimulated by the GPR30 agonist ICI 182,780. This provides novel information about the hormonal control of GnRH.

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