THE REGULATION OF OXYTOCIN RECEPTORS IN PORCINE ENDOMETRIAL TISSUE

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The Regulation of Oxytocin Receptors in Porcine Endometrial Tissue

A Thesis
Presented for the
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Jamie Eugene Mells

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DEDICATION

This thesis is dedicated to Roger Dickson whose continued support

inspires me to reach for the stars

and

in loving memory to Troy Collins "my baby brother"

who left this world too soon. You will

be missed.

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ABSTRACT

Oxytocin (OT) is the most potent natural substance stimulating uterine contractions; the mechanism for regulating oxytocin receptors (OTRs) is not fully understood in swine. Research suggests that there is a positive relationship between OTR density and OT sensitivity. OTRs are strongly up regulated immediately before parturition. It has been proposed that the ratio of estrogen to progesterone is the determining factor in the regulation of uterine OTR expression. Thus the objectives of this study were: (1) to adapt a standard technique for culturing endometrial tissue so that specific hormonal treatments could be evaluated *in vitro*. (2) To determine the effect of E_2 and P_4 on OTR expression in porcine endometrium *in vitro*. Endometrial cell cultures were treated with varying concentrations of E_2 and P_4 and harvested to determine the total protein and OTR concentration. The effect of estrogen and progesterone on OTR expression could not be sufficiently determined by our *in vitro* model.

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Literature Review

The uterus is the site of implantation and maintenance of mammalian embryos. The uterine wall is composed of three layers: the endometrium, myometrium and the perimetrium. The endometrium is the inner lining of the uterus. The endometrium consists of a superficial epithelial layer; a woven layer of connective tissue richly supplied with maternal blood vessels, and branched uterine glands (1,2,3). These tissues form two layers of endometrium: the stratum functionalis, which in primates is shed during menstruation and the stratum basalis, which is the permanent layer that gives rise to a new stratum functionalis after each menstruation (3,4,5). The endometrium responds to endocrine changes that accompany the female reproductive cycle, pregnancy, and parturition. During pregnancy the endometrial tissue merges with the fetal membranes to form the placenta. The myometrium consists of an inner layer of smooth muscle, a highly vascularized middle layer, adjacent to a thin outer longitudinal layer (1,3). During parturition myometrial contraction is responsible for expulsion of the fetus. The perimetrium or serosa is formed from the peritoneum and envelops the uterus, providing a protective covering.

Oestrous cycle

The purpose of the oestrous cycle is to provide a uterine environment that will support embryonic development. In swine, the cycle length ranges from 19 to 23 days (1). The oestrous cycle can be divided into two phases: follicular and luteal.

The follicular phase is divided into two subphases, proestrus and estrus. The

proestrus phase is a period of ovarian stimulation resulting in growth and maturation of the uterine environment in preparation for release of mature ova from the ovary (1,3). During proestrus, vascularity of the uterine endometrium is increased, surface epithelium is composed primarily of simple columnar cells, and there is a marked increase in uterine gland growth (1,3). Estrus is the phase of the cycle, commonly referred to as "heat," in which the female will accept copulation (1,2,3,6). Estrus persists for 2-3 days, although a variation in length of 1-4 days is not uncommon (1). During estrus, preovulatory follicles ovulate and liberate mature ova into the oviduct (3). The process of ovulation involves physical rupture of the distal wall of the preovulatory follicle, which is brought about by a series of biochemical and vascular changes (3). In swine, the majority of ova are released twenty-four to thirty-six hours after onset of estrus, thereby increasing the probability of fertilization (1). Following ovulation, the follicular wall collapses and hemorrhaging occurs within the central cavity of the ruptured follicle. The cavity, filled with clotted blood, is referred to as a *corpus haemorrhagicum.* (1,2). Follicular cells of the stratum granulosa increase in number and size forming the corpus luteum (1,3,6). Ovulation and subsequent development of the corpus luteum characterize the beginning of the luteal phase of the oestrous cycle.

The luteal phase is divided into two subphases, metestrus (or "interestrus") and diestrus. The length of the luteal phase is directly dependent upon occurrence of mating, fertilization, and subsequent implantation of the ovum. The corpus luteum is considered to be a transient endocrine gland that secretes hormones responsible for a

variety of functions in the luteal phase of the oestrous cycle. The primary function of the corpus luteum is to prepare the uterus for subsequent implantation and nourishment of the developing embryo (3,6). During interestrus, the corpus luteum begins secreting progesterone in preparation for implantation (1,3). In the event of fertilization and implantation, the corpus luteum persists and grows significantly larger. The enlarged corpus luteum, referred to as the *corpus luteum verum* during pregnancy, releases progesterone that acts at the pituitary level to modulate lutropin (LH) secretion, by enhancing the negative feedback loop caused by relatively low amounts of estrogen (3,7). In addition, the corpus luteum prevents repeated ovulation by inhibiting estrogen positive feedback mechanism (1,2,6,7). Diestrus occurs when fertilization does not take place. The corpus luteum spurium (of the estrous cycle) regresses into a white pale body known as the corpus albicans.

The Relationship of Progesterone and Estrogen in the Maternal Recognition of Pregnancy

The transformation from cyclic to pregnant endocrine state is referred to as the "maternal recognition of pregnancy." The "maternal recognition of pregnancy" occurs through a series of biological signals emitted from the corpus luteum and embryo. A key element in this recognition process is the maintenance of the corpus luteum vernum. Two hormones involved in this process are estrogen and progesterone.

Estrogens are steroids secreted from ovarian preovulatory follicles and conceptuses of domesticated mammals (3,8). Estrogens are known to promote growth

of endometrial glands, induce behavioral estrus (heat), stimulate mammary gland growth, enhance water and electrolyte movement, promote placental expansion, cell permeability, uterine blood flow, myometrial sensitivity/excitability, and cause secretory activity in the oviduct (1,3,8,9). Estradiol-17β (E_2) is the most potent of the estrogens.

In swine the "maternal recognition of pregnancy" occurs between days 10 and 12 after the start of oestrus (10,11). Estrogen production increases significantly and peaks during day 10 and 11 in swine (11). It is hypothesized that estrogens secreted by the blastocysts act as luteotrophic agents by redirecting the luteolytic prostaglandins from the endometrium (3,10,11). In cyclic sows the endometrium secretes prostaglandins in an endocrine direction, toward the myometrium and into the uterine vasculature, thereby transporting prostaglandins to the corpus luteum causing luteolysis and regression of the corpus luteum (3,10,12). Conversely, in pregnant sows the conceptus produces estrogens, which reorients prostaglandin release in an exocrine direction, causing prostaglandins to be released into the uterine lumen, where they have no effect on the corpus luteum (3,10,12). Indeed, experiments by Geisert *et al*, demonstrated that, in pigs, systemic injections of estrogens during Days 11-13 of the estrous cycle delays luteal regression (10).

The corpus luteum in swine and goats is the primary source of progesterone throughout gestation, unlike primates, sheep and horses in which the placenta is the primary source of progesterone for most of the pregnancy (3). In swine and goats the combination of estrogen and progesterone is necessary for the maintenance of gestation (1,3,6,10,11). Progesterone has several roles in the uterus, but its primary function is to promote secretory changes in the uterine endometrium. These "progestational changes" include marked growth of the uterine glands, proliferation of the uterine

stroma, and increased secretory activity in the oviduct and from the endometrial glands (3,6). In addition, progesterone functions to: prevent uterine contractibility, regulate secretions of pituitary gonadotropins, promote secretory changes in the mucosal lining of the fallopian tubes, enhance sodium, chloride and water reabsorption in the kidney, and promote development of alveoli cells in the mammary glands (1,2). In swine, rabbits, and guinea pigs, progesterone concentrations remain high until the initiation of labor. Experiments by Lundin-Schiller *et al.* revealed that progesterone levels dropped significantly during the interval between 90-day gestation levels and the occurrence of milk letdown (8). Progesterone withdrawal has been shown to be a principal factor in initiation of parturition. Indeed, in rabbits it has been shown that parturition could be delayed or prevented by the administration of progesterone just before the end of pregnancy (6).

The Interrelationship of Oxytocin and Prostaglandins in the Uterine Environment

Oxytocin (OT) is a nine amino acid peptide secreted by the posterior pituitary gland (3). The neurohypophysis is the primary source of OT (7,13). OT is generally produced within the axonal endings of the pars nervosa and plasma OT concentrations increase around the time of luteolysis (7,13). Although the role of OT in the initiation of labor in ruminants has been largely established, it is still not fully understood in swine. It is considered the most potent natural substance stimulating uterine contractions. As demonstrated in guinea pigs and humans, the frequency and

amplitude of OT induced uterine contraction are identical with those occurring during spontaneous labor (14,15). OT induces uterine contractions both directly, by binding to receptors on myometrial cells and thus stimulating contractions, and indirectly, by binding to endometrial/decidual cells and inducing prostaglandin production.

In addition it has been shown in experiments by Alexandrova *et al.*, that electrical stimulation of the posterior pituitary gland, which in theory causes OT release to the blood stream, induces labor contractions in guinea pigs (14,16). However, a consistent increase in OT concentration in peripheral maternal circulation prior to labor has not been observed, and for this reason OT may not have a direct physiological role in the early events of labor (8,14).

Prostaglandins are eicosanoid hormones consisting of a group of 20-carbon polyunsaturated fatty acids (3,7). Prostaglandins are derived primarily from arachidonic acid and are produced within the plasma membrane. Prostaglandins are not secreted from any one gland; many cell types in the body have the capacity to convert fatty acids into prostaglandins. The conversion of precursor fatty acid molecules can come from endocrine, nervous, or chemical stimulation. Prostaglandins can be divided into four groups: A, B, E, and F, which differ in the cyclopentane ring substituent and double bonds in the molecule (3,7). Prostaglandins are powerful stimulants of smooth muscle. In general, prostaglandin E (PGE) relaxes smooth muscle and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) contracts smooth muscle (1). Prostaglandins also play an integral role in the oestrous cycle. In the porcine endometrium, pulsatile secretion of endometrial $PGF_{2\alpha}$ is stimulated by OT during

days 14-16 postestrus (17,18,19). It has been widely established that $PGF_{2\alpha}$ is responsible for corpus luteum regression in cyclic sows, and ewes (13,17,18). Recent experiments by Gregoraszczuk *et al.* have shown that PGE_2 has a luteotrophic effect on corpus luteum by increasing its production of progesterone (20).

OT binds to specific oxytocin receptors (OTRs) on the endometrium to stimulate phosphoinositide hydrolysis, thereby activating the inositol triphosphate (IP3)-diacylgylcerol (DAG) second-messenger system mobilizing intracellular calcium and activating protein kinase C (7,17,18,21,22). Experiments by Mirando *et al.,* Whiteaker *et al.*, and Tysseling *et al.*, established this cascade of reactions promotes pulsatile $PGF_{2\alpha}$ secretion within the porcine endometrium (17,21,22). This OT induced $PGF_{2\alpha}$ production occurs during corpus luteum regression in swine and ruminants in normal parturition (3,8,17,18,23). $PGF_{2\alpha}$ plays an important role in the initiation of parturition by stimulating luteolysis and thereby initiating progesterone withdrawal (1). Nevertheless, as previously mentioned, the mechanism that both inhibits progesterone secretion and/or increases OT secretion, which in turn causes $PGF_{2\alpha}$ release, is still not fully understood.

The Role of Oxytocin Receptors in Parturition

The oxytocin receptor, a seven transmembrane domain, G protein linked receptor molecule is essential to parturition (24). It has been well established that OTRs are strongly up regulated immediately before parturition. It has been proposed that the ratio of estrogen to progesterone is the determining factor in the regulation of

uterine OTR expression; however, conflicting data suggest there are variations between species (25). In the rat, experiments by Alexandrova *et al.* correlated a proportional increase in estrogen receptor concentration in the myometrium, with the increase in OTR concentration (26). Further experiments, by Larcher *et al.* revealed that treatment of ovariectomized rats with E_2 lead to a significant increase in both OTR mRNA levels and OT binding. In ewes, Zhang *et al.* reported that progesterone and E_2 caused downregulation of endometrial oxytocin receptors, and only when progesterone was withdrawn, similar to that which occurs during luteolysis, did receptor density increase (27). In swine, Lundin-Schiller *et al.* reported that concentrations of E_2 were positively correlated with OT binding site concentrations, and progesterone concentrations were negatively correlated (8). The shift in estrogen: progesterone ratio occurred after day 90 and by 2 days prior to delivery (8). Similar conclusions were drawn from Lau *et al.*, who found that ovariectomized ewes treated with progesterone (P_4) alone or in combination with $PGF_{2\alpha}$, reduced OTR density. This relationship between OTR density and the ratio of estrogen/progesterone has also been established in the ventromedial nuclei of the hypothalamus of rats (28). Therefore the body of research suggests that OTR's are strongly up-regulated in response to E_2 administration (8,21,25). Progesterone induces down-regulation of OTRs, however OT downregulation is not accompanied by a decrease in OTR gene expression (8,21,25,29).

An increased uterine sensitivity to OT may play a key role in the initiation of parturition. The concentrations of OTRs in the rat, guinea pig, and human

myometrium have been shown to rise abruptly several hours before labor (14,16). Enhanced OT sensitivity is a consequence of an increase in the density of OTRs on myometrial plasma membranes. It has been reported that OTR concentration of human deciduas and expression of myometrial gap junctions increase during pregnancy and peak in early labor (8,15,29). Husslein *et al.*, have shown that the uterine response to OT is directly correlated with the number of binding sites in individual uteri (30). In rats the concentration of OTRs in the myometrium was found to rise abruptly several hours before labor, reach maximal levels during labor, and then decline significantly 24 hours after parturition (14). In addition experiments by Soloff *et al.* on women showed that 100 milliunits (mU) of OT infused per minute was needed to elicit uterine contractions in nonpregnant women. In pregnant women, 16 mU/min was sufficient to elicit contraction at 20 weeks of pregnancy, and 2mU/min at 32 weeks, and 1 mU/min at term. In addition, the number of myometrial OTRs was more than 150 times greater during labor than in uterine tissue non-pregnant myometrium (14). In swine, endometrial tissue expressed high numbers of OTRs during late gestation, labor, and shortly following parturition (7,8). Specifically, experiments by Lundin-Schiller *et al.* reported that endometrial and mammary tissue expressed acute increases in OT binding site concentrations, whereas myometrial tissues displayed a more gradual increase in comparison (8). Experiments by Whiteaker *et al.* have shown that swine endometrial tissue possess functional OTR's, making porcine endometrium ideal for studying the regulation of OTR (18). These experiments provide empirical data on the fundamental importance of OTR

density, specifically that there is a positive relationship between OTR density and OT sensitivity (8,16).

Objective and Hypothesis

Thus the objectives of this study were: (1) to adapt a standard technique for culturing endometrial tissue so that the effect of specific hormonal treatments on OTR expression could be evaluated *in vitro*. (2) To determine the effect of E_2 and P_4 on OTR expression in porcine endometrium *in vitro*. The working hypothesis is: treatment with P_4 followed by E_2 will increase OTR expression in endometrial tissue.

Materials and Methods

Tissue collection and culturing

Uteri were obtained at the time of slaughter from Hampton's Meats, Hopkinsville, KY, and transported to the laboratory in Incomplete Hanks Balanced Salt Solution (IHBSS, Ca^{2+} and Mg^{2+} free, pH 7.4) containing 20 µl/ml of antibiotics and antimycotics with the stock concentration being 10,000 units/ml penicillin G sodium, 10,000 µg/ml streptomycin sulfate and 25 µg/ml amphotericin B in .85% saline [ABAM]. Endometrium was cultured by the method of Davis and Blair (32). Briefly, endometrium was sharply dissected from myometrium and rinsed three times in IHBSS containing ABAM. The endometrium was then incubated in the enzyme dispase (4.6 mg/ml) and pancreatin (.0125 g/ml with a stock solution of 25 g pancreatin and 8.5 g NaCl per liter) for two hours at room temperature. Luminal endometrial cells were isolated by centrifugation (800 xg) and plated in 35 mm culture dishes at a density of 3×10^6 cells/plate. Cultures were maintained in RPMI 1640 without phenol red containing charcoal stripped Fetal Calf Serum (10%), ABAM, and insulin (2.5 µg/ml) in a humidified atmosphere of 5% CO_2 in 95% air at 37° C. Media were changed every 48 hours and experiments conducted on confluent cultures. Materials were purchased from Gibco, (Grand Island NY), and Sigma (St. Louis MO).

Experimental Design

At confluence endometrial cell cultures were treated with experimental media as outlined below, and then harvested to determine total protein and OTR concentration. In all experiments steroids (Sigma) were solubilized in 95% ethanol. Control medium contained an equal volume of ethanol as the steroid-containing treatment.

Experiment A

Question 1: Does E_2 treatment increase OTR expression *in vitro*? Question 2: Does P_4 priming in conjunction with E_2 increase OTR expression? Plates of cells were exposed to control or P_4 (15 ng/ml) for 48 hours. Media were removed and replaced with either control or E_2 10^{-8}M. Each treatment combination was done on six plates of cells so that at the end of the incubation period OTR assays could be conducted in triplicate (three nonspecific binding plates and three total binding plates). Additional concentrations of E_2 (10^{-9} and 10^{-10} M) were employed if cell harvests were large enough. Protocol 1 as described below was used for determination of OTR expression. This experiment was conducted on three different cultures.

Experiment B

Question: Does the concurrent administration of P_4 and E_2 change OTR expression? Cultures were exposed to 0 (control), P_4 (15 ng/ml), or P_4 (15 ng/ml) plus E_2 at varying concentrations for 48 hours. Media were removed and replaced with control

and varying concentrations E_2. Each treatment combination was done on six plates of cells so that at the end of incubation period OTR assays could be conducted in triplicate (three nonspecific binding plates and three total binding plates). Additional concentrations of E_2 (10^{-9} and 10^{-10} M) were employed if cell harvests were large enough. Protocol 1 as described below was used for determination of OTR expression. This experiment was conducted on three different cultures.

Experiment C

Question: Does the concurrent administration of P_4 and E_2 change OTR expression? Cultures were exposed to 0 (control) or P_4 (15 ng/ml) plus E_2 10^{-9}M. Media were removed and replaced with control, P_4, $E_2 10^{-10}$M, or $E_2 10^{-10}$M, respectively. After treatment when cells were harvested, cell samples from like treatments were pooled. The goal was to achieve 100µg/100ml from the plates. This allowed OTR assays to be conducted in duplicate on as large a cell population sample as possible. Protocol 2 described below was used for the determination of OTR expression. This experiment was conducted on four different cultures.

OT Binding Assay

Protocol 1

The OT binding assay is a modified version of the procedure presented by Adachi & Oku (33). OT binding has been analyzed using the following procedure. Plated cells are rinsed three times with Tris Buffer (50 mM, pH 7.6). Then each treatment is incubated for sixteen hours at 4° C with Total Binding solution using a

concentration of 1 nM ^{3}H-OT in 50 mM Tris buffer (pH 7.6) plus 0.1% bovine serum albumin and 5 mM $MnCl_2$, or Nonspecific Binding solution containing 1 nM ^{3}H-OT in 50 mM Tris Buffer (pH 7.6) plus 0.1% bovine serum albumin and 5 mM $MnCl_2$, and

1 μM of cold OT. Following incubation, the binding solutions are removed and the cells are rinsed six times with cold 50 mM Tris buffer (pH 7.6). Cells are scraped from the plates into 1 ml of 0.1N NaOH and placed in scintillation vials with 10 mls of scintillation fluid. Disintegration per minute will be determined for five minutes on a Packard Instruments Tri-Carb 1600CA scintillation counter. Total binding solution contains only ^{3}H-OT. Therefore all binding that occurs can be attributed to OT binding to its receptor and random or nonspecific binding. Nonspecific binding solution contains ^{3}H-OT and an excess amount of non-labeled OT. Thereby any binding found can be attributed to binding that occurs on substances other than OTR's. Specific Binding is determined by subtracting Nonspecific Binding from Total Binding values.

Protocol 2

OT binding was analyzed by a second procedure adapted from Adachi & Oku (32). Cells are grown to confluence, and then placed on a bed of ice. Cells are rinsed three times with 0.25 M glycine-HCl (pH 2.8) at 4°C. Cells are then frozen in 1 ml of 10 mM Tris-HCl plus 2.5M EDTA (pH 7.6) and stored at -20°C. Cells are thawed, scraped with buffer and incubated for 15 minutes. The cells are then sonicated. The cellular homogenate is centrifuged at 80,000Xg for 1 hour. The supernatant is

discarded, and the pellet is rinsed three times with 50 mM Tris-HCl (pH 7.4) making sure not to disrupt the pellet. The pellet is resuspended in Tris buffer and stored at -80°C until assayed. At the time of assay, the crude membrane is thawed and diluted with 50 mM Tris-HCl with 5mM $MnCL_2$, 0.1 mg/ml BSA, pH 7.4 to achieve approximately 100μg of protein per 100μl of solution. The crude membrane solution is then incubated in 12 x 27 mm polysterene test tubes for 36 hours at 4°C, with gentle agitation, with ^{3}H-OT (1nM)± cold OT (1μM) in duplicate or triplicate as follows: 100 μl crude membrane preparation, 100μl 3 nM ^{3}H-OT and 100 μl 3μM cold OT or plain Tris-HCl, pH 7.4. After incubation is complete, samples are filtered over Whatman GF/F glass filters using the Millipore vacuum manifold. The filters are rinsed four times with 1 ml of Tris-HCl, pH 7.4, then placed in scintillation vials with scintillation fluid and left overnight. Disintegrations per minute (DPM) are determined for 5 minutes. Specific binding will be determined by subtracting nonspecific binding from total binding values.

Protein Assay

Total cell protein is sampled in 50 μls aliquots from the solubilized cells. The concentration of protein was determined by the method of Lowry *et al.* (33). Specific binding is normalized for total cell protein, by dividing specific binding by total protein, resulting in specific binding in femtomoles OT bound per milligram of protein.

Statistical Analysis

Statistical analysis of all results was as follows. A mean number of OT binding sites for each group was determined. Nonspecific and Total Binding values were measured in triplicate. All statistical tests were generated by the JMP v. 5 The Statistical Discovery Software (34). The following non-parametric tests were performed: the Tukey-Kramer test determined if any group differed significantly from another, Dunnett's Method specifically tested if the treatment groups differed from control, and the Wilcoxon/Kruskal-Wallis was an additional test that also determined significant differences between means.

Results

Experiment A

Figures 1[1], 2, and 3 show the data from the three cultures in which experimental design A was used. The graphs show specific binding of ^{3}H-OT in femtomoles/microgram total protein (fmol/μg protein) for each treatment group. Specific binding was determined in triplicate as previously described as protocol 1. The figures show there was no consistent trend observed in the three cultures. The Tukey-Kramer[2] test in Tables 1, 2, and 3 showed that the means of the groups were not significantly different from each other at the 0.05 alpha level. The Dunnett's method confirmed these results and showed that the treatment groups were not significantly different from control at the 0.05 alpha level. The Wilcoxon/Kruskal-Wallis Test failed to reject the null hypothesis that there was no significant difference between groups at the 0.05 alpha level.

Experiment B

Figures 4, 5, and 6 and Tables 4, 5, and 6 show data from three cultures in which experimental design B was used. The graphs show specific binding of ^{3}H-OT in fmol/μg protein for each treatment group. Specific binding was determined in triplicate as previously described as protocol 1. The Figures 4, 5, and 6 show there was no consistent trend observed overall in the three cultures. In Table 4 the Tukey-Kramer test revealed that there was a significant difference between the treatment

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1 All figures and tables are reported in the appendix section.

2 Negative binding values were reported, however a negative specific binding value indicated no detectable specific binding.

groups. Levels not connected by same the letter are significantly different from each other at the 0.05 alpha level. The control group had significantly more specific binding of OT than the E_2 10^{-8}M and control/P_4 (15 ng/ml) treatment groups. The Dunnett's Method revealed that the control group specific binding was significantly different than all treatment groups. Figure 4 shows that the treatment groups appeared to have significantly lower binding of ^{3}H-OT binding than the control group. The Wilcoxon/Kruskal-Wallis non-parametric test rejected the null hypothesis. The groups were significantly different at the 0.05 alpha level. The Tukey-Kramer test in Tables 5 and 6 showed that the means of the groups were not significantly different from each other at the 0.05 alpha level. The Dunnett's method confirmed these results and showed that the treatment groups were not significantly different from control at the 0.05 alpha level. The Wilcoxon/Kruskal-Wallis Test failed to reject the null hypothesis that there was no significant difference between groups at the 0.05 alpha level.

Experiment C

Figures 7, 8, 9, and 10, and Tables 7, 8, 9, 10 show data from four cultures in which experimental design B was used. The graphs show specific binding of ^{3}H-OT in fmol/μg protein for each treatment group. Specific binding was determined in duplicate as previously described as protocol 2. Cultures were treated for a total of 96 hours with media changes occurring every 48 hours. There were four treatment groups used for each experiment: during the initial 48 hours, cultures were treated

with control media, or P_4 15 ng/ml and E_2 10^{-9}M administered concurrently. During the last 48 hours the groups were treated as follows: control media, E_2 10^{-9}M, and E_2 10^{-9}M respectively. Statistical analysis was as follows. Figure 7 shows that P_4 15 ng/ml treatment group appears to have more specific binding of ^{3}H-OT than the group that received P_4 15 ng/ml and E_2 10^{-9}M initially. Table 7 confirms that P_4 15 ng/ml treatment group had significantly more specific binding than the P_4 15 ng/ml + E_2 10^{-9}M group. The Dunnett's Method analysis showed that the P_4 15 ng/ml group and the P_4 15 ng/ml + E_2 10^{-9}M treatment group were significantly different than the control group. However it should be noted that (see Table 7) the control group had a negative mean, which again suggests no detectable binding occurred in the cultures. The Wilcoxon/Kruskal-Wallis test rejected the null hypothesis at 0.05 alpha level. Figure 8 results were similar to those reported by Figure 4, in which the treatment groups appear to have less binding as compared with the control group. Table 8 shows the control group had significantly more binding of OT than the treatment groups. The Dunnett's Method analysis confirmed these results. All treatment groups were significantly different from control at the 0.05 alpha. The Wilcoxon/Kruskal-Wallis test rejected the null hypothesis at the 0.05 alpha level. Figure 9 results were similar to those reported by Figure 7, except that both P_4 15 ng/ml and the E_2 appeared to have more binding than the treatment group in which P_4 15 ng/ml + E_2 10^{-9} M were administered concurrently. Table 9 reveals that there was no significant difference amongst groups with positive binding values. The Dunnett's Method revealed that all treatment groups were significantly different from the control group. However it

must be noted that the control group had negative mean average. The Wilcoxon/Kruskal-Wallis test rejected the null hypothesis at the 0.05 alpha level. Figure 10 had relatively low amounts of specific binding. The P_4+E_2 10^{-9}M was the only treatment group that had detectable binding occur. Table 10 shows that the control group had significantly more specific binding of OT than all the steroid treated groups. The Dunnett's Method also confirmed that control group is significantly different than all steroid treated groups. The Wilcoxon/Kruskal-Wallis test rejected the null hypothesis.

Discussion

The data for this study are presented chronologically in order to explain the mode of thought and relative comparative studies, followed by an overall analysis of this study.

The purpose of experiment A was to evaluate the experimental design and to determine if any trends were present. Although the statistical tests revealed no significant differences between the groups, Figure 1 showed that the E_2 treatments seemed to increase the amount of binding occurring, but not statistically significant. Hypothesizing that E_2 10^{-8} was not optimal for OT binding; in experiment B the concentrations of E_2 were varied. In addition, a progesterone pretreatment protocol was added to mimic the environment that occurs *in vivo* prior to labor in the uterine environment. Experiments by Edgerton *et al.* revealed that the uterine secretion of prostaglandin $F_{2\alpha}$ in response to OT in sows increased in response to the concurrent administration of P_4 and E_2 (35). Similar experiments in the ewe by Lau *et al.* also suggested that OTR expression increased due to the withdrawal of P_4 (36). Figures 4, 5, and 6 and Tables 4, 5, and 6 reveal that there was no consistent trend presented throughout the experiments. All three cultures failed to duplicate the results found in the previously mentioned studies.

In designing experiment C, the goal was to increase the amount of OTRs available for binding and standardize the experiments so they could be analyzed together. Experiments by Adachi *et al.* revealed that OTRs once expressed might disappear from the cell surface in the presence of exogenous OT, due to OTR

internalization (33). Consequently, cell cultures were sonicated in order to expose the internalized OTRs. The results for experiment C were again mixed, with no trend seen in each experiment. Figures 7, 8, 9, and 10 actually had less specific binding than all of our previous experiments, so the attempt to increase the amount of OTR's available for binding was unsuccessful.

In Figure 8 and 10 the control group had significantly more binding than the treatment groups. However in Figure 7 and 9, both P_4 and E_2 treatment groups had more OTR expression.

In analyzing this study in its entirety, there are four hypotheses that might explain the varied results garnered in this study.

1. OTR expression *in vitro* is dependent upon the estrous stage of the gilt at the time of slaughter.
2. Exposure to OT for extended period causes the disintegration of both the internal and external OTRs.
3. The *in vitro* model may be too restrictive to adequately reflect the complex nature of the uterus.
4. The high level of non-specific binding that occurred may mask the small differences in specific binding.

OTR expression in vitro is dependent upon the estrous stage of the gilt at the time of slaughter.

"The 'maternal recognition of pregnancy' can be defined as the method by which the conceptus prolongs the functional lifespan of the corpora lutea established after ovulation" (10). The uterine endometrium is the source of the luteolysin and in cyclic pigs the endometrium secretes prostaglandins in an endocrine direction, and that only through the actions of E_2 and P_4 is the corpus luteum maintained (3,10,12). However it is possible that the sensitivity of the endometrial tissue to estradiol and progesterone my have been significantly influenced by the stage of the oestrous cycle the gilt was in at the time of slaughter. Experiments by Geisert *et al.* showed that endometrial nuclear estrogen receptor sites increased from Day 0 to 12 and declined from Day 15 to 18, and were comparatively similar in sows and gilts (37). In obtaining the uteri from Hampton's Meats, it was difficult to ascertain the physiological status of pig in the slaughterhouse. Therefore the sensitivity of the tissue to E_2 may have been influenced significantly by the day of slaughter. Another item that needs to be addressed is the amount of OTRs present at the time of slaughter. OTR gene expression appears to be controlled at the transcriptional level, and some studies suggest sex steroids appear to have an indirect effect on that expression (29). These studies show that OTR gene expression is suppressed *in vivo* by interferon-[gamma] and in creating an *in vitro* model the suppression is removed. Thus causing any effects observed by sex steroid treatment to be obfuscated.

> "Despite the presence of steroid receptors in bovine endometrial cells, the level of OT receptor mRNA could neither be affected by progesterone or estradiol nor by a progesterone withdrawal protocol.

> The only factor that affected the OT receptor mRNA level was interferon- [gamma] (29)."

Consequently, the actual amount of OTR present may have been determined by the day of slaughter and less by the actual steroid treatment. Again this would explain the varied results garnered in our experiments.

Exposure to OT for extended period causes the disintegration of both the internal and external OTRs.

Experiments by *Adachi et al.* in humans revealed that when 1nM OT was added to myometrial monolayer cultures a decrease of 70% in surface OTRs was observed 24 hours after exposure (33). Prolonged exposure to OT leads to the disappearance of intracellular OTRs (33). Experiments by Hu *et al.* reported that OT was secreted by luminal epithelial cells, and that the OT secreted acted in an autocrine and/ or paracrine manner in pig endometrium (38). Consequently, OTR disintegration could have occurred throughout the entire culturing process. At this time there are no published studies that examine effect of prolonged exposure OT on OTR disintegration or internalization in pigs.

The in vitro model may be too restrictive to adequately reflect the complex nature of the uterus.

In reviewing the literature currently available, it is readily apparent that the majority of the studies being conducted are *in vivo* models. One simple explanation to the varied results could be that the series of biological events necessary to trigger

adequate OTR expression may be more complex than an *in vitro* model (at this time) can sufficiently simulate. Cofactors, alternate pathways of control, or some other unknown protein could all be present *in vivo* models and may be necessary for proper analysis.

The high level of non-specific binding that occurred may mask the small differences in specific binding.

The majority of the cultures in this study reported negative specific binding values for at least on group. One obstacle inherent in the experimental design was the use ^{3}H-OT, which has a low specific activity. As a result of its relative low specific activity, millimole quantities (i.e. pharmalogical amounts) were used in the assay. This issue combined with the fact that there exists some cross reactivity with other receptors caused our non-specific binding numbers to be exceedingly high. Figures 7, 8, 9, and 10 illustrate this idea. In exposing internal receptors during the sonication process, actually reduced the amount of OTR expression observed. Future experiments should try using ^{125}I-OT or ^{125}I-OT analog.

In conclusion, the effect of estrogen and progesterone on OTR expression could not be sufficiently determined by our *in vivo* model. Further study is warranted, the development of an effective *in vivo* model is critical to the full understanding of parturition in swine.

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Appendix
Figures

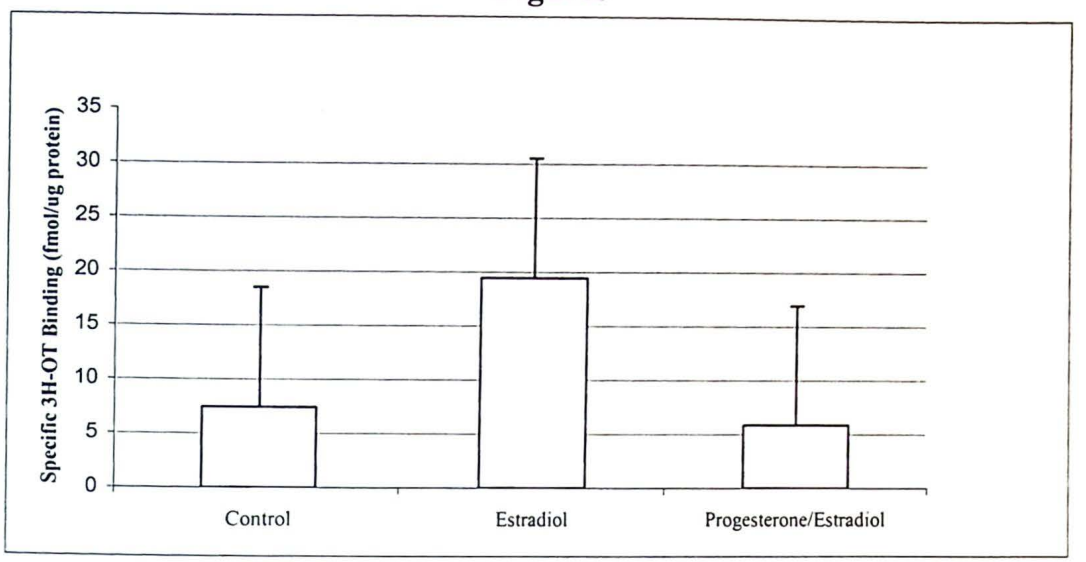

Figure 1. Specific binding of ^{3}H-OT to surface of luminal endometrial cells grown to confluence in vitro. (Mean+SEM, n=3). Cultures were treated for 48 hours with control, E_2 (10^{-8}M), and P_4 (15ng/ml). After 48 hours media were removed and replaced with control, E_2 (10^{-8}M), and E_2 (10^{-8}M) respectively.

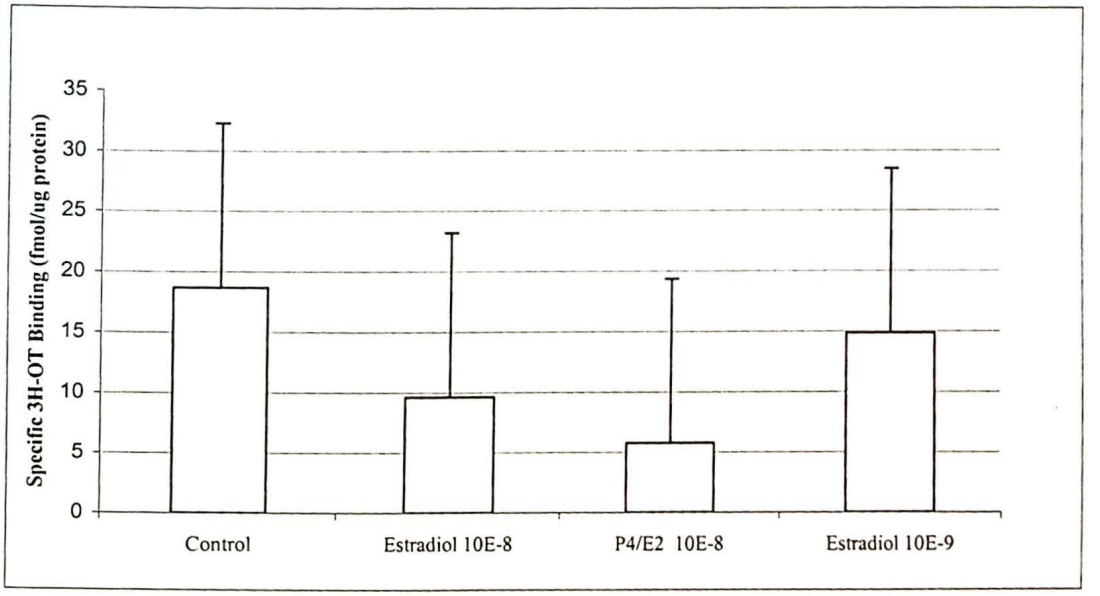

Figure 2. Specific binding of ^{3}H-OT to surface of luminal endometrial cells grown to confluence in vitro. (Mean+SEM, n=3). Cultures were treated for 48 hours with control, E_2 (10^{-8}M), E_2 (10^{-9}M) and P_4 (15ng/ml). After 48 hours media were removed and replaced with control, E_2 (10^{-8}M), E_2 (10^{-9}) and E_2 (10^{-8}M) respectively.

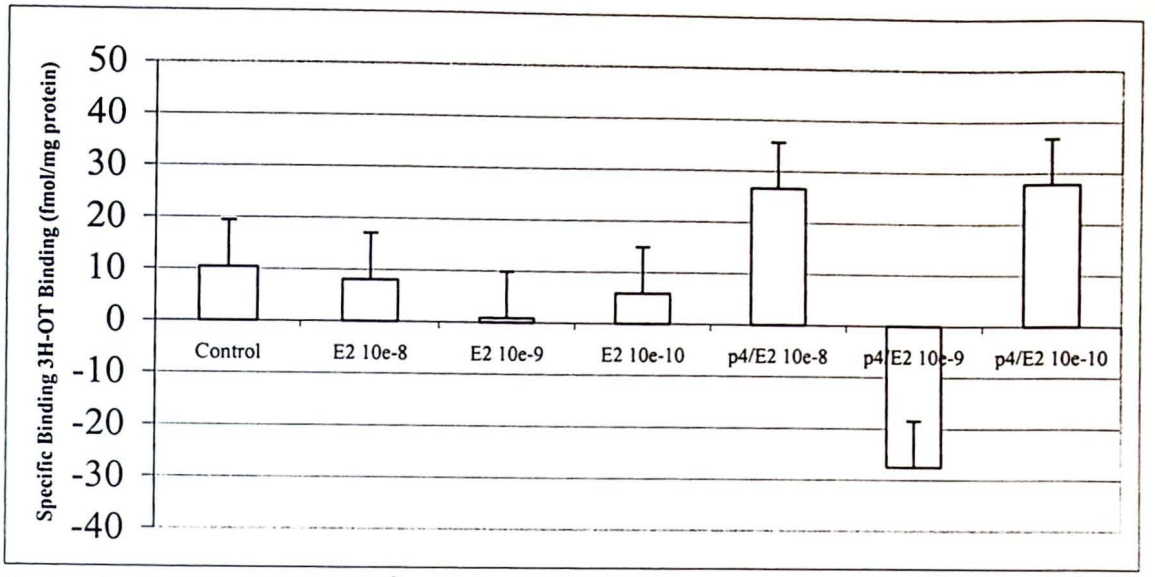

Figure 3. Specific binding of ^{3}H-OT to surface of luminal endometrial cells grown to confluence in vitro. (Mean+SEM, n=4). Cultures were treated for 48 hours with control, E_2 (10^{-8}M), E_2 (10^{-9}M), E_2 (10^{-10}) or P_4 (15ng/ml). After 48 hours media were removed and replaced with control, E2 (10^{-8}M), E_2 (10^{-9}) or E2 (10^{-10}) respectively.

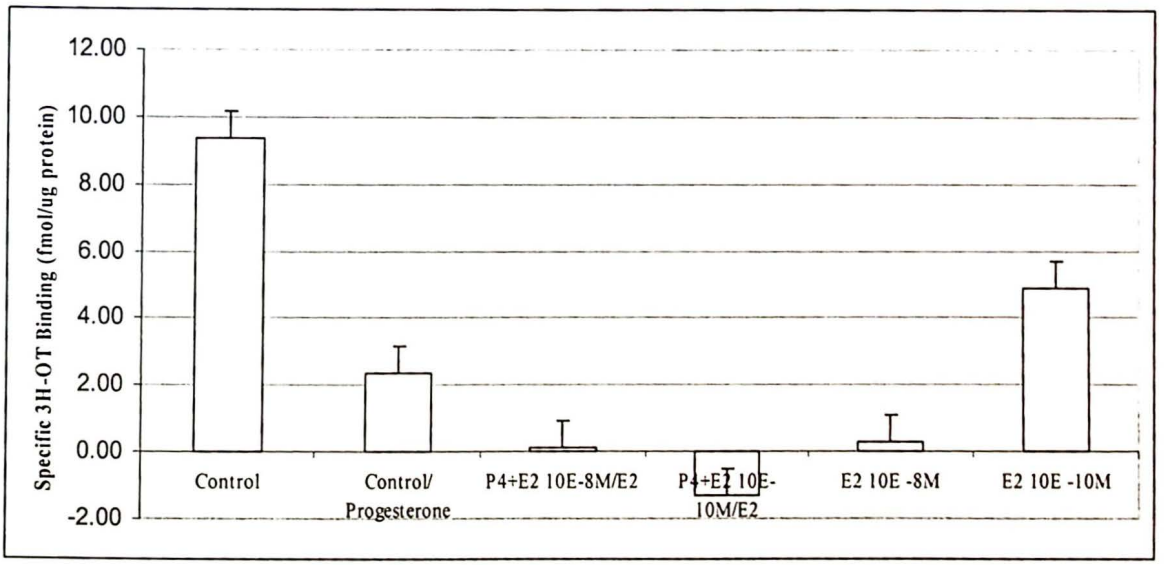

Figure 4. Specific binding of ^{3}H-OT to surface of luminal endometrial cells grown to confluence in vitro. (Mean+SEM, n=3). Cultures were treated for 48 hours with control, control, P_4 (15ng/ml)+ E_2 (10^{-8}M), P_4 (15ng/ml)+ E_2 (10^{-10}M), or E_2 (10^{-8}M) and E_2 (10^{-10}M). After 48 hours media were removed and replaced with control, P_4 (15ng/ml), E_2 (10^{-8}M), or E_2 (10^{-10}M) respectively.

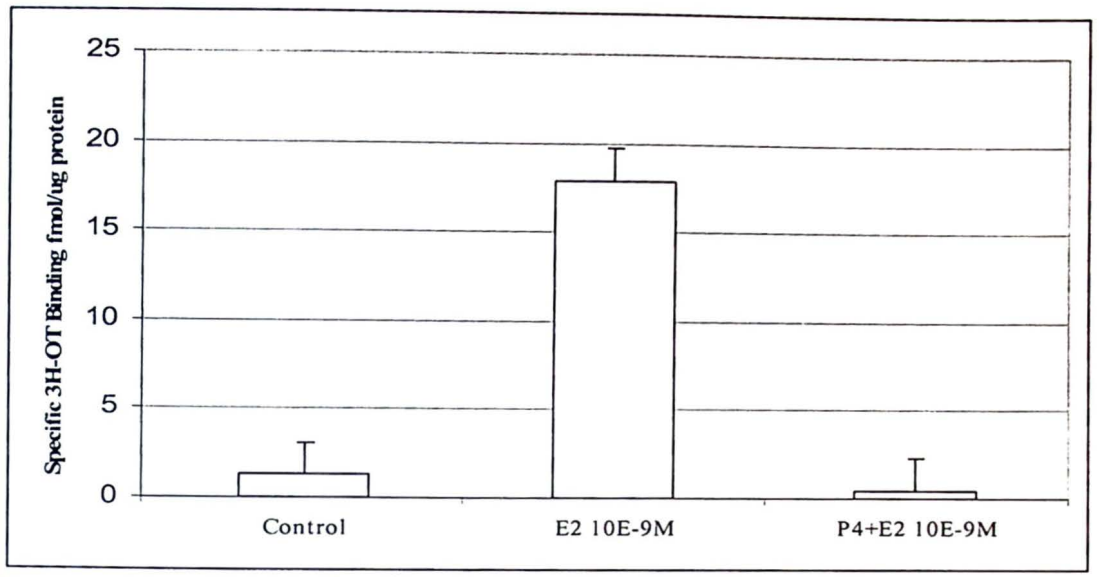

Figure 5. Specific binding of ^{3}H-OT to surface of luminal endometrial cells grown to confluence in vitro. (Mean+SEM, n=3). Cultures were treated for 48 hours with control, or E_2 (10^{-9}M) or P_4 (15ng/ml)+ E_2 (10^{-9}M). After 48 hours media were removed and replaced with control, E_2 (10^{-9}M), or E_2 (10^{-9}M) respectively.

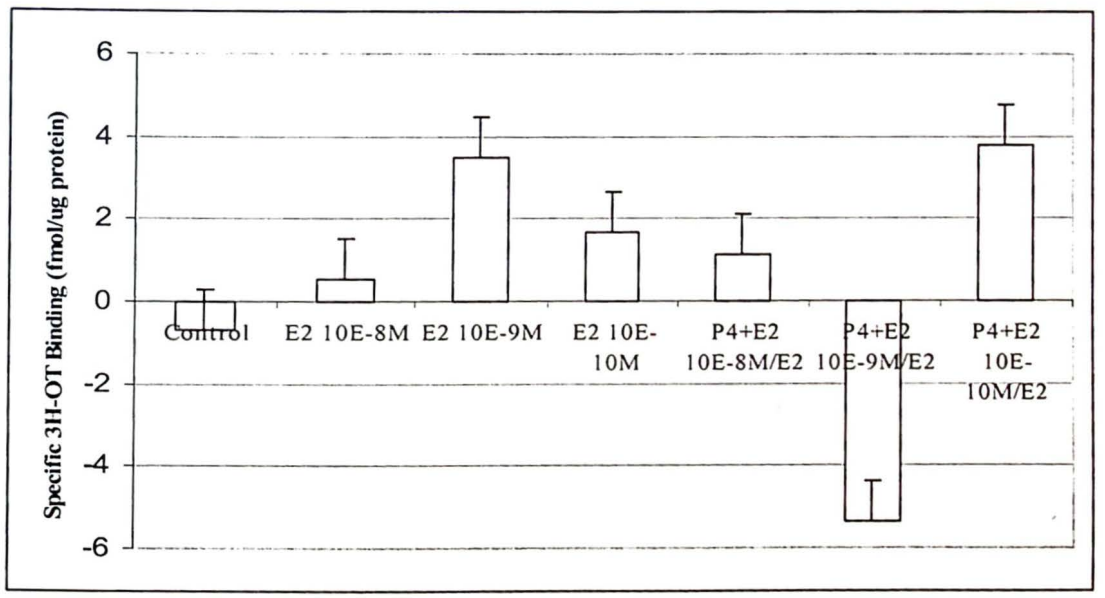

Figure 6. Specific binding of ^{3}H-OT to surface of luminal endometrial cells grown to confluence in vitro. (Mean+SEM, n=3). Cultures were treated for 48 hours with control, E_2 (10^{-8}M), E_2 (10^{-9}M), E_2 (10^{-10}) or P_4 (15ng/ml) + E_2 (10^{-8}M), E_2 (10^{-9}M), or E_2 (10^{-10}). After 48 hours media were removed and replaced with control, E2 (10^{-8}M), E_2 (10^{-9}) or E2 (10^{-10}) respectively.

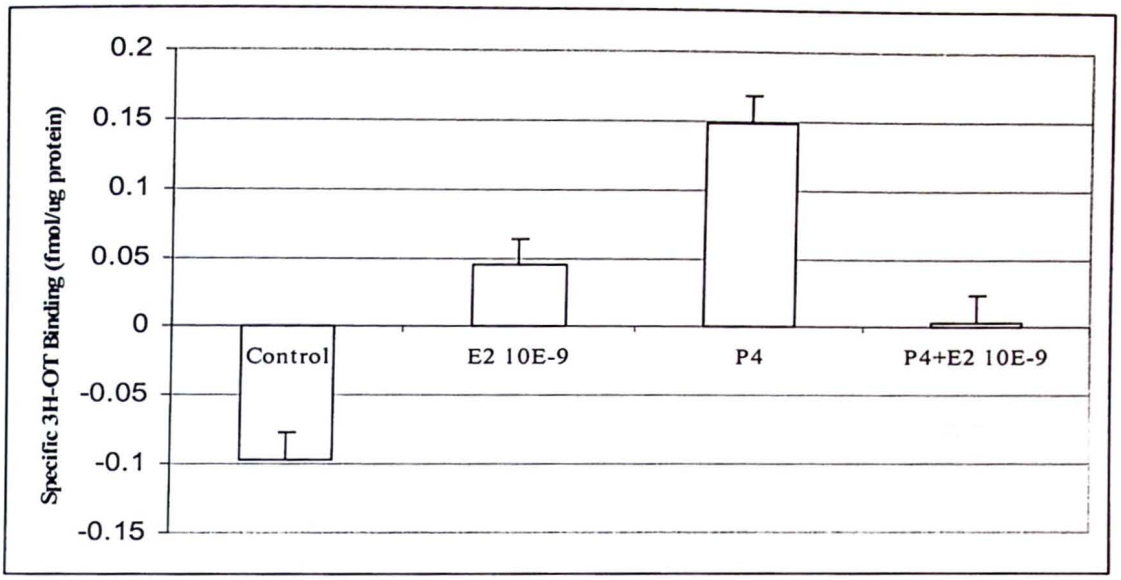

Figure 7. Specific binding of ^{3}H-OT to both surface and internal OTR's of luminal endometrial cells grown to confluence in vitro. (Mean+SEM, n=2). Cultures were treated for 48 hours with control, or P_4 (15ng/ml)+ E_2 (10^{-9}M). After 48 hours media were removed and replaced with control, E_2 (10^{-9}M), P_4 (15ng/ml) or E_2 (10^{-9}M) respectively.

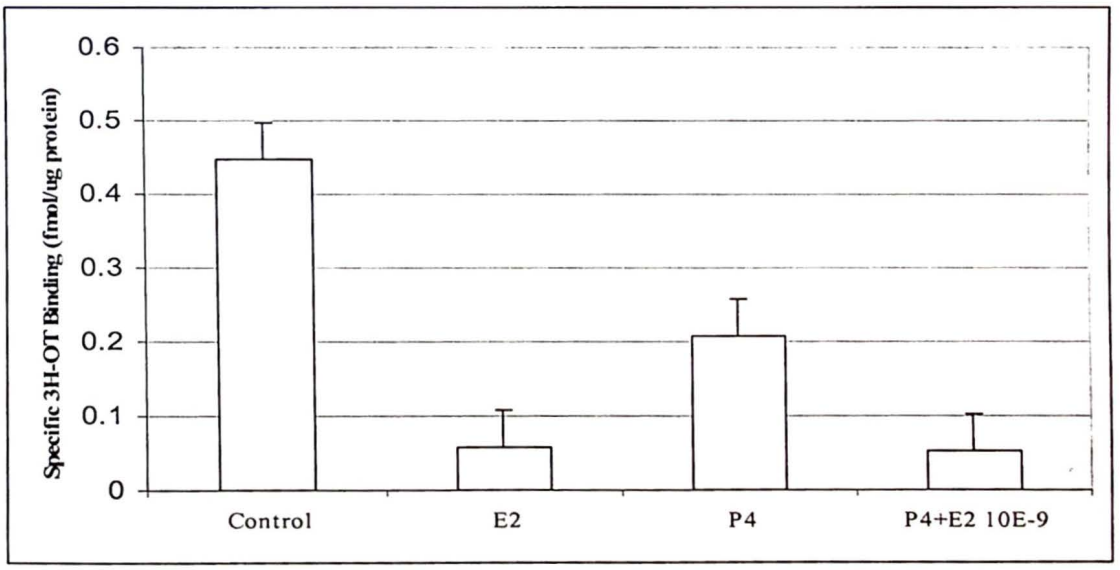

Figure 8. Specific binding of ^{3}H-OT to both surface and internal OTR's of luminal endometrial cells grown to confluence in vitro. (Mean+SEM, n=2). Cultures were treated for 48 hours with control, or P_4 (15ng/ml)+ E_2 (10^{-9}M). After 48 hours media were removed and replaced with control, E_2 (10^{-9}M), P_4 (15ng/ml) or E_2 (10^{-9}M) respectively.

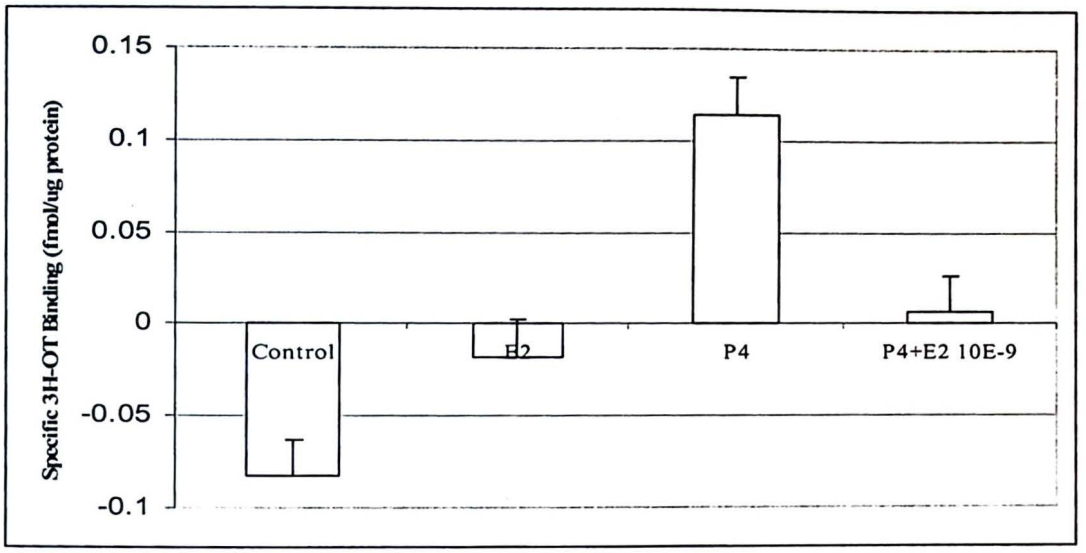

Figure 9. Specific binding of ^{3}H-OT to both surface and internal OTR's of luminal endometrial cells grown to confluence in vitro. (Mean+SEM, n=2). Cultures were treated for 48 hours with control, or P_4 (15ng/ml)+ E_2 (10^{-9}M). After 48 hours media were removed and replaced with control, E_2 (10^{-9}M), P_4 (15ng/ml) or E_2 (10^{-9}M) respectively.

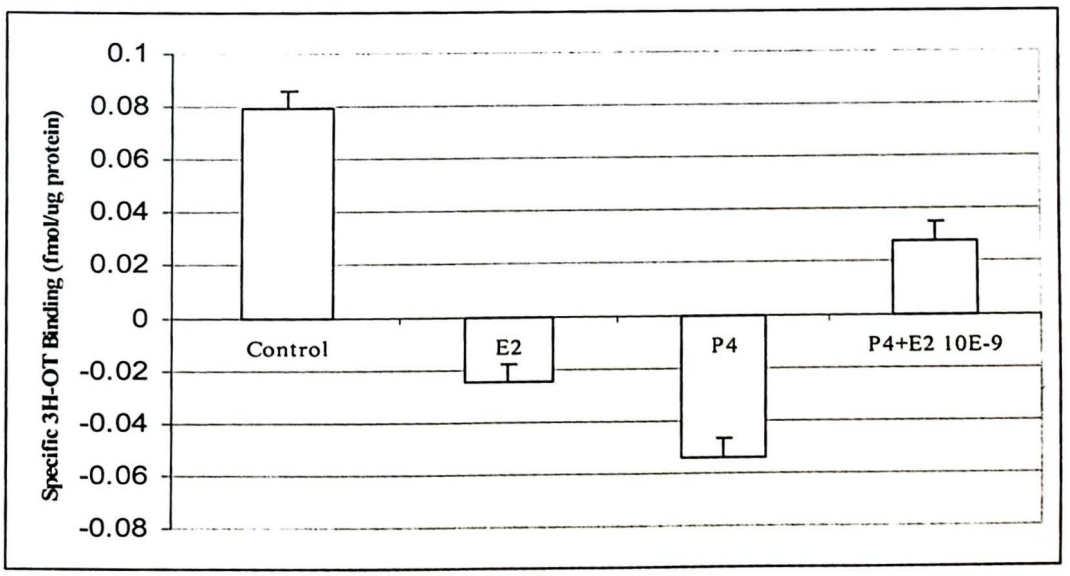

Figure 10. Specific binding of ^{3}H-OT to both surface and internal OTR's of luminal endometrial cells grown to confluence in vitro. (Mean+SEM, n=2). Cultures were treated for 48 hours with control, or P_4 (15ng/ml)+ E_2 (10^{-9}M). After 48 hours media were removed and replaced with control, E_2 (10^{-9}M), P_4 (15ng/ml) or E_2 (10^{-9}M) respectively.

Appendix 2
Tables

Table 1. Porcine Endometrial Culture (Pec) 15 Tukey-Kramer Mean Analysis

LEVEL		MEAN
E_2 (10e-9)	A	19.46667
Control	A	7.436667
P_4/E_2	A	6.343333
*Levels not connected by the same letter are significantly different from each other at alpha .05 level.		

Source: Data generated by JMP.
SAS Institute. *JMP. The Statistical Discovery Software, Version 5.* Cary, North Carolina, 2002.

Table 2. Pec 16 Tukey-Kramer Mean Analysis

LEVEL		MEAN
Control	A	18.696667
E2 (10e-9)	A	14.880000
E_2 (10e-8)	A	9.616667
P_4/E_2 10e-8	A	5.773333
*Levels not connected by the same letter are significantly different from each other at alpha .05 level.		

Source: Data generated by JMP.
SAS Institute. *JMP. The Statistical Discovery Software, Version 5.* Cary, North Carolina, 2002.

Table 3. Pec 24 Tukey-Kramer Mean Analysis

LEVEL			MEAN
P_4+E_2 (10e-10)	A		27.82250
P_4/E_2 (10e-8)	A		26.64500
Control	A	B	10.34667
E_2 (10e-8)	A	B	8.05250
E_2 (10e-10)	A	B	5.86750
E_2 (10e-9)	A	B	.87750
P_4/E_2 10e-9		B	-23.29750
*Levels not connected by the same letter are significantly different from each other at alpha .05 level.			

Source: Data generated by JMP.
SAS Institute. *JMP. The Statistical Discovery Software, Version 5.* Cary, North Carolina, 2002.

Table 4. Pec 29 Tukey-Kramer Mean Analysis

LEVEL					MEAN
Control	A				9.38
E_2 (10e-10)	A	B			4.86
Control/P_4		B	C		2.38
E_2 (10e-8)		B	C	D	.266
P_4+E_2 (10e-10)			C	D	-1.31
P_4+E_2 (10e-8)				D	-3.81

*Levels not connected by the same letter are significantly different from each other at the alpha .05 level.

Source: Data generated by JMP.

SAS Institute. *JMP. The Statistical Discovery Software, Version 5.* Cary, North Carolina, 2002.

Table 5. Pec 30 Tukey-Kramer Mean Analysis

LEVEL		MEAN
E_2 (10e-9)	A	8.1950000
Control	A	1.2666667
P_4+E_2 (10e-9)	A	.4520000

*Levels not connected by the same letter are significantly different from each other at alpha .05 level.

Source: Data generated by JMP.

SAS Institute. *JMP. The Statistical Discovery Software, Version 5.* Cary, North Carolina, 2002.

Table 6. Pec 31 Tukey-Kramer Mean Analysis

LEVEL			MEAN
P_4+E_2 (10e-10)/E_2	A		3.763
E_2 (10e-9)	A		3.483
E_2 (10e-10)	A	B	1.673
P_4+E_2 (10e-8)/E_2	A	B	1.110
Control	A	B	-.7100
E_2 (10e-8)	A	B	-1.413
P_4+E_2 (10e-9)E_2		B	-3.893

*Levels not connected by the same letter are significantly different from each other at alpha .05 level.

Source: Data generated by JMP.

SAS Institute. *JMP. The Statistical Discovery Software, Version 5.* Cary, North Carolina, 2002.

Table 7. Pec 43 Tukey-Kramer Mean Analysis

LEVEL			MEAN
P_4	A		.1483
E_2	A	B	.0444
P_4+E_2		B	.0032
Control		B	-.0975
*Levels not connected by the same letter are significantly different from each other at alpha .05 level.			

Source: Data generated by JMP.

SAS Institute. *JMP. The Statistical Discovery Software, Version 5.* Cary, North Carolina, 2002.

Table 8. Pec 44 Tukey-Kramer Mean Analysis

LEVEL			MEAN
Control	A		.4480
P_4		B	.2068
E_2		B	.0582
P_4+E_2		B	.0539
*Levels not connected by the same letter are significantly different from each other at alpha .05 level.			

Source: Data generated by JMP.

SAS Institute. *JMP. The Statistical Discovery Software, Version 5.* Cary, North Carolina, 2002.

Table 9. Pec 45 Tukey-Kramer Mean Analysis

LEVEL			MEAN
E_2	A		.1205
P_4	A		.1141
P_4+E_2	A	B	.0062
Control		B	-.0827
*Levels not connected by the same letter are significantly different from each other at alpha .05 level.			

Source: Data generated by JMP.

SAS Institute. *JMP. The Statistical Discovery Software, Version 5.* Cary, North Carolina, 2002.

Table 10. Pec 46 Tukey-Kramer Mean Analysis

LEVEL				MEAN
Control	A			.0792
P_4+E_2		B		.0280
E_2			C	-.0248
P_4			C	-.0540
*Levels not connected by the same letter are significantly different from each other at the alpha .05 level.				

Source: Data generated by JMP.

SAS Institute. *JMP. The Statistical Discovery Software, Version 5.* Cary, North Carolina, 2002.

VITA

Jamie Eugene Mells was born in Fort Lauderdale, Florida on September 27, 1973. As part of a military family he attended numerous elementary schools in Florida, Virginia, Missouri, and in the country of Belgium. He graduated from Kaiserslautern American High School (Germany) in June, 1991. He received an Associates Degree in Liberal Arts from the University of Maryland at the Munich/Augsburg Germany Campus in May, 1993. He went on to receive a Bachelor of Science Degree in Biology, from Austin Peay State University, Tennessee, in May, 1996. He continued his education in Biology and in received a Master of Science degree in Biology in December 2004, also from Austin Peay State University.

Jamie is currently the Director of Minority Affairs at Tennessee Tech University, Cookeville.