INTRODUCTION

1.1 Bovine Estrous Cycle

The reproductive cycle of nonprimate mammals is referred to as the estrous cycle. In the cow, the estrous cycle is 21 days long with day 0 being designated as estrus. Estrus is the only phase during the estrous cycle when the cow is receptive to mating. Sperm deposited in the anterior vagina by a bull, if mating occurs, is transported by contractions of the reproductive tract and enters into the oviduct to await the arrival of the ovum. During estrus, which lasts 12-15 hours, there is release of gonadotropin releasing hormone (GnRH) from the hypothalamus. This hormone acts on the anterior pituitary to stimulate a surge release of luteinizing hormone (LH). Luteinizing hormone acts on mature ovarian follicles to cause ovulation, the release of an ovum. Fertilization occurs in the oviduct and the developing embryo enters the uterus where it will develop into a calf to be born 283 days later.

Following ovulation, the empty follicle begins to undergo angiogenesis. This production of blood vessels is necessary to form the corpus luteum (CL), a temporary endocrine gland (figure 1). By the time it is fully formed at approximately Day 6 to 8, the CL is made up of more than 50% endothelial cells, the cells that make up blood vessels, meaning that the CL contains more endothelial cells than any other organ in the body. The CL releases hormones, such as progesterone, that are necessary for the uterus to maintain an embryo. If fertilization does not occur, the CL begins to degenerate or regress on approximately Day 18. During its existence, the CL also produces and releases the hormone oxytocin.

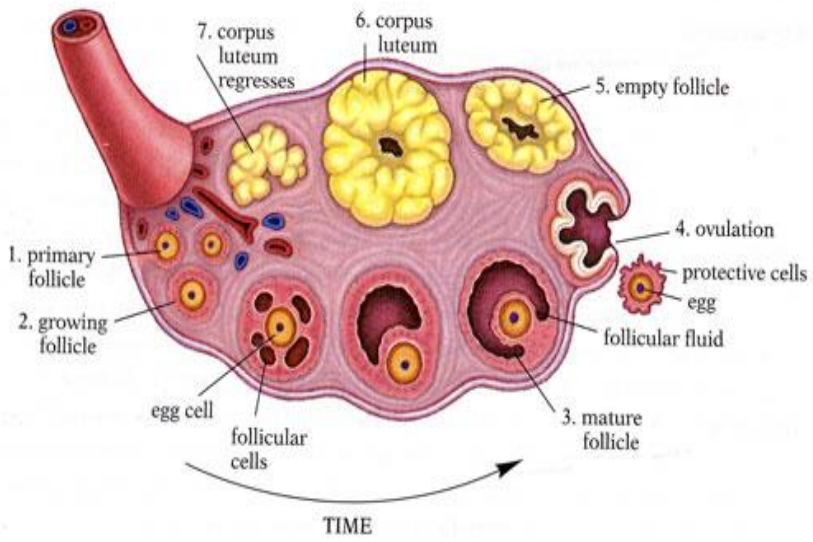

Figure 1. The growth and formation of a follicle and, subsequently, a corpus luteum in the ovary. (Cornell University, Biology 1105-1106 website)

Oxytocin is a hormone normally synthesized by neurons in the hypothalamous and stored and released from the posterior pituitary gland. It is known to cause uterine contractions during childbirth and is released upon stimulation of the mammary glands by suckling or milking to assist in milk let-down. However, OT is also secreted by the bovine CL in large amounts. Secretion of OT begins following ovulation and continues until the CL is fully formed around Day 7 or 8 of the estrous cycle, at which point levels of the hormone decrease (figure 2). It was hypothesized that oxytocin produced by the CL may act in a paracrine fashion to enhance luteal angiogenesis. An experiment was conducted to determine whether luteal endothelial cells are endowed with OT receptors.

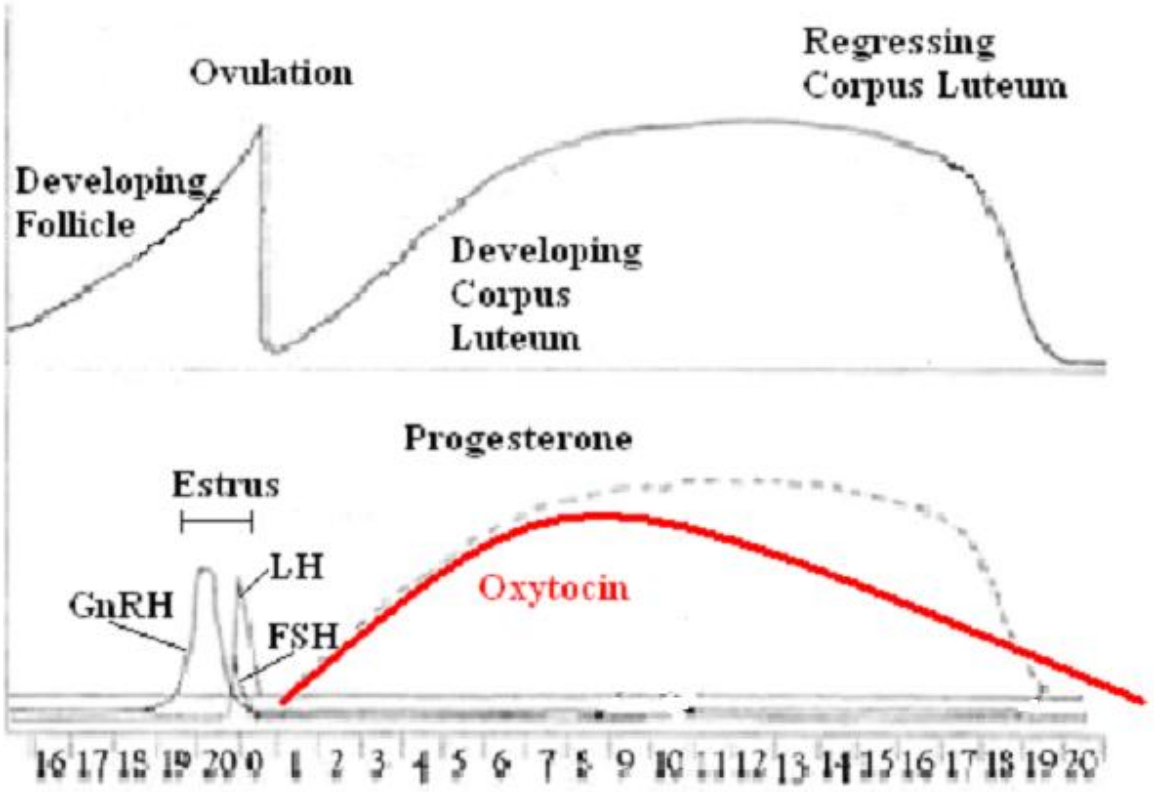

Figure 2. The physical and hormonal changes during the bovine estrous cycle. (Ibrihim and Eley, 2009)

IMPORTANCE

Growth of the CL in humans is similar to that in the cow- in humans, the menstrual cycle lasts 28 days with the CL forming from an follicle following ovulation and degenerating if fertilization of the ovum does not occur. Further research into the development of the bovine CL is appealing for two primary reasons: (1) The CL is necessary for the growth of an embryo. Anti-angiogenesis of the CL would subsequently prohibit the release of progesterone, thereby inhibiting the uterus from maintaining a pregnancy; and (2) With exception to the case of injury, in the adult, angiogenesis and the production of blood vessels only occurs in females during the reproductive cycle and in both sexes during the

growth of tumors. Tumors are also made up of roughly 50% endothelial cells and their growth is almost identical to that of the CL. If OT is what is causing angiogenesis to occur in the CL, it is possible that it is also causing angiogenesis to occur in tumors. If that is the case and there can be found a way to block those OT receptors, then it might be possible to prevent the growth of tumors.

LITERATURE REVIEW

Many researchers have investigated the role of oxytocin (OT) in the proliferation of endothelial cells. Many early studies exist that contradict each other as to the type of OT receptor that exists in endothelial cells and what their precise action is and the result of the activation of such receptors. Thibonnier *et al*. (1999) suggested that the confusion was due to OT agonists and antagonists used in the studies losing their selectivity and specificity when used at high concentrations. Furthermore, Thibonnier *et al*.(1999) clearly characterized the nature of the OT receptor. First, ECV304 EC, a spontaneously transformed immortal endothelial cell line that had been established using the vein of a normal human umbilical cord, was found to possess a single class of receptors belonging to the OT subtype. The nucleotide sequence amplified with degenerate primers matched the OT receptor sequence similar to the one present in uterine and mammary glands. Furthermore, stimulation of these endothelial OT receptors was found to lead to the mobilization of intracellular calcium and various kinases, which also seemed to be instrumental in the activation of the nitric oxide (NO) pathway. It was deduced that this activation of the NO pathway is related to the supported hypothesis that administered OT produced a peripheral vasodilating effect (Hendricks and Brenner, 1970). This theory was

established when the hypotensive effect of large doses of OT was found in correlation to the severe hypotensive episodes reported in hemorrhagic postpartum situations. But previous studies have also found NO to have conflicting functions, with both angiogenic and antiangiogenic properties. Thibonnier *et al*. (1999) suggests that these differences are the result of the use of different cell lines and cells from different vascular beds, as well as differing experimental conditions. While Thibonnier *et al.* (1999) did not come to any definitive conclusions as to the exact cause of the activation of the OT receptors on endothelial cells, the study suggests that the receptor operates in a calcium dependent and possibly a NO dependent fashion to activate a vasodilatory pathway that stimulates cell proliferation.

A second study by Cattaneo *et al*. (2007) attempted to further define the role of the oxytocin receptors on human umbilical vein endothelial cells (HUVEC). The study found that OT stimulated HUVEC migration and invasion, which further suggests that OT may contribute to angiogenesis *in vivo*. Cattaneo *et al.* (2007) findings suggest that OT is equally influential in angiogenesis as vascular endothelial growth factor (VEGF), a hormone previously thought to act independently to initiate endothelial cell proliferation. The study further found that there are two types of OT receptors coupled to G-proteins, G_q and G_i, which explains the contradictory findings in previous studies relative to the role of OT in regulating angiogenesis.

The following study attempted to further define the role of oxytocin released by bovine luteal cells during the growth of the corpus luteum, a time marked by significant angiogenesis requiring the proliferation and migration of endothelial cells.

HYPOTHESIS

Bovine luteal oxytocin binds locally to oxytocin receptors present on the proliferating endothelial cells within the vascular system of the developing corpus luteum.

MATERIALS AND METHODS

1.1 Removal of Corpus Luteum on Day 8 of Estrous Cycle

Five heifers were checked for estrus twice each day (morning and evening) using a vasectomized bull. Estrus was determined when intromission occurred. Pre and post-estrus were determined when mounting occurred without intromission. A medium was prepared by dissolving 2 – 1 L packets of Ham's F-12 (GIBCO) in 1.8 L of distilled water then adding 2.35 g sodium bicarbonate and 11.92 g of Hepes. The medium was then brought to 2 liters and filter sterilized using a 0.2 μm filter. The pH was checked to ensure the medium was at 7.35 pH. Eight days following the second observed estrus the corpus luteum was removed from the ovary via a trans-vaginal lutectomy. The tissue was then taken back to the laboratory in medium on ice to preserve the cells.

2.1 Separation of Luteal Endothelial Cells with Tosyl-Activated Magnetic Beads

The corpus luteum was weighed and diced into small (1 mm) cubes and then placed into fresh Ham's F-12 medium containing 0.5% bovine serum albumin (BSA). A small portion of tissue was saved and separated on ice in medium for immunohistochemistry (described below). Two samples each of 9.09 mg of DNAse with activity 550 U/mg and collagenase (equation 1) were weighed out and refrigerated until use. The remaining tissue was heated in a water bath at 35°C for 2 minutes. One sample

of each DNAse and collagenase were added and the mixture was stirred for 45 minutes at 35°C. The supernatant was transferred to centrifuge tubes and placed on ice and the procedure was repeated with the remaining tissue until it was completely digested. The supernatant was centrifuged at 146 *x g* for ten minutes at 4°C and the supernatant removed. Ten ml of Ham's F-12 medium with 0.1% BSA was added to each tube to break up the pellet and the sample was recentrifuged two more times at 88 *x g* and 65 *x g* respectively, for 10 minutes each at 4°C. The cells were resuspended in 1 ml of 0.1% BSA medium and filtered through a 53 micron nylon mesh cloth. A small sample was taken and diluted 1:10 with trypan blue to visualize the cells using a microscope. The mixture was vortexed quickly and allowed to sit for one minute before cells were counted using a hemocytometer in order to determine the total quantity of live cells in the sample.

Dynabeads M-450 Tosylactivated (Dynal Inc., Lake Success, NY), were used to separate the endothelial cells from other non-endothelial luteal cells. Dynabeads M-450 are tosylactivated magnetic beads which contain reactive sulphonyl esters that bind covalently to ligands when incubated with them. To prepare the beads, they were first resuspended in their packaging vial by vortexing for one minute. The volume to be used was then immediately added into a 15 ml tube with a cap. The tube was placed on top of a magnet for one minute. The magnetic beads were attracted to the magnet, and the supernatant was removed. Beads were resuspended in 3 ml of a 0.1 M borate buffer at pH 9.5 and mixed gently. Again the beads were placed in front of a magnet and the supernatant removed. Three ml of the 0.1 M borate buffer was then added. *Griffonia Bandeiraea Simplicifolia lectin 1* (BS-1), a sugar binding protein with an affinity for endothelial cells, was added to a 0.5 M borate solution in the amount of 0.15 mg/ml and

then filtered with a 53 micron mesh cloth. Equal amounts of beads and BS-1 stock were pipetted into a capped tube and placed on a shaker for 24 hours at room temperature. The mixture was then washed three times with 1 ml of phosphate buffered saline (PBS) + 1 % BSA for 10 minutes at room temperature on a rocking platform. The beads were washed one more time with 1 ml of PBS + 1% BSA for 30 minutes on the rocking platform. The bead mixture was then stored in the refrigerator at 4°C until use.

Tosylactivated magnetic beads covalently bound to BS-1 were added to the cell sample in a ratio of 20 beads per steroidogenic luteal cell and incubated for 25 minutes on a rocking platform at 4°C to allow the endothelial cells to bind to the beads (Christenson and Stouffer, 1996). For each experiment conducted, there were not enough tosylactivated magnetic beads to add at 20 beads per cell, so the entire amount of beads was added to the cell sample. This totaled to approximately 4×10^8 beads per run. The mixture was then placed in front of a magnet for 1 minute and the supernatant containing the BS-1 negative, non-endothelial cells was removed. The bead-endothelial cell sample was rinsed three times with 3 ml of PBS + 1% BSA to ensure the removal of all non-endothelial cells from the sample. Three ml of a 0.1 M fucose solution were then added and the mixture incubated on a rocking platform for 10 minutes in order to allow the endothelial cells to dissociate from the beads. The sample was again placed in front of a magnet for 1 minute and the supernatant containing the BS-1 positive, endothelial cells removed. The remaining beads were re-incubated with fucose three more times and the supernatant removed to guarantee endothelial cell separation. The supernatant was centrifuged for 8 minutes at 1127 *x g*. The pellet was resuspended in 2 ml of medium and

re-counted using the hemocytometer to ensure separation of endothelial cells, non-endothelial cells and beads.

2.2 Oxytocin Binding Radioreceptor Assay

The endothelial cells were analyzed for oxytocin receptors using a modified procedure from Hazzard and Stormshak (1997). Following counting of cells, both endothelial and nonendothelial cell samples were centrifuged for 5 minutes at 1127 *x g.* The pellet was resuspended in 5 ml of 1 M Tris-HCl at pH 7.4 (buffer #2) and vortexed for 30 seconds. The cells of each type were homogenized separately using a Dounce glass homogenizer (20 strokes each) in order to lyse them and centrifuged at 282 *x g* for 10 minutes at 4°C in order to separate out the nuclei. The supernatant containing the cell membranes was then concentrated by ultracentrifucation at 100,000 *x g* for 1 hour and 5 minutes at 0°C. The remaining pellets were gently rinsed 3 times and then resuspended in 0.5 ml of buffer #2. The samples were recentrifuged at 180 *x g* and the pellet was discarded.

The protein concentration of the cell membranes could then be determined using a spectrophotometer. A spectrophotometer produces light of a certain wavelength, and a photometer that measures the intensity of the light. Small cuvettes are filled with liquid and placed so that the light passes through the liquid and can be read by the photometer. When the cuvettes contain a colored liquid, it absorbs some of the light passing through. The absorbance can then be read by the photometer.

A bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL) was used to determine sample protein levels. Protein contains peptide bonds which have the ability to reduce Cu^{+2} to form Cu^{+1}. When BCA is added to protein, two of the BCA molecules chelate one Cu^{+1} cation, forming a purple color during the reaction. The depth of the

color differs depending on the amount of protein present and can be read in the spectrophotometer. Standards containing known amounts of protein were read on the machine first, in order to determine a plot of wavelength versus concentration of protein. The standard curve helped to determine the amount of protein present in the unknown samples of endothelial and non-endothelial cell plasma membranes.

When the amount of protein in each sample was determined, the higher concentration sample was diluted so that both endothelial and non-endothelial membrane samples were equal in concentration.

A 2 nM sample of [^{3}H]-Oxytocin with a specific activity of 46.3 Ci/mmol was prepared as well as 1.6 µM sample of cold oxytocin. The oxytocin was then added to the membrane samples as follows:

#1 2 tubes [^{3}H]-Oxytocin (Endothelial and Non-endothelial each)	#2 2 tubes Cold + [^{3}H]-Oxytocin (Endothelial and Non-endothelial each)
50 µL [^{3}H]-OT + 50 µL OT buffer + 100 µL cell membranes	50 µL [^{3}H]-OT + 50 µL Cold-OT + 100 µL cell membranes

The solutions were incubated at room temperature for 1 hour to allow binding. A diluting buffer was prepared earlier by combining 7.5 ml of 1M Tris-HCl at pH 7.4 and 1.5 ml of 2% sodium azide. Then 0.6 g BSA were added and the mixture was chilled overnight at 4°C. In the morning, 0.75504 g anhydrous manganese chloride (MW 125.84) were added and the buffer was brought to 300 ml with distilled water. When the samples were finished incubating, they were washed in the diluting buffer to remove unbound oxytocin.

The solutions were added to Ecolume (a scintillation cocktail), where the radioactive material in the unknown samples release beta particles that interact with the cocktail to form photons of light. The radioactivity was then measured using a liquid

scintillation counter (LSC), a commonly used technique for detecting and quantifying radioactivity via the energy of the photons, to determine the amount of [^{3}H]-oxytocin bound to the membranes in each sample in counts per minutes (CPM).

With only [^{3}H]-oxytocin present in the first sample, if oxytocin receptors were present in endothelial cells, then the hormone would bind to the receptors, as well as nonspecifically to the membrane surfaces. But with an almost 200 fold excess of cold versus [^{3}H]-oxytocin in the second sample, the cold oxytocin would overwhelm the receptors, and the [^{3}H]-oxytocin would only be able to bind nonspecifically to the membrane surfaces (figure 3). Therefore, the LSC reading would show greater CPM for the endothelial cell membrane samples with only [^{3}H]-oxytocin present. It was predicted that specific binding would not occur on the non-endothelial cells, therefore there would be no difference in the amount of [^{3}H]-oxytocin present in both samples of the non-endothelial cell membranes (figure 4).

a. b.

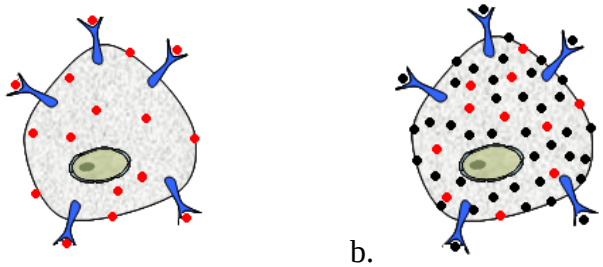

Figure 3. An example of the proposed binding of oxytocin to endothelial cells; a) a cell sample exposed to only [^{3}H]-oxytocin (red) and b) a cell sample exposed to both unlabeled oxytocin (black) and [^{3}H]-oxytocin.

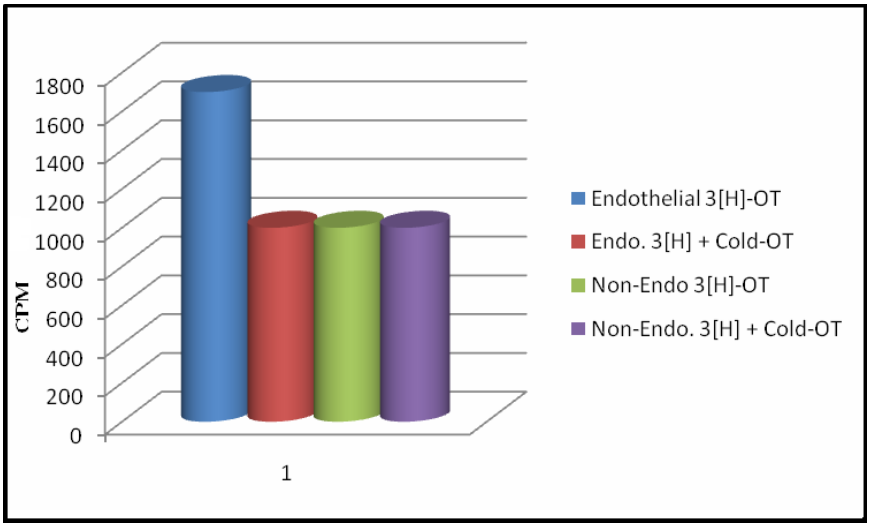

Figure 4. Predicted results given by LSC if oxytocin was binding specifically to corpus luteum endothelial cells- the sample containing endothelial cells and only [^{3}H]-oxytocin would have a greater amount of specifically bound ligand.

3.1 Immunohistochemistry Tissue Stabilization

Part of the luteal tissue was saved in Hams F-12 medium at 4°C for immunohistochemistry (IHC). The saved tissue was cut into approximately 3-4 mm thick cubes and placed into 0.5 ml Hanks Solution + 0.7% Hepes at pH 7.3. A 300 ml beaker of water was placed in the rear corner of a microwave oven and heated at full power for two minutes. The tissue was then placed into the microwave with the water and heated at the lowest possible setting for 7 seconds. The sample was immediately moved into cold PBS buffer + 10% sucrose. Tissue Tek II OCT (Miles Inc. Elkhart, IN) was used to make a small pond in a half inch square piece of aluminum foil. The tissue was blotted on a paper towel to remove excess moisture and then placed into the OCT pond until fully saturated.

Liquid propane was prepared by flowing propane gas into a volumetric flask chilled in liquid nitrogen. A small Dewar flask was half filled with the liquid nitrogen, into which was placed a 400 ml polypropylene beaker. The liquid propane was transferred into the chilled suspended beaker and stirred occasionally to prevent freezing. The foil containing OCT and tissue was then folded up and frozen as a block in the liquid propane. Once removed it was placed on a block of dry ice and stored in a foil envelope in the -80°C freezer to prevent thawing. The tissue samples were sent to the Oregon Health and Sciences University Primate Laboratory for the IHC to be completed.

3.2 Immunohistochemistry

A PAPF fixative was prepared by combining 75ml of 1.22% picric acid in water (PA) with 10 g paraformaldehyde (PF). The PAPF mixture was heated to 60ºC to dissolve the PF. The mixture was then cleared with 5N sodium hydroxide, cooled, filtered into a phosphate buffer and brought to a volume of 500 ml with water.

Fixed and frozen tissue samples were cut into 5-7 μm thick slices on a cryostat and mounted onto slides. The mounted tissues were then rinsed with PBS. A weighing tray filled with ice was put into the microwave (prepared the same as above) and the slide placed on top. The microwave was set for 2 seconds and the slide then immediately removed and immersed in a fixative of PAPF + 1.5% polyvinyl-pyrrolidone (PVP) at room temperature for 10 to 15 minutes. The slide was then rinsed twice in 85% ethanol + 1.5% PVP at 4°C for two minutes and then rinsed three times with PBS + 1.5% PVP at 4°C for 3 minutes each. The slides were washed again, twice with 0.37% glycine in PBS/PVP for 7 minutes and 3 times with PBS + 1.5% PVP at 4°C for 3 minutes each. The slides were placed in glucose oxidase and incubated for 45 minutes at room

temperature. Finally, the slides were rinsed three times with PBS + 1.5% PVP with 0.1% gelatin at 4°C for three minutes each and placed into a blocking serum for 20 minutes at 4°C. The slides were then drained off and a primary goat polyclonal antibody diluted at 5-26 µg/ml (Abnova, Taiwan) was added and incubated overnight. It was anticipated that this primary antibody would bind to the oxytocin receptors present in the bovine corpus luteum.

Day two, the slides are rinsed with three changes of PBS + 0.1% gelatin at 4°C for three minutes each and placed in a blocking serum for 20 minutes at 25°C. The slides were drained and a biotinylated secondary antibody was added to visualize the cellular location of the primary antibody-antigen complex. The slides were left to incubate for 30 minutes at room temperature. During this time an avidin biotin complex (ABC) solution and Tris-HCl buffer were prepared and allowed to sit for 30 minutes. The 0.025% 3,3'-diaminobenzideine, tetrahydrochloride (DAB) solution was prepared in the hood at this time as well. The DAB (Dojindo Laboratories, Japan) was combined with 150 ml Tris (0.038 M) and brought to pH 7.6 with sodium hydroxide. Hydrogen peroxide (0.001%) was then added.

The slides were then prepared as follows: (1) Rinsed three times with PBS + 0.1% gelatin at 25°C for three minutes each. (2) ABC solution centrifuged down at 10,000 rpm for 10 minutes and then added and incubated for 60 minutes at room temperature. (3) Step 1 repeated. (4) Rinsed three times with Tris-HCl buffer for 3 minutes each. (5) DAB added and incubated for 10-15 minutes under the hood. (6) Step 4 repeated. (7) Rinsed three times with reverse osmosis (RO) water for three minutes each. (8) 0.026% of OSO_4

added and incubated for 1 minute. (9) Step 7 repeated. (10) Post fixation performed by leaving 3 minutes in PAPF. (11) Rinsed several times in RO water.

The slides were counterstained with hematoxylin to visualize the tissue without the antibody-antigen complex. Dehydration was performed by rinsing the slides for five minutes in each of the following: 30, 50, 70, 85, 95 (twice), and 100% (twice) ethanol. The slides were cleared with 100% xylene three times for 5 minutes each and mounted with permount.

RESULTS

1.1 Oxytocin Binding Radioreceptor Assay

During the first two trials of the experiment only endothelial cell membranes were tested. The amounts of oxytocin present in the samples as determined by LSC were 1086.5 CPM and 792.25 CPM, respectively, for the samples containing only [^{3}H]-oxytocin and 843.125 CPM and 406.36 CPM, respectively, for the samples containing both the [^{3}H]-oxytocin and unlabeled oxytocin. Both runs resulted in a higher amount of [^{3}H]-oxytocin in the first sample, indicating that there might be specific binding occurring on the endothelial cells.

In the subsequent studies non-endothelial cells were tested as well to provide a reference. Each time, there was a greater amount of [^{3}H]-oxytocin bound in the endothelial samples containing only labeled oxytocin, as was seen in the previous studies, but the specifically bound oxytocin was neglible. The total amount of [^{3}H]-oxytocin bound in the samples of non-endothelial cells was much greater than nonspecifically bound OT (figure 5). This difference was much greater than the difference in the

endothelial samples (figure 6), suggesting that there is specific binding occurring in non-endothelial cells and either none or very little specific binding occurring in the endothelial cells.

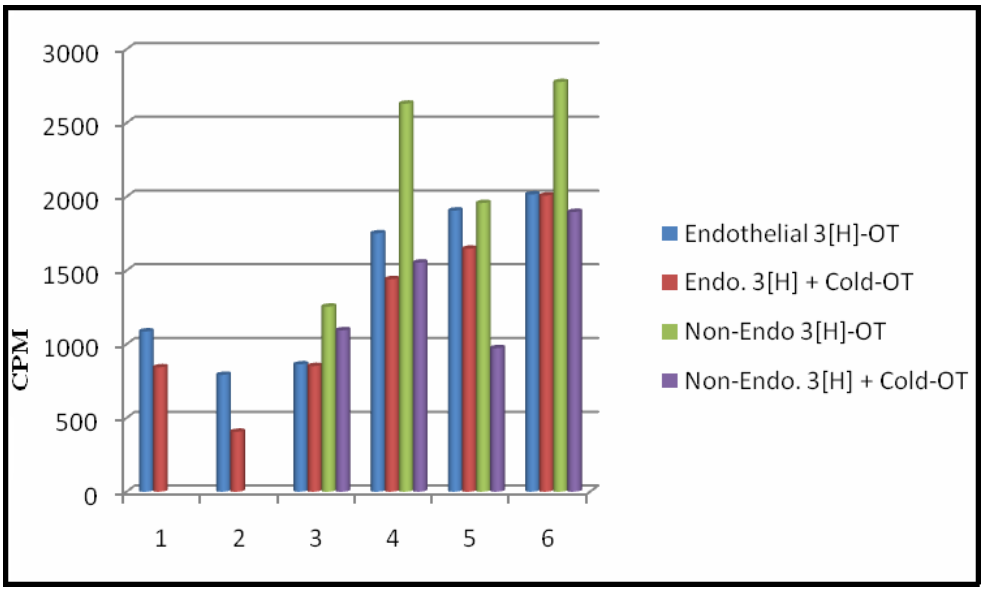

Figure 5. Results showing specific binding of [^{3}H]-oxytocin occurring on non-endothelial cells.

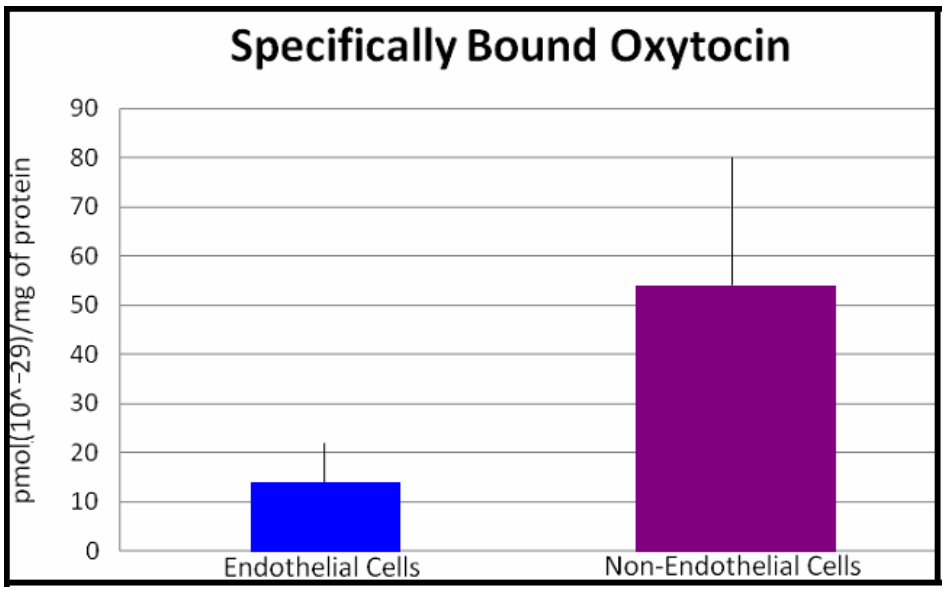

Figure 6. A summary of the amount of specifically bound oxytocin present in the endothelial cells versus the non-endothelial cells.

2.1 Immunohistochemistry

The IHC support the findings of the radioreceptor assay (figure 7). The results of the staining in the sample taken from Cow 148 and the monkey mammary gland control show possible binding to endothelial cells. However, the sample stained from Cow 617 shows no such binding to blood vessels in the tissue. Tissue of Cow 617 does show binding to the surface of the CL large luteal cells.

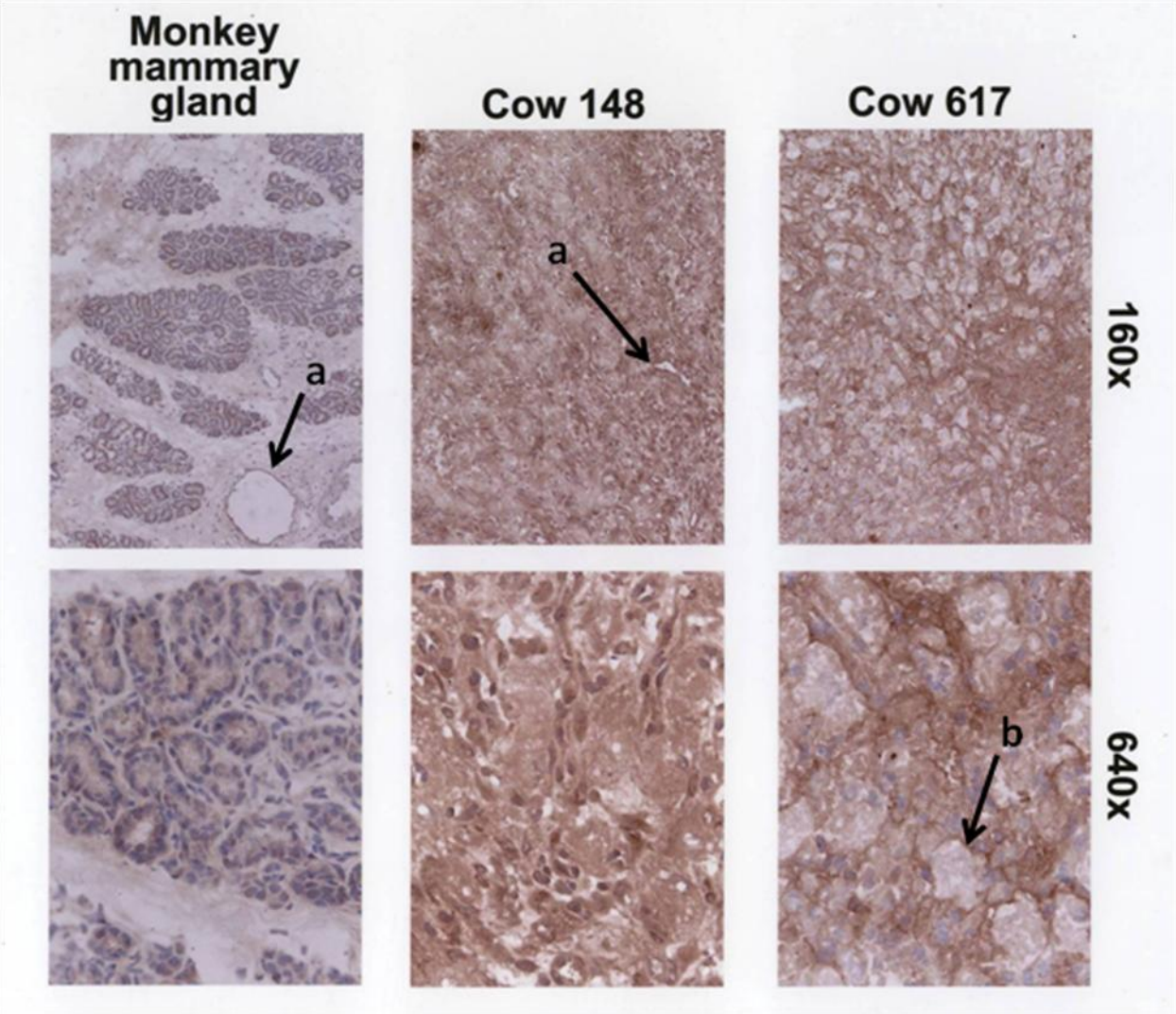

Figure 7. Results from the immunohistochemistry assay. Blue shows non-binding of the antibody-antigen complex. Brown shows binding. (a) Binding to endothelial cells of small capillaries. (b) Binding to large luteal cells.

DISCUSSION

The results of these studies show that oxytocin does not appear to be binding specifically to endothelial cells in the bovine corpus luteum. However, the data suggests

that the hormone is binding elsewhere in the corpus luteum. Two of the possibilities as to the role of OT in the CL are worth contemplating.

The IHC showed possible binding of OT to the surface of large luteal cells. It is established that large luteal cells are the source of luteal OT (Rogers et al., 1984), thus presenting the possibility that OT is acting in an autocrine negative feedback loop. This hypothesis would explain how the CL regulates the amount of OT released during the estrous cycle.

Another cell type is currently under scrutiny by researchers trying to discover its role in angiogenesis. Along with providing structural support to small vessels, there have been many proposed functions as to how pericyte cells and endothelial cells interact. Among these, it is thought that pericyte cells may contact numerous endothelial cells and thus integrate the signals along the vessel length. At present it is not known how pericyte cells may signal the surrounding endothelial cells, however, the cells have been shown to release vascular endothelial growth factor in the ovine CL (Beckman et al., 2006) as well as ANG1, or angiopoietin, a protein growth factor that promotes angiogenesis (Gerhardt and Betsholtz, 2003). In the uterus and mammary glands, oxytocin has the effect of stimulating cells via kinases which activate the NO pathway (Thibonnier et al., 1999). Interestingly, pericyte cells have been shown to release VEGF in the same NO pathway. At present the activation of this pathway is unknown. For further research as to the effect of OT in the bovine CL, the unknown cause of pericyte VEGF secretion should be taken into account.

ACKNOWLEDGEMENTS

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