EFFECT OF EXOGENOUS OXYTOCIN ON ALPACA LUTEAL FUNCTION

By

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EFFECT OF EXOGENOUS OXYTOCIN ON ALPACA LUTEAL FUNCTION

Abstract

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Pregnancy maintenance depends on the maternal recognition of pregnancy (MRP), a physiological process by which the lifespan of the corpus luteum is prolonged. This mechanism is not well characterized in camelids. The objectives of the research present here were to determine if exogenous oxytocin prolongs the corpus luteum activity in alpacas and to evaluate expression and localization of oxytocin receptors within the endometrium at 9 and 14 days post-mating. In the oxytocin studies plasma progesterone profile were determined after ovulation in the same alpacas on 2 cycles: one cycle without oxytocin treatment and one cycle with oxytocin treatment. Oxytocin was administered daily by intramuscular injections at a dose of 20 IU (experiment 1, n=6) or 60 IU (experiment 2, n=8) from day 3 through day 10 after induction of ovulation with GnRH IM. There was no significant difference in the length of the luteal phase (i.e. corpus luteum lifespan) between the treated and control cycle using either dose of oxytocin. In the final experiment, uteri from open and pregnant alpacas (n=4 per group) at 9 and 14 days post-mating were evaluated for expressions of oxytocin receptors by immunohistochemistry. No significant difference ($P \leq 0.05$) in the expression of oxytocin receptors was observed between open and pregnant animals in either staining intensity or tissue localization. We conclude that oxytocin is not involved in luteolysis and early MRP in alpacas.

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Dedication

This thesis is dedicated to my little brother Andrea, who fought for his life and won!

CHAPTER 1

LITERATURE REVIEW

Llamas and alpacas are important economic resources for people living in the high plains of the Andes Mountains. Llamas (*Lama glama*), alpacas (*Vicugna pacos*), guanacos (*Lama guanicoe*) and vicuñas (*Vicugna vicugna*) are grouped under the name "South American camelids" (SACs) because for centuries they have been confined to the Andes in South America. Llamas and alpacas are the domesticated species while guanacos and vicuñas are the wild one (Wheeler, 1995). Llamas are used as transport animals, whereas alpacas are more important for meat and fiber production. There is an increasing interest as livestock animals in other countries including North America mainly as companion animals and for the quality of their fleece, which is very thin (24 micrometers in diameter, half of the sheep one), soft and light. Its thermal properties are superior to those of mohair and cashmere, attracting the attention of textiles industries. Despite being relatively easy to manage, breeding management strategies for alpacas can be challenging. Reproductive studies in domestic SACs are very limited if compared with the other livestock species. Research on reproductive physiology of SACs has increased in the last 20 years. However, there are still several fundamental aspects that need further studies. One of the main challenges to reproductive efficiency in camelids is the high incidence of early pregnancy loss. Embryo mortality rate of up to 35% has been documented in alpacas in the first 35 days after breeding (Bravo et al., 2010). Understanding the basic mechanism of Maternal Recognition of Pregnancy (MRP) in alpacas may be the key to developing therapeutic strategies to prevent early embryonic death. In other domestic species, oxytocin was demonstrated to be involved in the mechanism of luteolysis and MRP. There are no studies in camelids regarding the role of this peptide. This study wants to shed

light on the role of oxytocin in these physiologic mechanisms so that a treatment protocol can be developed to reduce the incidence of early embryonic death in this species.

1.1 Female alpaca reproductive anatomy and uterine histology

Camelids ovaries are globular, with different sizes according to the structures present on them. In adult animals, numerous follicles are present on the surface. The ovarian bursa completely surrounds the ovaries. The oviduct is tortuous and embedded within the mesosalpinx. The uterotubal junction is characterized by a prominent papilla protruding in the uterine horn. The uterus of alpacas and llamas is bicornual and with a "Y" shape. The body of the uterus is very short and the left horn is often larger than the right, even in nulliparous females. The uterus is located in the pelvic area when the animal is not pregnant. The cervix is 2 to 5 cm long and 2 to 4 cm wide. Three incomplete rings are present in it. The broad ligament has a ventral attachment. The vagina is between 13 and 15 cm long and 5 cm in diameter. The vulva is very small with well-delineated external labia. It doesn't change in appearance according to the stage of the cycle.

The uterine histology is not different from the one of other species. The endometrium is the glandular layer of the uterus and the innermost layer. It can be divided in surface epithelium and a glandular section. Both the inner and the glandular epithelia are single tall columnar with variations during the receptive state of the female. The endometrial stroma consists of fibroblasts, vessels, few mast cells, and lymphocytes. The uterine glands are tubular and fewer in number if compared to other domestic species. These glands appear sparser during the follicular phase and more tortuous during the luteal phase. Edema can be observed in the subepithelium space during the period of receptivity of the female. During the pregnancy, the blood supply increases and the

uterine glands increase in number. On the epithelium, maternal crypts are visible. The cervical epithelium is cuboidal and there are no glands in the cervical submucosa. Two layers of myometrium are present and the serosa is called perimitrium (Powers et al., 1990).

1.2 Overview of reproductive physiology in alpacas

Alpacas are induced ovulators; therefore, they exhibit continuous overlapping follicular waves in the absence of mating. A follicle larger than 5 to 6 mm in diameter producing estradiol-17β, can be found at any given time in camelids making them be receptive to the male for more than 30 days. Short stages of male rejection interrupt their receptivity (Walter Bravo and Sumar, 1989a). Mating activity stimulates a neuroendocrine signal that elicits the Gonadotropin Releasing Hormone (GnRH) production from the hypothalamus and the subsequent Luteinizing Hormone (LH) surge resulting in the ovulation of the dominant follicle. Two are the main components to induce ovulation: The irritation of the endometrium by the penetration of the penis in the uterine horns during copulation and the presence of a β-nerve growth factor in the seminal plasma. Normal consequence of the introduction of the penis in the uterus is abrasion and excoriation of the endometrium, which may facilitate the absorption of the β-nerve growth factor. This mechanism was demonstrated in a study where disruption of the uterine mucosa by curettage increased the ovulatory effect of seminal plasma (67% of females ovulated compared with 24% in the group that received intrauterine infusion without curettage) (Ratto et al., 2005; Adams et al., 2005). However, in the same studies support for a systemic endocrine mechanism was found since intramuscular administration of seminal plasma resulted in ovulation in 94% of the alpacas (Ratto et al., 2005; Adams et al., 2005).

Spontaneous ovulations are rare. Their incidence is between 3.5% and 10%. Their mechanism is still unknown (Walter Bravo and Sumar, 1989a). The luteal phase is very short and last only 9 to 10 days in this species. Females are receptive again approximately 12-14 days after a sterile mating. Luteolysis is driven by the pulsatile production of Prostaglandin F2alpha (PGF2α) from the endometrium in non-pregnant alpacas (Aba et al., 2000). The PGF2α production from the left uterine horn has both systemic and local actions, affecting both ovaries. Removal of the left horn prolongs luteal activity in animal carrying a Corpus Luteum (CL) on the left ovary. Conversely, removal of the right uterine horn in females with the CL on the right ovary causes only a slight delay in luteal regression demonstrating that the right uterine horn has only local luteolytic activity. This mechanism it is very important in relation to the maternal recognition of pregnancy in camelids. An embryo derived from an ovulation from the right ovary has to migrate to the left horn to avoid the lysing of the CL on the right ovary (Fernandez-Baca et al., 1979). Both ovaries are equally active but 98% of the pregnancies are established in the left uterine horn (Fernandez-Baca et al., 1970a). This is probably why embryos descended in the right horn will migrate to the left side (Picha et al., 2013). The corpus luteum is the only source of progesterone throughout pregnancy (Sumar, 1988).

The events of oocyte maturation, sperm capacitation, and acrosome reaction, leading to fertilization have not been described in camelids in detail and are assumed to be similar to processes in other livestock (Vaughan and Tibary, 2006). The embryo enters the uterus approximately 5-6 days after ovulation at the early-hatched blastocyst stage (Picha et al., 2013). Maternal recognition of pregnancy (MRP) occurs shortly after between day 6 and 9 post ovulation. This is a very short time frame when the conceptus needs to signal its presence to the dam. The mechanism of maternal recognition of pregnancy is still unknown in this species. Elucidation of

the MRP in camelids will allow a better understanding of the embryo loss mechanisms and could further advance reproductive technologies, such as embryo transfer.

1.2.1 Follicular growth pattern

In absence of mating or an exogenous stimulus to induce ovulation such as administration of GnRH, alpacas present overlapping follicular waves. The duration of each follicular wave is widely variable. The average duration of follicular development, maintenance, and atresia is 12 days (Walter Bravo and Sumar, 1989b). Another study showed interwave intervals ranging from 12 to 22 days (Vaughan et al., 2004).

Follicular recruitment is when for the first time small follicles appear in a previously inactive ovary. In cattle, it is known that increasing circulating Follicular Stimulating Hormone (FSH) leads to the beginning of the folliculogenesis in the ovaries or growth and differentiation of the oocyte and associated cells. This mechanism is still not clear in alpacas. A follicular wave involves recruitment and synchronous emergence of a cohort (8–10) of antral follicles approximately 2–3 mm in diameter, followed by growth of usually one (selected follicle), but sometimes two or three follicles up to 3–5 mm in diameter. The follicle destined to become dominant continues growing, while the others in the cohort (subordinate follicles) regress by atresia. The small follicles in alpacas grow 0.4 mm/day and their size rage between 8 and 12 mm in diameter (Vaughan et al., 2004). When the dominant follicle is selected, it will continue to maturate until reaching the pre-ovulatory size (>7 mm). It is suggested that it is the LH that stimulates the growth of the dominant follicle in camelids as in cattle. Based on a laparoscopic study on ovarian activity in alpacas by Bravo *et al.,* the ovulation size is reached after 4 days of

growth and the pre-ovulatory follicle continued to be present in the ovary between 2 and 8 days (Walter Bravo and Sumar, 1989b).

1.2.2 Mechanism of ovulation in induced ovulators

In species with spontaneous ovulation, the ovarian cycle occurs at regular intervals. Such intervals are controlled by the hypothalamus-pituitary-gonadal axis. The dominant follicle present in the ovary during the estrus phase produces estradiol 17β, which stimulates the LH surge resulting in ovulation, developing of a corpus luteum and beginning of the luteal phase. In contrast, ovulation is induced by copulation in camelids. The number of matings seems not to play a role on ovulation rate or LH levels in alpacas as in the cat, another induced ovulating species (Bravo et al., 1992). During copulation, the penis passes through the cervix and semen is deposited deep into each uterine horn. This causes an irritation by the cartilaginous process of the tip of the penis. The endometrial inflammation potentiates the ovulation-inducing factor (OIF, β-Nerve Growth Factor) present in seminal plasma improving its absorption and endocrine action on the hypothalamus (Ratto et al., 2012). Ovulation occurs in the majority of females within 30 hours of mating. Ovulation only occurs in female alpacas that have cued follicles between 7 and 12 mm in diameter (Bravo et al., 1991). Lack of ovulation after copulation has been reported between 10 and 30% (Bravo, 1994). However, in our recent study, ovulation occurred in more than 90% of the females after mating (Campbell et al., 2015). The same study showed that multiple ovulations and conception of twins are a common phenomenon in alpacas. In a retrospective study by Campbell *at al.,* twin pregnancies were identified between 25 and 30 days in 47.1% of double-ovulating females. There were more twins from bilateral ovulations (62.5%) than from unilateral ovulations

(37.5%). Twin pregnancies were either reduced to a singleton (62.5%) or lost completely (37.5%). One set of twins remained viable until Day 52 in gestation. In the prospective study by the same author, double ovulation occurred in 18.8% of the cycles. Two embryos were collected from 2 of the 4 double-ovulating females (Campbell et al., 2015).

1.2.3 Endocrinology of the ovarian activity

In anovulatory camelids regular growth and atresia of follicles are observed. Estradiol-17β is the major hormone produced and its plasma concentration is proportional to the follicular size and number. It was reported that peak of estradiol-17β plasma concentration occurs 8 days after the beginning of the growing phase in alpacas (Vaughan and Tibary, 2006). Since follicular waves overlap, the level of estrogens remains elevated, giving a long period of receptivity (approximately 30 days). Follicles will develop until a dominant size and then become atretic or anovulatory. Anovulatory follicles are described as persistent follicular structures of more than 12 mm in diameter that can reach sizes of up to 70 mm. They can become hemorrhagic or luteinize. Ovarian follicular activity is conserved but animals with luteinized anovulatory follicles can present persistent rejection of the male because of significant progesterone production.

The exogenous administration of human Chorionic Gonadotropin (hCG) or Gonadotropin Releasing Hormone analogs (GnRHa) mimic the endogenous release of GnRH after mating, leading to the LH surge. The LH surge is stimulated within 15 minutes of copulation and last 7 to 8 hours. Similar response has been observed by exogenous treatments with GnRH (Bravo et al., 1991). An additional breeding 6 and 24 hours after the first does not cause another LH surge (Bravo et al., 1992). Little is known about the endocrinologic profiles of FSH in alpacas except for a large

individual variability.

After mating, a corpus luteum will form and produce progesterone, which is going to rise and reach 2ng/mL by day 6 post-breeding and have the maximum level by day 8 (Fernandez-Baca et al., 1970a). It will continue to increase until day 8-9 and then sharply decrease by day 13. Measuring progesterone level is a good indication that the ovulation occurred. In South American Camelids the size of the CL ranges between 11 and 20 mm. Cavitary CLs are normal (Tibary et al., 2007). Luteolysis is due to the production of PGF2α by the endometrium in absence of the embryo.

1.2.4 Hormonal induction of ovulation

Exogenous administration of hormones to induce ovulation is the most common treatment in camelids breeding management. Two main hormones are used for this purpose: hCG and GnRH or its synthetic analog gonadorelin diacetate tetrahydrate (Fernandez-Baca et al., 1970b). An acute release of LH occurs after the administration of these drugs. The administration of 500 to 750 IU of hCG has been used in alpacas but less commonly than GnRH because of its intravenous administration route. The GnRH dose varies from 25 to 50 mcg intramuscularly. Recently Silva *et al.* demonstrated a direct correlation between the dose of GnRH and the magnitude of LH surge and ovulatory response in llamas. Llamas administered with 50 mcg of GnRH released more LH and the LH surge had longer duration than those given 25, 12.5, or 6.25 mcg. However, subsequent CL development and plasma progesterone concentrations were not affected (Silva et al., 2012).

1.2.5 Mechanism of luteolysis

In most mammals, luteolysis, which occur in absence of a conceptus, represents the regression of the CL and resumption of cyclicity with a new opportunity to breed. Luteolysis is described as functional when it is evidenced by a decline in serum progesterone levels. Structural luteolysis describes the morphological change of the corpus luteum (regression, apoptosis).

As previously mentioned, the corpus luteum in alpacas has a slow development and a sudden death in absence of pregnancy. The presence of a CL can be established by serum progesterone level or by ultrasonographic examination of the ovaries. Ultrasonographic visualization is difficult in the first 4 days post mating. However, it becomes easily identifiable after day 6 post-ovulation due to its size and echogenicity. The corpus luteum reaches its maximum size (12-14 mm) on day 8 or 9 after breeding. Its complete regression is between day 9 and 10 after mating (Aba et al., 2000). Luteolysis is suspected ultrasonographically when the CL size and its blood flow are decreased. The pulsatile production of PGF2α from the endometrium is responsible for luteolysis in camelids. PGF2α production begins on day 7 or 8 after mating. Luteolysis is completed by day 9 or 10 post-breeding in non-pregnant llamas and alpacas. Even though there is not difference in the frequency of ovulation between the left and right ovary, a different luteolytic effect has been found between the two horns in camelids. In fact removal of the left horn in females that have the CL on the left ovary prolongs the life of the CL, conversely, the removal of the right horn with CL on the right ovary leads luteolysis anyway (Fernandez Baca S et al., 1979). An embryo that derives from an ovulation on the right ovary has to migrate to the left horn and signal its presence avoiding luteolysis. This is the reason why the 98% of the pregnancies are carried in the left horn in this species.

1.3 Maternal recognition of pregnancy in large domestic species

Maternal recognition of pregnancy (MRP) is the biological process that allows the corpus luteum, therefore the gestation, to be maintained. The female recognizes the presence of the conceptus and remodels the endocrine, histological and secretory function within the reproductive tract. The signaling between the embryo and the uterus promotes a successful implantation. This process involves different actions that ultimately influence the corpus luteum activity: luteotropic, antiluteolytic, or luteostatic. Luteotropic is when the signal directly promotes the corpus luteum function. Antiluteolytic is when the signal prevents the release of luteolytic prostaglandin $F_{2\alpha}$ ($PGF2\alpha$). Luteostatic is a re-distribution of $PGF2\alpha$ so that it does not reach the CL.

MRP has been studied intensively in ruminants (Spencer et al., 2007; Spencer et al., 2008) swine (Franczak and Bogacki, 2009; Bazer and Johnson, 2014) and to a lesser extend in horses (Klein and Troedsson, 2011) and camelids (Tibary et al., 2007). Below is a literature review of our knowledge on MRP in domestic large animal species.

1.3.1 Ruminants

In Ruminants, the blastocyst needs to hatch from the zona pellucida around day 8 to 10 before to start elongating. The elongation is necessary for the MRP in fact during this phase giant binucleate cells of the trophoblast produce IFNτ, a protein responsible for the maintenance of the pregnancy. In Ruminants, IFNτ has an antiluteolytic action. The critical period for recognition is between 15 and 16 days and 13 and 14 days post ovulation in cattle and sheep, respectively. IFNτ

acts in a paracrine manner on the endometrium to prevent luteolysis by down-regulating oxytocin receptors (OTR) gene both in the cow and in the ewe (Xiao et al., 1999). It was initially hypothesized that IFNτ prevented luteolysis by inhibiting the estrogens receptors (ER) and blocking oxytocin receptors (OTR) but more recent studies showed that inhibition of oxytocin induced PGF2α from the endometrium does not require ER down-regulation (Robinson et al., 2008). The untiluteolytic effects of IFNτ inhibit transcription of the estrogen receptor alpha (ESR1) gene in sheep and the OTR gene in both sheep and cattle in the luminal epithelium of the uterus. In cattle, IFNτ down-regulates OTR and prostaglandins-generating enzyme prostaglandin synthase 2 (PTGS2; formerly cyclooxygenase 2 or COX2) expression, which results in a reduced expression of PGF synthase and consequently reduced PGF2α secretion (Xiao et al., 1999). The expression of PTGS2 and secretion of prostaglandins was found in pregnant and non-pregnant sheep (Charpigny et al., 1999). In the presence of a conceptus, luteolysis is prevented by inhibition of transport of endometrial prostaglandins by the IFNτ (Banu et al., 2010). IFNτ acts on the endometrium also to induce or enhance the expression of IFN-stimulated genes (ISG), which are important for embryonic elongation and implantation (Bauersachs et al., 2006; Johnson et al., 1999). Several types I INF-stimulated genes (ISG) have been discovered in cattle in response to IFNτ. In sheep, it appears that interferon regulatory factor 2 (IRF2) in the luminal epithelium restricts IFNt induction of most classical ISGs to the uterine stroma and glandular endometrium. IFNt has also been shown to exert systemic effects that may affect CL function (Hansen et al., 2013). In cattle, it is very important that the ovarian progesterone is produced in order to drive the conceptus to elongation and establish the uterine receptivity to implantation between 7 and 13 (Forde and Lonergan, 2012). Progesterone in fact, induces the expression of many conceptus elongation and implantation-related genes including those coding for attachment and migration

factors, PTSG2, protease inhibitors, cell proliferation factors, adhesion protein insulin-like growth factors, prostaglandin G/H synthase, hidroxysteroid (11β) dehydrogenase, proteases, candidate cell proliferation factor, glucose transporters, and cationic amino acid transporter. This gene expression pattern occurs in association with loss of progesterone receptors between Day 10 and 12 in luminal endometrium and between Days 12 and 16 in the glandular endometrium after the onset of estrus (Forde and Lonergan, 2012).

1.3.2 Swine

In swine, as in ruminants, the blastocyst needs to hatch and elongate to signal its presence in the uterus. During the MRP (day 11 to 15 post-ovulation) the production of PGF2α is not decreased but metabolized in the uterine lumen. The porcine blastocysts, specifically its trophoblast, has a very high aromatase activity producing estrogens which re-orient PGF2α release away from the luteolytic endocrine direction (exocrine direction) (Bazer, 2013). The administration of exogenous estradiol to gilts between days 11 and 15 post ovulation produced a state of pseudopregnancy and increase the chances of maintenance of pregnancy after the transfer of a single embryo (Kawarasaki et al., 2012). In pigs oxytocin may play a role in the MRP, however, the specific mechanism is still unclear. Endometrial oxytocin levels increase up to 50-fold during the time of MRP in pigs (Trout et al., 1995). Intrauterine oxytocin administration on days 10 to 16 post estrus decreases endocrine PGF2α secretion on Days 14 to 16 and delayed luteolysis (Sample et al., 2004). Therefore, it is speculated that oxytocin may contribute to the luteal maintenance by decreasing the endocrine secretion of PGF2α allowing its exocrine release with a luteostatic mechanism.

1.3.3 Equine

In horses, the exact mechanism of maternal recognition of pregnancy is still unclear. Transuterine embryonic migration is required for the inhibition of luteolysis. Recent studies suggested that the mechanism is primarily antiluteolytic with the suppression of PGF2α production due to reduced PTGS2 (prostaglandins-generating enzyme prostaglandin-endoperoxidase synthase 2, formerly COX-2) gene expression, accompanied by suppression of OTR gene expression (de Ruijter-Villani et al., 2015). During the early pregnancy the conceptus and the endometrium produces PGE2, which may be another factor inhibiting luteolysis (Boerboom et al., 2004). The critical period for recognition is between 12 and 14 days post-ovulation in the mare.

1.3.4 Camelids

In this species, the signal for the MRP is still unknown. As described above, the timing of MRP in these species occurs in a very short time frame after the descent of the embryo in the uterus (Tibary et al., 2007). It is important to note that despite the presence of a conceptus, a pulsatile release of PGF2α was observed between Day 7 and 15 post-mating. This release of PGF2α results in a transient decrease in progesterone concentrations by Day 9 post-mating (Aba et al., 2000).

Similarly to the equine and pigs embryos, camelids embryos have a high aromatizing ability producing estradiol-17β between days 10 and 15 of pregnancy (Skidmore et al., 1994). The increase in estradiol production occurs during the blastocyst elongation (days 10 to 13). It is possible that elongation plays an important role in MRP as in ruminants and pigs (Picha et al., 2013). However, no detectable protein, or INFt were found in camelids during the maternal

recognition of pregnancy as in ruminants (Skidmore et al., 1998). Powell *et al.* in 2007 demonstrated that llama blastocysts produce increasing quantities of estradiol from day 7 through day 15 of gestation. The peak of estradiol production was around day 13, which is too late as signal involved in suppression of the PGF2α surge. However, these authors noticed that the blastocysts start producing estradiol by day 9 and the level are three to four-fold greater than at day 7. Therefore, this may be an adequate signal to prolong the lifespan of the CL (Powell et al., 2007). Estradiol benzoate (10 mg, IM) administered from Day 7 to Day 15 after induction of ovulation to females transiently prolonged luteal lifespan and increased progesterone secretion during the period when luteolysis would have been completed (Powell et al., 2007). In another study on alpacas, administration of estradiol on days 8 and 9 post-ovulation improved embryo survival by 30 to 50% (Chipayo et al., 2003). The involvement of estrogens in MRP in camelids is further supported by the increase in estrogen receptor alpha (ESR1) between Days 8 to 12 post-mating in pregnant animals and a reduction in the expression of progesterone receptors by Day 12 post-mating (Bianchi et al., 2013). However, attempts to prolong luteal lifespan in cyclic camels by administering a range of types and doses of exogenous estrogens at various times during the luteal phase were unsuccessful (Skidmore et al., 1994).

Migration of the embryo from the right to the left uterine horn is another mechanism that may be involved with the MRP in camelids (Picha et al., 2013). However, this migration is different from the transuterine embryo migration observed in the equine. In the mare, the embryo remains spherical and moves around in the uterus whereas in camelids the embryo elongates. The migration of the embryo from the right to the left horn and the subsequent preponderance of left-horn pregnancies in camelids are attributed to a difference in PGF2α release between the two uterine horns. PGF2α release from the right uterine horn is local whereas its release from the left

horn is systemic (Tibary et al., 2007). The migration of the embryo originating from an ovulation in the right ovary to the left horn is therefore required to prevent PGF2α release into the general circulation and may even exert a luteotropic effect making possible the survival of the embryo (Picha et al., 2013). Because all embryos migrate to the left horn, a greater expression of ER in the right horn to promote its contraction was expected but could not be demonstrated in our previous study (Campbell, 2015). We couldn't find any difference in thickness of the endometrium and myometrium between the right and left uterine horns for each alpaca weather open or pregnant. Despite the lack of evidence of histologic differences, it is possible that significant changes may be occurring at the molecular level. This suggests that a more complex mechanism with differential expression of ER subtypes may be involved in embryo migration from the right to the left horn. In addition, it is possible that oxytocin, as in other species, plays a role in this mechanism.

1.4 Role of oxytocin in the Maternal Recognition of Pregnancy in large domestic species

Oxytocin was discovered by Sir Henry Dale in 1906 when he found that extracts from the human posterior pituitary gland contracted the uterus of a pregnant cat. It was also Sir Henry Dale who named oxytocin from the Greek words ωκνξ, τοκοχξ, meaning "swift birth." Oxytocin was the first peptide hormone to be sequenced and synthesized by Vincent du Vigneaud in 1953 who was awarded the Nobel Prize in 1955 for this scientific achievement (Du Vigneaud, 1956).

Oxytocin is a nonapeptide hormone synthesized by the magnocellular neurons located in the paraventricular and supraoptic nuclei of the hypothalamus where it is packaged into secretory granules (Lincoln and Wakerley, 1974). Oxytocin is then stored by the neurohypophysis.

Oxytocin is also synthetized by several other tissues (placenta, amnios, uterus, testis, and heart) (Gimpl and Fahrenholz, 2001a). The oxytocin sequence of amino acids is Cysteine-Tyrosine-

Isoleucine-Glutamine-Asparagine-Cysteine-Proline-Leucine-Glycinamide, with a sulfur bridge between the two cysteines.

Oxytocin has multiple and various effects according to its timing and degree of release. It is important for the regulation of the hypothalamo-pituitary-adrenal axis in response to stress, pregnancy, luteal function, maternal behavior, cell proliferation, modulation of emotional relationships, sexual behavior, erectile function, ejaculation, antinociception, cardiovascular function, osteoporosis, and neuropsychiatric disorders (Viero et al., 2010). However, the most important roles of oxytocin in domestic mammals are milk ejection, uterine contraction during parturition (Ferguson reflex), and luteolysis.

The oxytocin receptor (OTR) belongs to the rhodopsin-type class I G-protein-coupled receptor (GPCR) superfamily. The OTR can also bind to other molecules similar to oxytocin, such as arginine vasopressin (AVP), oxytocin agonists or antagonists. Activation of the OTR occurs when oxytocin binds its receptor on the cellular surface. This stimulates several intracellular signal pathways resulting in the final effect. The oxytocin receptor is coupled to a guanosine triphosphate (GTP) binding protein (Gq). For example, in order to have the contraction of myometrial muscles or mammary myoepithelial cells, the oxytocin needs to bind its receptor, this will activate the phospholipase C which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to InsP3 and diacylglycerol (DAG) (Smith, 2007). InsP3 releases calcium ions from the intracellular space and DAG activates protein kinases type C, which further phosphorylates other proteins. Calcium ions bind to calmodulin and this complex activates the myosin light-chain kinase causing the contraction (Gimpl and Fahrenholz, 2001b). OTR is also coupled with other G-proteins, which result in various cellular effects (Valeria Rimoldi et al., 2003).

Oxytocin has a luteolytic effect in some species. This is regulated by the mitogen-activated protein kinase (MAPK) and the Rho kinase pathways. Activations of both OTR and MAPK result in increased cytosolic phospholipase A2 (cPLA2) activity. cPLA2 hydrolyzes phospholipids and liberates arachidonic acid, which results in increased production of prostaglandins via cyclooxygenase-2 (COX-2) (Soloff et al., 2000).

Despite the fact that PGF2α is the primary hormone responsible of the actual luteolysis, it is important to emphasize that its release is controlled by the oxytocin, at least in pigs, ruminants, and horses (Bazer, 2013; Mc Cracken et al., 1996; Betteridge et al., 1985).

1.4.1 Role of oxytocin in MRP in swine

During luteolysis in pigs, oxytocin is secreted primarily from the neurohypophysis and the uterine endometrium (Bazer, 2013). Very little amount of oxytocin is produced by the corpus luteum. Inter-estrus interval can be decreased when oxytocin is administered systemically before the luteolysis occurred. During MRP endometrial oxytocin secretion in swine increases up to 50-folds (Trout, 1995). This event coincides with the embryonic production of estrogens and the exocrine release of PGF2α. Oxytocin has luteostatic effect in pigs.

1.4.2 Role of oxytocin in MRP in ruminants

In ruminants, towards the end of the luteal phase, endometrial progesterone and estrogens receptors are respectively down and up regulated (Mccracken et al., 1996). This event leads to the increase of the endometrial oxytocin receptors and oxytocin release from the hypothalamus. This initial burst of oxytocin stimulates a subluteolytic production of PGF2α from the uterus, which in

turn allows the production of oxytocin from the corpus luteum (Mc Cracken et al., 1996).

Luteolytic levels of PGF2α are then produced when levels of oxytocin become sufficient. Current evidence from sheep and cattle suggests that progesterone from the corpus luteum and estradiol from developing follicles act in tandem to control the development and sensitivity of receptors for oxytocin in the endometrium (Mann and Lamming, 2006). In cattle, exogenous oxytocin given between day 4 and 8 after ovulation provoke premature regression of the corpus luteum (Armstrong and Hansel, 1959); however, Gilbert demonstrated that the infusion of oxytocin by an osmotic minipumps implanted subcutaneously from day 10 after estrus extended the luteal phase in non-pregnant cows. This suggests that oxytocin may be involved in down-regulating its own receptor when given at the end of diestrus (Gilbert et al., 1989). In presence of an embryo, interferon tau inhibits the development of oxytocin receptors in the endometrium and, by a local action on the uterine epithelium, inhibits oxytocin-induced luteolytic-type episodes of PGF2α release (Payne and Lamming, 1994).

1.4.3 Role of oxytocin in MRP in equine

Based on these findings in the cow, similar studies have been carried out in mares to identify a possible role of oxytocin in luteolysis. However, when oxytocin was given to mares from day 4 to day 8 post ovulation, premature luteolysis was not observed (Neely et al., 1979). Also, there is no oxytocin production from the CL in the mare. The neurohypophysis is the sole source (Mc Cracken et al., 1996). For these reasons, for many years it was widely accepted that there is no oxytocin involvement in luteolysis in the mare. This concept was changed when Betteridge et al. demonstrated that the administration of oxytocin to mares during late diestrus induced immediate rise in peripheral 13,14-dihydro-15-keto PGF2α, the principal metabolite of

PGF2α (Betteridge et al., 1985). Today it is well known that in non-pregnant mares, oxytocin induces the secretion of PGF2α from the endometrium causing luteolysis between day 14 and 15 post ovulation. Release of oxytocin by the neurohypophysis concomitantly with an increased number of endometrial oxytocin receptors in late diestrus is responsible for release of PGF2α from the endometrium and luteolysis. In contrast, studies by Vanderwall et al. demonstrated that the administration of supraphysiologic doses of oxytocin in mid-diestrus in mares prolongs diestrus (Vanderwall et al., 2016). In 2007, the same group reported that administration of 60 IU of oxytocin twice a day, intramuscularly (IM) on days 7 to 14 after ovulation maintained progesterone concentration >1 ng/mL continuously through day 30 (Vanderwall et al., 2007). In 2012, another study from the same group showed that once daily administration of the 60 IU IM was as effective as twice-daily administration, simplifying the treatment protocol (CL function was prolonged in 60% to 70% of mares in both groups) (Vanderwall et al., 2012). However, this effect of oxytocin seems to have a different mechanism from the one in the cow. In the cow, in fact, there is a down-regulation of the oxytocin receptors, which it is not present in the mare. Vanderwall at al. reported that oxytocin treatment in the mare doesn't alter late diestrus oxytocin receptors concentration (Vanderwall et al., 2012). Ball et al. inferred that an inhibition of COX-2 might be a mechanism by which PGF2α release is suppressed in mares experiencing luteostasis secondary to oxytocin treatment, which supports previous researches that showed the importance of COX-2 activity in equine luteolysis (Atli et al., 2010).

1.4.4 Role of oxytocin in MRP in camelids

As discussed earlier, in absence of pregnancy, camelids exhibit a relatively short luteal

phase of only 8-10 days. In presence of a conceptus, luteolysis is prevented and the corpus luteum is maintained as the sole source of progesterone for the entire gestation.

Similarly to the equine and pigs embryos, camelids embryos have a high aromatizing ability producing estradiol-17β between days 10 and 15 of pregnancy (Skidmore et al., 1994). The increase in estradiol production occurs during the blastocyst elongation (days 11 to 13). Based on these similarities with other species, we hypothesized that oxytocin may play a role in the luteolysis and in the maternal recognition of pregnancies in alpacas as well. In a recent study in llamas by Bianchi et al., endometrial expression of estrogen, progesterone, oxytocin receptors and cyclooxygenase-2 (COX-2) was evaluated. Based on the immunoexpression in endometrium, they could speculate that estrogens from the ovulatory follicle promote an increase in the population of estrogens and progesterone receptors. Similar observations have been reported in ewes (Spencer et al., 2007), mares (Hartt et al., 2005), cows (Robinson et al., 1999) and llamas (Bianchi et al., 2010). However, unlike ruminants, an increase (or a cyclical regulation) of endometrial oxytocin receptors population before and during luteolysis could not be observed in llamas (Bianchi et al., 2010). Nevertheless, similar to ewes, estrogens receptors expression was up regulated at the beginning of the process of luteolysis in llamas, probably in relation to an increase in plasma estradiol-17β concentrations from the new dominant follicle. Nevertheless, changes in the population of oxytocin receptors seem not to be involved in the initiation of luteolysis in llamas.

1.5 Conclusion

Pregnancy loss is one of the most common complaints in camelid Theriogenology. Early embryonic loss can account for up to 50% in the first 45 to 60 days of gestation in cameldis. Our

knowledge of MRP in camelid is limited compared to other domestic species, particularly ruminants. Understanding the mechanism of maintenance of the corpus luteum in camelids is fundamental to further improve our knowledge on causes of early embryo loss and its prevention. Several studies have been performed by our laboratory to answer specific questions regarding ovulation, early embryo development and embryo/uterus crosstalk. The ultimate goal of these studies is to determine the mechanisms of MRP in in this species.

1.6 References

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CHAPTER 2

RESEARCH DESIGN AND METHODOLOGY

2.1 Background

Oxytocin secreted by the corpus luteum (Wathes et al., 1996, 1983) plays an essential role in luteolysis and maternal recognition of pregnancy (MRP) in domestic ruminants (Flint and Sheldrick, 1986). Oxytocin functions to simulate endometrial PGF2α synthesis. Through a unique counter-current mechanism, endometrium-derived PGF2α induces the further release of oxytocin from the corpus luteum (CL) (Flint and Sheldrick, 1982), as well as release from the hypothalamus (Silva et al., 1991) via positive feedback on these structures. Administration of exogenous oxytocin to cows between day 4 and day 8 after ovulation induces luteolysis (Armstrong and Hansel, 1959).

However, oxytocin administration to cyclic heifers from day 10 by subcutaneous osmotic mini-pumps lengthened the luteal phase (Gilbert et al., 1989). Similar results were obtained in ewes by Flint and Sheldrick (1985), delaying the return to estrus by 7 days when oxytocin was administrated intravenously from day 13 to day 21 after estrus. These authors attributed the results to the high levels of circulating oxytocin preventing the expression and cell surface display of endometrial oxytocin receptors, thus preventing the PGF2α release necessary for luteolysis (Flint and Sheldrick, 1985). These observations suggest that oxytocin is involved in down-regulating its own receptor when given at the end of diestrus in ruminants (Gilbert et al., 1989). In the presence of an embryo, interferon tau inhibits the expression of oxytocin receptors in the endometrium and, by a local action on the uterine epithelium, inhibits oxytocin-induced luteolytic-type episodes of PGF2α release in sheep (Payne and Lamming, 1994). Similarly, in non-pregnant mares, oxytocin induces the secretion of PGF2α from the endometrium causing luteolysis between day 14 and 15

post ovulation (Allen, 2000). Release of oxytocin by the neurohypophysis concomitantly with an increased number of endometrial oxytocin receptors in late diestrus is responsible for the release of PGF2α from the endometrium and induction of luteolysis. However, studies in mares demonstrated that the administration of high doses of oxytocin in mid-diestrus prolongs diestrus (Vanderwall et al., 2016). Administration of 60 IU of oxytocin twice a day, intramuscularly (IM) on days 7 to 14 after ovulation maintained progesterone concentration >1 ng/mL continuously through day 30 (Vanderwall et al., 2007). The same group reported similar result with once daily administration of the 60 IU of oxytocin IM. Luteal function was prolonged in 60% to 70% of treatment mares in these experiments (Vanderwall et al., 2012). However, the mechanism that mediates the actions of oxytocin are likely distinct from that in the cow. In the cow, oxytocin receptors are down-regulated, which does not appear to be the case in the mare. Oxytocin treatment in the mare does not alter the number of oxytocin receptors in late diestrus (Vanderwall et al., 2012).

In camelids, the role of PGF2α as a luteolysin is well established. The pulsatile production of PGF2α from the endometrium is responsible for luteolysis beginning on day 7 or 8 and finishing by day 9 or 10 after mating in non-pregnant llamas and alpacas (Aba et al., 2000). However, it remains to be determined whether or not oxytocin plays a role in these physiologic mechanisms. The camelid embryo enters the uterus on day 6.5 to 7 after fertilization allowing for only a few days to signal its presence and avert luteolysis (Picha et al., 2013). In the presence of the conceptus, luteolysis is prevented and the corpus luteum is maintained in the alpaca as the sole source of progesterone for the entire gestation. Similarly to the equine and porcine embryos, camelid embryos have a high aromatase capacity and produce estradiol-17β between days 7 and 15 of pregnancy (Skidmore et al., 1994 and Powell et al 2007). This increase in estradiol production

occurs during blastocyst elongation (days 11 to 13). It is possible that camelid elongation plays an important role in MRP as in ruminants and pigs (Picha et al., 2013). In llamas, although the peak of estradiol production is not reached until day 13, blastocysts estradiol production starts at 7 days and by day 9 the levels are three to four-fold higher (Powell et al 2007). This may provide an adequate signal to prolong luteal lifespan (Powell et al., 2007). Estradiol benzoate (10 mg, IM) administration from days 7 to 15 after induction of ovulation results in prolonged luteal lifespan and increased progesterone secretion during the period when luteolysis normally occurs (Powell et al., 2007). Others authors reported that administration of estradiol (200 μg/day IM) on days 8 and 9 post-ovulation to embryo recipients improved alpaca embryo survival by 30 to 50% (Chipayo et al., 2003). The involvement of estrogens in MRP in camelids is further supported by the increase in estrogen receptor alpha (ESR1) in the endometrium between Days 8 to 12 post-mating in pregnant animals and a reduction in the expression of progesterone receptors by Day 12 post-mating (Bianchi et al., 2013). However, attempts to prolong luteal lifespan in cyclic camels by administering a range of types and doses of exogenous estrogens at various times during the luteal phase were unsuccessful (Skidmore et al., 1994).

Migration of the embryo from the right to the left uterine horn is another mechanism that may be involved in the MRP in camelids (Picha et al., 2013). However, this migration is different from the transuterine embryo migration observed in the equine. In the mare, the embryo remains spherical and moves around in the uterus whereas in camelids the embryo elongates. The migration of the embryo from the right to the left horn and the subsequent preponderance of left-horn pregnancies in camelids are attributed to a difference in PGF2α release between the two uterine horns. PGF2α release from the right uterine horn is local whereas its release from the left horn is systemic (Tibary et al., 2007). The migration of the embryo originating from an ovulation in the

right ovary to the left horn is therefore required to prevent PGF2α release into the general circulation and may even exert a luteotropic effect making possible the survival of the embryo (Picha et al., 2013).

There are no studies on the role of oxytocin at the time of MRP in camelids. The present experiments were designed to determine if oxytocin plays a role in luteolysis in alpacas. Two approaches were taken. The first approach consisted of studying if there is any difference in luteal phase duration in alpacas following treatment with oxytocin after ovulation. The second approach was to determine if there is a difference in oxytocin receptors expression between pregnant and non-pregnant animals at 9 and 14 days post-mating.

2.2 Rationale

Understanding the MRP mechanism in Camelids can prevent early pregnancy and economic losses. One of the recent discoveries in other species is the involvement of the oxytocin in this process. It is still unknown if oxytocin plays a role in luteolysis in camelids. There are no studies looking at the oxytocin as luteotropic hormone as it is in equine and bovine. The work presented in this thesis is part of a research started previously with the effort to study early embryo development, maternal recognition of pregnancy, as well as factors involved in early pregnancy loss.

2.3 Hypotheses

Two hypotheses were developed:

- The administration of 20 IU or 60 IU of oxytocin IM during the luteal phase, from day 3 to day 10 post-ovulation, will prolong the life span of the corpus luteum in alpacas.

- A difference in the expression of oxytocin receptors will be observed in the pregnant and non-pregnant endometrium of alpacas.

2.4 Objectives

Three experiments were designed to test these hypotheses.

- In experiment 1, the objective was to determine if 20 IU of oxytocin given intramuscularly (IM) from day 3 to day 10 post-ovulation prolongs the corpus luteum activity in alpacas justifying its possible role in luteolysis and MRP in alpacas.
- In experiment 2, the objective was the same of the first experiment but testing the effect of 60 IU of oxytocin given intramuscularly (IM) from day 3 to day 10 post ovulation.
- In experiment 3, the objective was to evaluate the expression and localization of oxytocin receptors within the endometrium in pregnant and non-pregnant uteri at 9 and 14 days post-mating.

2.5 Materials and Methods

2.5.1 Animals

All alpacas used for this study were healthy, non-pregnant, non-lactating, between 5 and 16 years of age. Animals enrolled were assessed through an initial physical examination and breeding soundness examination. Alpacas were maintained in outdoor paddocks and fed grass hay and water ad libitum. All procedures were completed in accordance and approval from the

institutional animal care and used committee at Washington State University.

2.5.2 Experiment 1

Thirteen alpacas were evaluated by transrectal ultrasonography using an Exago ultrasound machine (Exago, Universal Imaging, New York, USA) equipped with a 7.5 MHz linear array transducer mounted on a polyvinyl chloride (PVC) extension rod to determine the presence of a dominant follicle in the ovaries. Ovulation was induced with GnRH (50 µg) (Cystorelin®, Merial, Duluth, GA, USA) intramuscularly (IM) when a follicle between 7 and 12 mm in diameter was present in the ovaries and the uterus was toned and edematous. Seven days after the treatment, the animals were evaluated by transrectal ultrasonography to assess the presence of a CL in the ovaries.

Only alpacas that had ovulated (n=7) were enrolled in the experiment. Each animal was its own control. Females were monitored over two cycles. The first cycle served as a control (no treatment). In the second cycle, all females received oxytocin (20 IU, IM) daily from day 3 post-induction of ovulation until day 10. Blood samples were collected by jugular venipuncture every other day from day 0 (day of induction of ovulation) until day 14. Blood sampled were allowed to clot then centrifuged to harvest serum which was stored at -20°C until assayed for progesterone.

The ovarian and uterine status was verified by transrectal ultrasonography on day 7 and day 14 after induction of ovulation. The presence, size and blood flow (Color Doppler flow) of the corpus luteum was recorded in a subset of animals.

2.5.3 Experiment 2

Ovulation was induced as in the first experiment. Eight out of 13 animals responded and ovulated. Females were monitored over two cycles. The first cycle served as a control (no treatment). In the second cycle, the oxytocin dose was increased since no prolongation of the CL life was observed in experiment 1. All alpacas were treated with 60 IU IM from day 3 to day 10 post-ovulation. Blood samples were scheduled and handled as in experiment 1.

For Experiments 1 and 2 serum progesterone was analyzed using radioimmunoassay (RIA) at the University of Idaho, Department of animal science. The sensitivity of the assay was 0.05 ng/mL; values less than the assay sensitivity values were assigned a value equal to the sensitivity value. The intra- and inter-coefficient of variation were <10%. The standard curve correlation coefficient was 0.9992.

2.5.4 Experiment 3

Reproductively sound sexually mature female alpacas (n=16) that were scheduled for uterine and ovarian sampling as part of a larger experiment to study MRP were included in this experiment. All females were mated to a male of proven breeding capacity. Following mating, females were assigned randomly to 4 groups. Groups 1 and 2 alpacas were scheduled for postmortem collection of the reproductive tract and embryos on either day 9 (n=4) or day 14 (n=4) of pregnancy. Groups 3 and 4 females were scheduled for pregnancy diagnosis on day 14 post-mating. All females in groups 3 and 4 received an injection of 250 µg of cloprostenol (Estrumate®, Merck Animal Health, De Soto, KS, USA) intramuscularly after pregnancy diagnosis. Following luteolysis, the follicular wave was monitored again and ovulation was induced with GnRH (50 µg,

IM) when a follicle greater than 7 mm in diameter was observed in the ovaries. Females from groups 3 and 4 were scheduled for postmortem collection of the reproductive tract at day 9 (n=4) or day 14 (n=4) post-administration of GnRH respectively. All females were euthanized using an overdose of barbiturates on day 9 or 14. The uteri were collected en bloc immediately after euthanasia. Samples were transferred to 10% buffered formalin and fixed for 48 hours. Samples were then transferred to 70% ethanol and paraffin embedded for immunohistochemical analyses as described below.

2.5.4.1 Immunohistochemistry

For the immunohistochemical analyses, whole uterine tissue sections were fixed in 4% buffered paraformaldehyde (24 h at 4°C) followed by paraffin embedding. Paraffin-embedded uterine sections (5 μm) were deparaffinized with xylenes followed by graded rehydration in ethanol (100, 95, 80 and 70%) and distilled water. After peroxidase quenching (10 min in methanol containing 0.3% hydrogen peroxide) antigens were unmasked by high-temperature protein denaturation (2 min boiling in sodium citrate buffer (pH 6.0)). The sections were cooled for 30-45 minutes. After a 5-minute wash in buffer, sections were exposed to a 45-minute non-immunoblock using diluted Normal Goat Serum (Vectastain®; Vector Laboratories, Burlingame, CA, USA) in buffer in a humidified chamber at room temperature. Details of the antibodies used for detection of each receptor and conditions are specified in Table 2.5. Sections were then washed (PBS, 3 10 min each) and incubated for 1h at room temperature with biotinylated secondary antibody (1:500; Santa Cruz Biotechnologies, Santa Cruz, CA) followed by washing and a final incubation with horseradish peroxidase-conjugated streptavidin for 45min at room temperature (Vector

Laboratories, Burlingame, CA). Sections were exposed to 3,3-diaminobenzidine substrate, counterstained with methyl green, dehydrated in ethanol and xylenes, and mounted for light microscopy. Antibody specificity was confirmed in negative control experiments in which primary antibodies were omitted. Test dilutions of primary antibody were performed prior to assay.

2.5.4.2 Image analysis

A microscopic evaluation of receptor immunostaining for oxytocin receptors (OTR) was performed. A subjective image analysis was performed to estimate the expression of OTR in different cell types, luminal epithelium, glandular epithelium, stroma and myometrium. Two independent observers blinded for treatment performed the evaluation. Ten microscopic fields of view were analyzed for each cell type at different magnifications (10x, 20x and 1000x). The staining was scored from 0 to 3 (0= no staining, 1= 1-10% cell stained, 2= 11-50% cell stained, and 3= >50% cell stained). The intensity of the stain was scored from 1 to 2 (1= weak, and 2= moderate/strong). The scores from the two evaluators were averaged and the final score was assigned by multiplying the staining and the intensity scores.

2.6 Statistical Analyses

All statistical analyses were performed with SAS software (SAS Institute, Inc., Cary, NC). In Experiments 1 and 2, progesterone levels from the alpacas in the control and treatment groups were compared using repeated measure ANOVA. In Experiment 3, a Student's t-test was used for simple groups comparisons. Regardless of statistical test used, mean values were considered

statistically different when P≤ 0.05.

2.7 Results

2.7.1 Experiment 1

One of seven control alpacas had a spontaneous prolongation of the CL **(Alpaca 9330 Table 2.1 and 2.2)**; therefore, she was excluded from the study. None of the treated alpacas had prolonged CL function by 14 days post ovulation **(Table 2.1 and 2.2).** There was no significant difference (P≤ 0.05) in the duration of the CL activity and progesterone levels at each sampling point between the control and the treatment groups **(Table 2.3).** All alpacas underwent luteolysis by day 10 post-ovulation and a dominant follicle was present by day 14 post-induction of ovulation. In all animals, the progesterone concentration returned to below 1 ng/mL by day 14 post-ovulation. No adverse effects or reactions to the oxytocin treatment were observed in any of the alpacas.

2.7.2 Experiment 2

None of the treated alpacas had prolonged CL function by 14 days post ovulation **(Tables 2.4 and 2.5).** There was no significant difference (P≤ 0.05) in the duration of the CL activity and progesterone levels between the control and the treatment groups **(Table 2.6).** All alpacas underwent to physiology luteolysis by day 10 post-ovulation and they had a dominant follicle on their ovaries by day 14 post-induction of ovulation.

2.7.3 Experiment 3

Oxytocin receptors immunoreactivity was observed in the cell membranes and mostly in the luminal and superficial glands epithelium**.** There was slight individual variation between alpacas in intensity of staining **(Table 2.7).** The percentage of immunoreactive area and the staining of 10 fields were averaged between the two observers. Based on blinded scoring by two individuals and statistical analysis no significant difference ($P \leq 0.05$) was observed between pregnant and open animal at 9 or 14 days post-mating **(Fig. 2.6)**.

2.8. Discussion

To our knowledge, this is the first study where the effect of exogenous oxytocin administration during the luteal phase was evaluated in relation to the onset of luteolysis in alpacas. In addition, by considering the variation in expressions of oxytocin receptors in open and pregnant alpacas uteri, this study provides new insights into the possible mechanism regulating the process of luteolysis and MRP. We found that administration of 20 IU or 60 IU IM of oxytocin from day 3 through day 10 post-ovulation was not effective in preventing luteolysis or prolonging the CL function in alpacas. This suggests that oxytocin may not be involved in these physiologic mechanisms of luteolysis or maintenance of the corpus luteum in this species. All alpacas underwent luteolysis at the expected time and had a dominant follicle and toned edematous uterus by day 14. In a subset of alpacas, the size and blood flow of the corpora lutea were evaluated by transrectal ultrasonography and power Doppler ultrasonography, respectively. No significant

differences in the sizes or blood flow were found between control and treatments groups (Vaughan and Tibary, 2006). Serum progesterone concentrations were below 1.0 ng/mL by day 12 in all alpacas.

Although we excluded from the study the alpaca that experienced prolonged luteal maintenance, it is important to note that spontaneous prolongation of the luteal phase occurs in camelids (Adam et al., 1989). However, this is often noted in camelids with uterine metritis or endometritis in the postpartum period. Based on our results, oxytocin is not involved in the mechanism of luteolysis and MRP in alpacas. Camelids have a slower development of the corpus luteum and a shorter luteal phase compared to other domestic species, so it is possible that the timing of oxytocin administration used in the present study may not have been optimal. However, we chose to treat the alpacas with oxytocin from day 3 to day 10 post-induction of ovulation based on prior studies in mares (Stout and Allen, 1999; Vanderwall et al., 2012) and cows (Gilbert et al., 1989) where oxytocin given in mid-luteal phase was able to prolong the life of the CL instead of causing luteolysis.

There are no pharmacokinetic or pharmacodynamics studies in camelids regarding oxytocin, therefore in the first experiment, we chose a dose of 20 IU, which is 30% of the dose used in mares. In the mare, the persistence of luteal function was obtained with a dose of 60 IU which was based on the dose originally used in continuous oxytocin infusion (27.5 IU/h on day 8 through 20) (Stout and Allen, 1999) and the dose identified as luteostatic (Goff et al., 1987). The route of administration of oxytocin seems to also play a role in its efficacy to prolong luteal function. In one study, 80% of mare receiving 10 IU of oxytocin intravenously has prolonged luteal function compared to only 16% of mares receiving the same dose intramuscularly (Gee et al., 2012). Based on these studies in mares we rationalized that a dose between 10 and 60 IU should be appropriate

for alpacas. Although results of our experiment showed that IM injection of either 20 or 60 IU of oxytocin to alpacas during diestrus does not prolong CL lifespan, it is important to note that timing of the injection may influence the results. Further studies with different timing of injection are needed before stating that oxytocin doesn't have a role of luteolysis. Additionally, dose and method of administration may need to be further investigated.

Given the short half-life of oxytocin in mares (6.8 minutes)(Paccamonti et al., 1999), multiple doses may be needed to achieve clinical efficacy in alpacas.

Our study on the expression of oxytocin receptors did not reveal any significant difference between uteri from pregnant and non-pregnant alpacas. When combined with our oxytocin dosing scheme in Experiments 1 and 2, this finding suggests that oxytocin receptor regulation in not involved in the MRP or luteolysis. A study in open cyclic llamas did not find any difference in oxytocin receptors expression before and during luteolysis (Bianchi et al., 2013). The present research extended this study by going beyond the luteolytic period. Based on our hypothesis we expected higher OTR expression in open than pregnant animals. However, no significant difference between the two groups was found at Day 9 post-mating when luteolysis and MRP should normally occur. Also, we hypothesized a lower OTR expression at 14 days when in non-pregnant alpaca a dominant follicle should be present in the ovary and females are receptive to the male but no significant differences were observed as well. Although no histological differences in the OTR expression were present between the left and right uterine horns in the present study, it is possible that significant changes may be occurring at the molecular level. Further studies should look at the expression of estrogens receptors (ESR1) in pregnant and open animals to understand if estrogens actually play a role in the MRP in this species. Because all embryos migrate to the left horn, a greater expression of ESR1 in the right horn to promote its contraction was expected in a recent

work in our lab, but could not be demonstrated (Campbell, 2015). This suggests that a more complex mechanism with differential expression of ESR subtypes may be involved in embryo migration from the right to the left horn, assuming that estrogens are involved in MRP in camelids.

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CHAPTER 3

CONCLUSION

Maternal Recognition of Pregnancy (MRP) is the physiologic signal coming from the embryo that prevents luteolysis and allows the maintenance of the gestation. In the majority of the domestic species, this signal was identified. Either the suppression of the uterine release of PGF2α or the production of a substance that protect the CL from the luteolytic action of PGF2α are the most common mechanisms of MRP. In domestic ruminants: sheep, goats, and cattle, INFt produced by the trophoblast has the antiluteolytic action. In pigs the MRP is luteostatic meaning that PGF2α are produced but in an exocrine manner so that the CL is protected by their luteolytic action. In equine, the exact mechanism is unclear but seems being primarly untiluteolytic with the suppression of the production of PGF2α from the endometrium. In camelids the MRP is still unknown. Research regarding this aspect is driven by the impact of early pregnancy loss on productivity in this species. Early embryonic loss can account for up to 50% in the first 45 to 60 days of gestation in cameldis. Understanding the biologic mechanism of MRP is important in deciphering factors affecting pregnancy loss.

Studies on camelids propose a potential role of estrogens in the MRP since the embryo had a high aromatase activity during the time of luteolysis. Administration of estradiol to camelids during the MRP period have had contrasting results.

No studies have been carried out looking at the role of oxytocin in MRP in this species.

Oxytocin is integral component of the molecular mechanism behind the MRP in the other domestic species so this study wanted to answer the question: Is oxytocin involved in corpus

luteum function and maternal recognition of pregnancy in alpacas?

We concluded that IM administration of either 20 or 60 IU of oxytocin to alpacas from day 3 to day 14 after induction of ovulation did not prolong the CL lifespan. These results suggest that oxytocin may not be a major player in luteolysis in alpacas and therefore may have a minimal role in MRP. The limited role if any of oxytocin in MRP of alpaca is further substantiated by our finding that there are no significant difference in oxytocin receptors in the uteri from pregnant and non-pregnant alpacas around the time of MRP (day 9 after ovulation and conception) or after completion of elongation (Day 14 after ovulation and conception). Although this study has been conducted on a limited number of alpacas, the results strongly show that other mechanisms may be involved in inhibition of PGF2α release and prevention of luteolysis in pregnant alpacas.

Comparative genes expression in uteri from pregnant and non-pregnant diestrous alpacas would be of a great benefit in shedding more light on the mechanism(s) of MRP in camelids.

Table 2.1 Experiment 1: Serum progesterone concentrations (ng/mL) from the day of ovulation (day 0) through day 14 in seven untreated control alpacas.

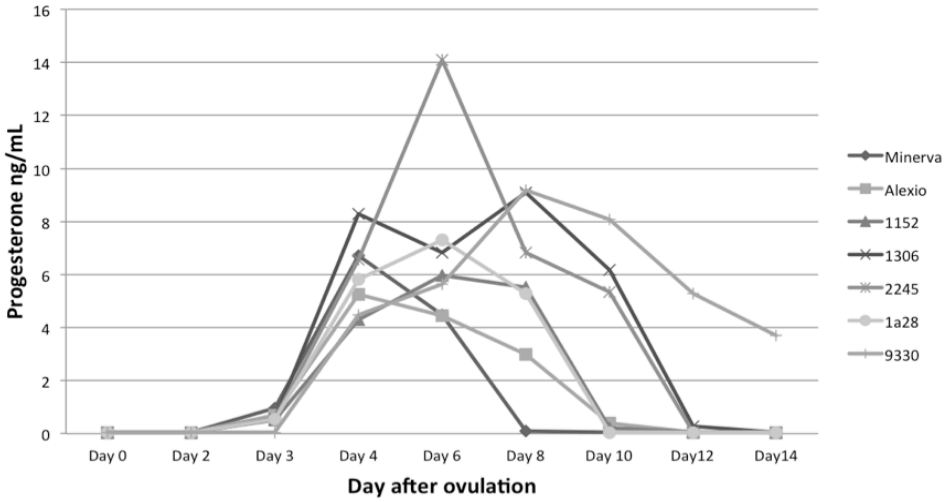

Table 2.2 Experiment 1: Serum progesterone concentrations (ng/mL) from the day of ovulation (day 0) through day 22 in seven alpacas treated with 20 UI of oxytocin intramuscularly daily from day 3 to day 10 post-ovulation. Notice the physiologic prolongation of the CL in Alpaca 9330.

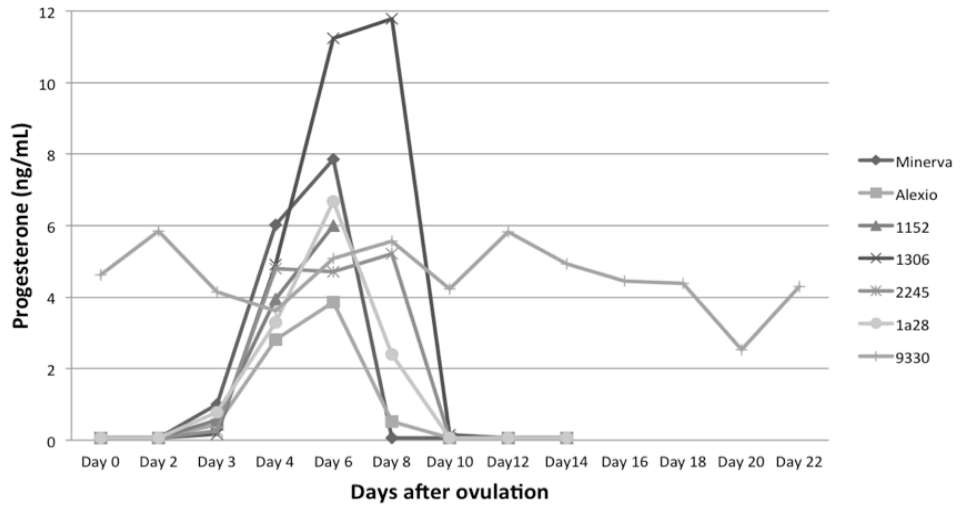

Table 2.3 Experiment 1: No significant differences (P< 0.05) in the serum progesterone levels (ng/mL) are present between the control and the animals treated with 20 IU of oxytocin.

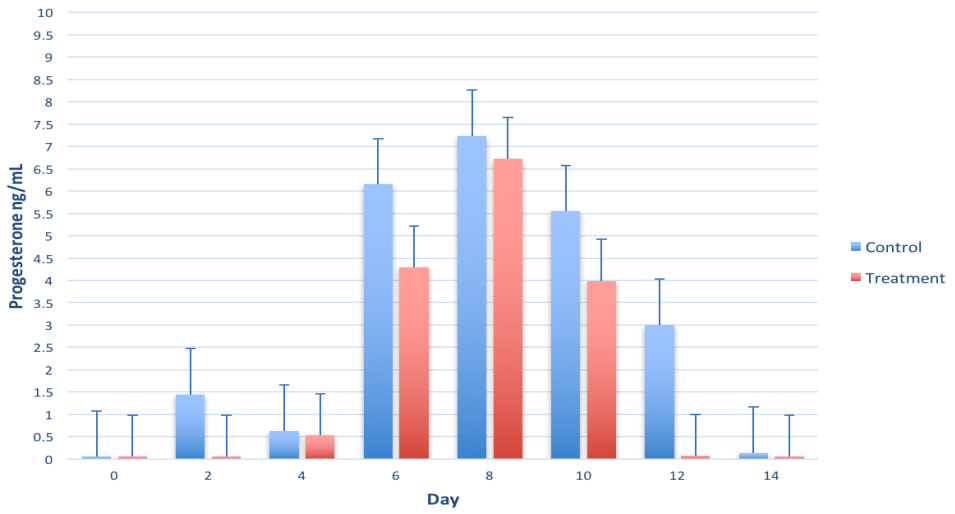

Table 2.4 Experiment 2: Serum progesterone concentrations (ng/mL) from the day of ovulation (day 0) through day 14 in eight untreated control alpacas.

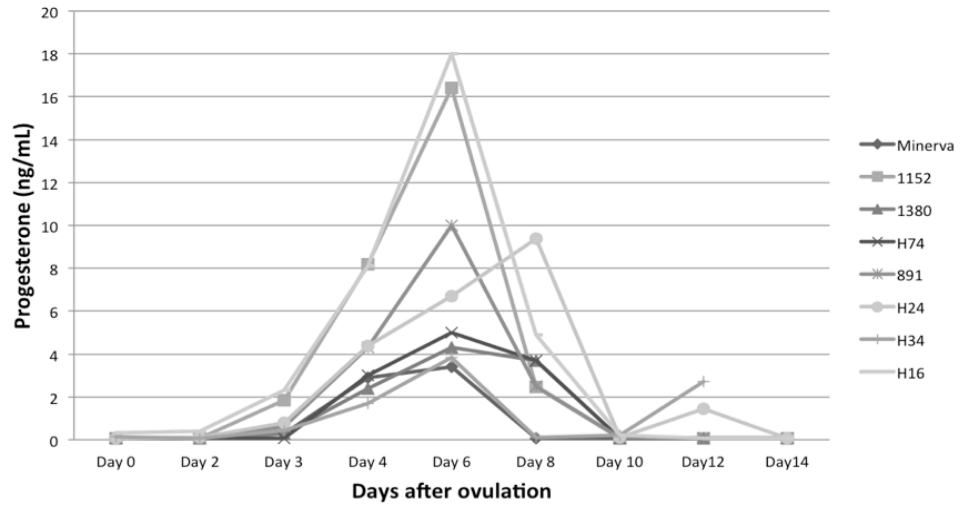

Table 2.5 Experiment 2: Serum progesterone concentrations (ng/mL) from the day of ovulation (day 0) through day 14 in eight alpacas treated with 60 UI of oxytocin intramuscularly daily from day 3 to day 10 post-ovulation.

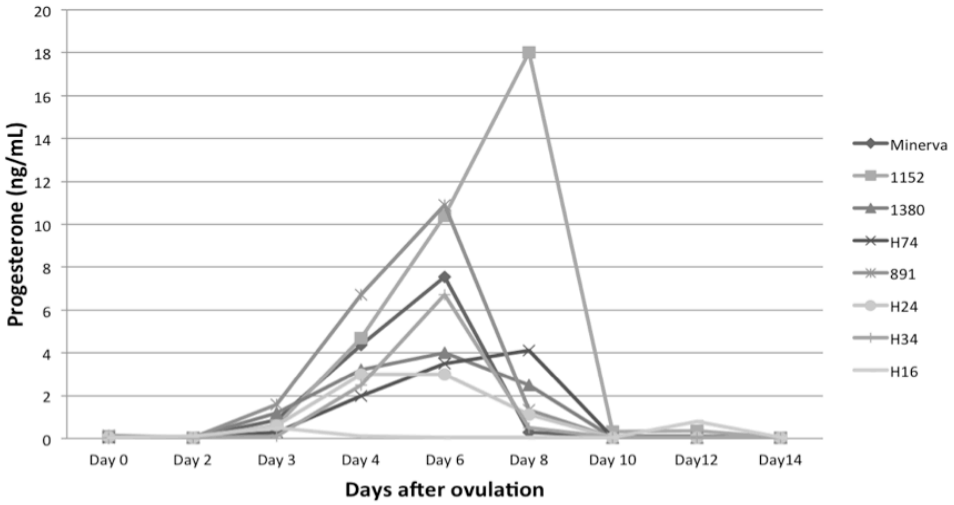

Table 2.6 Experiment 2: No significant differences (P<0.05) in the serum progesterone levels (ng/mL) are present between the control and the animals treated with 60 IU of oxytocin.

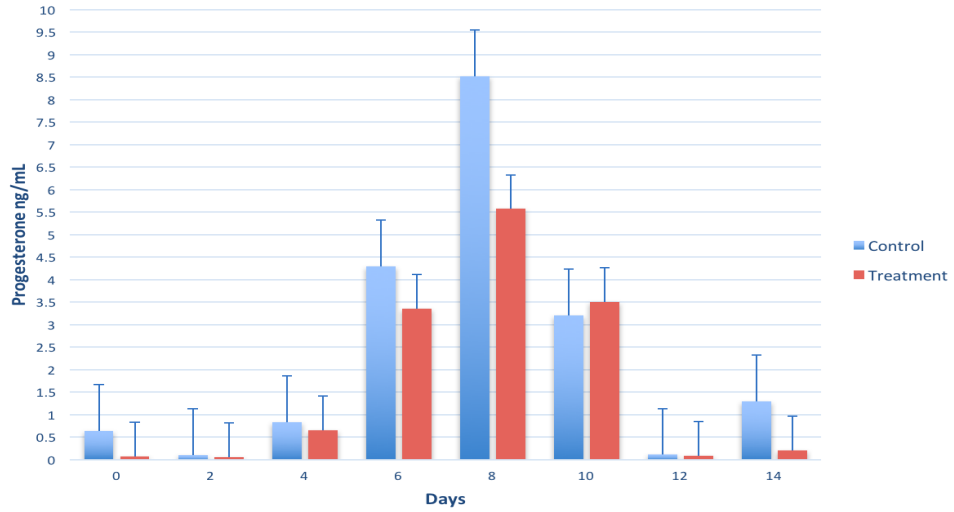

Table 2.7 Antibody used for the determination of endometrial OTR expression by immunohistochemistry

Receptor	Laboratory	Antibody	Dilution with PBS
OTR	Biorbyt LLC, California, USA	Polyclonal	1:100

Figure 2.1 Transrectal ultrasonography was used to monitor follicular development, confirmation of ovulation and luteal regression or persistency

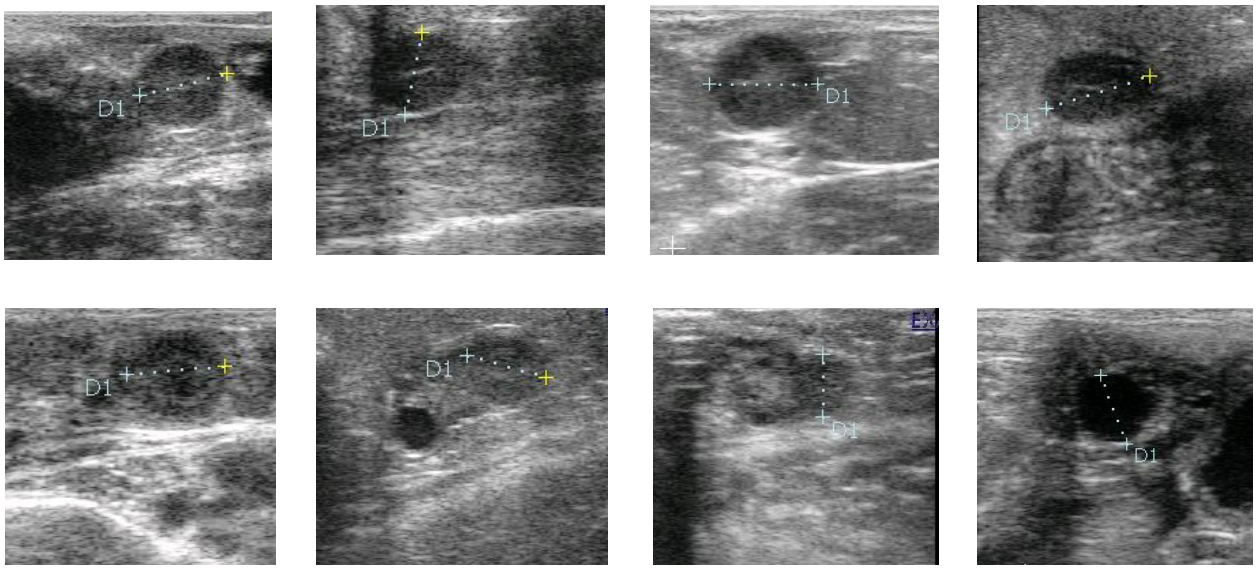

Figure 2.2 Transrectal ultrasonogram of ovaries demonstrating the presence of corpora lutea (CLs) and a dominant follicle in the last picture.

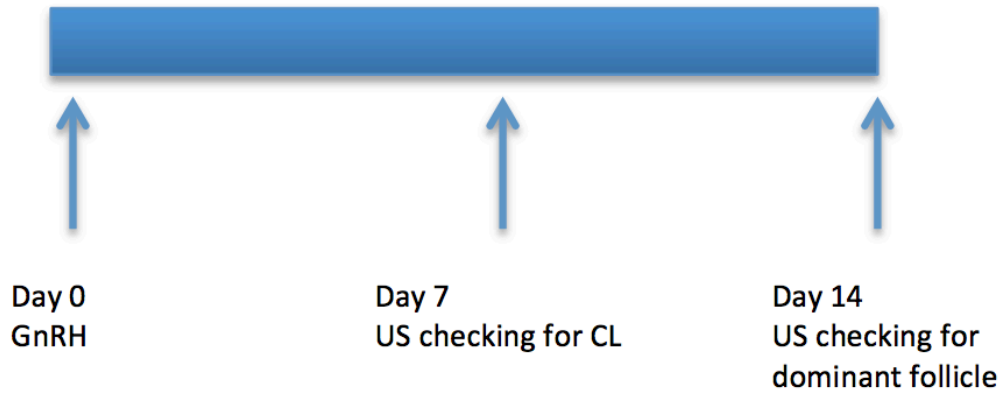

Figure 2.3 Protocol for the control groups.

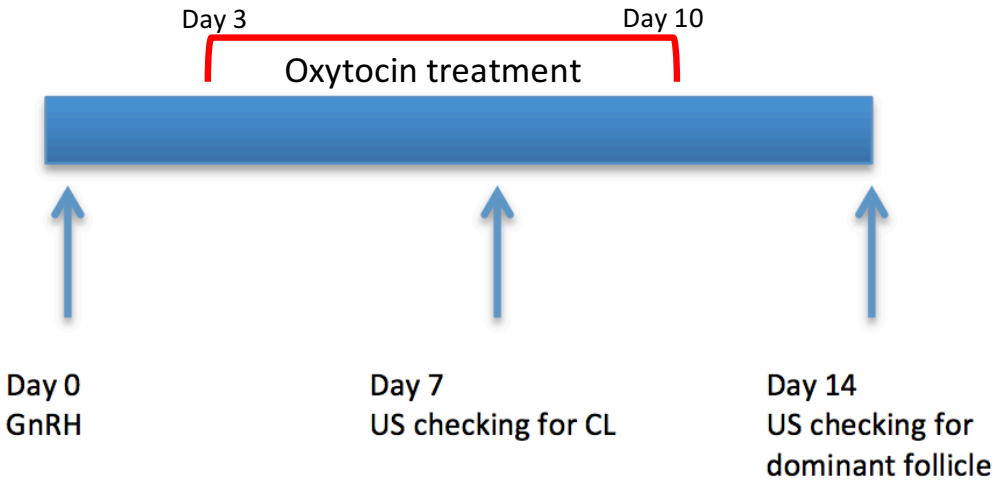

Figure 2.4 Protocol for treatment groups.

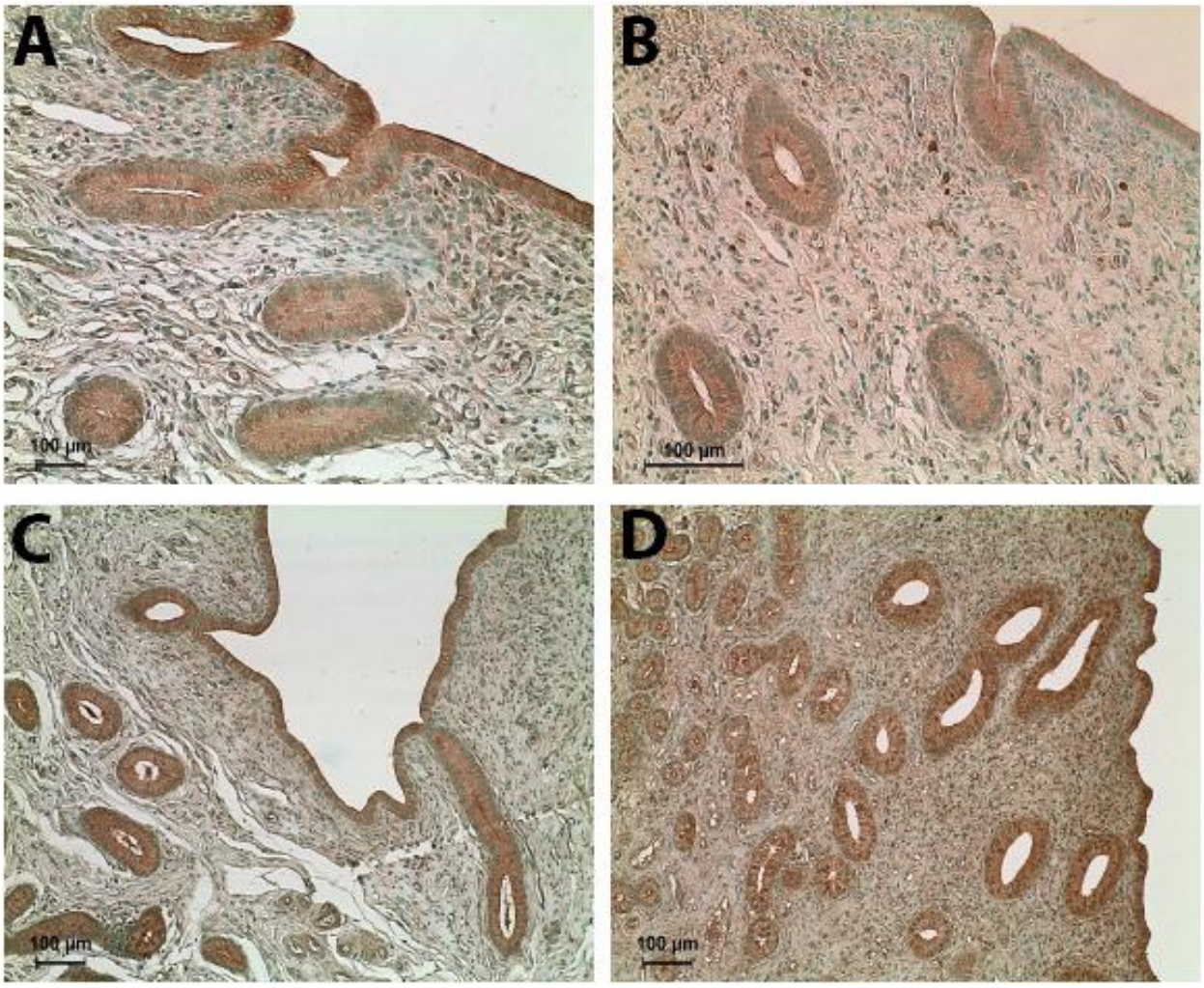

Figure 2.5 Experiment 3: Immunohistochemical localization of OTR in the endometrium in open (A and B) and pregnant (C and D) alpacas (n=4 per each group). (A) 9 days post-mating (200x); (B) 14 days post-mating (200x); (C) 9 days post-mating (100x); (D) 14 days post-mating (100x).

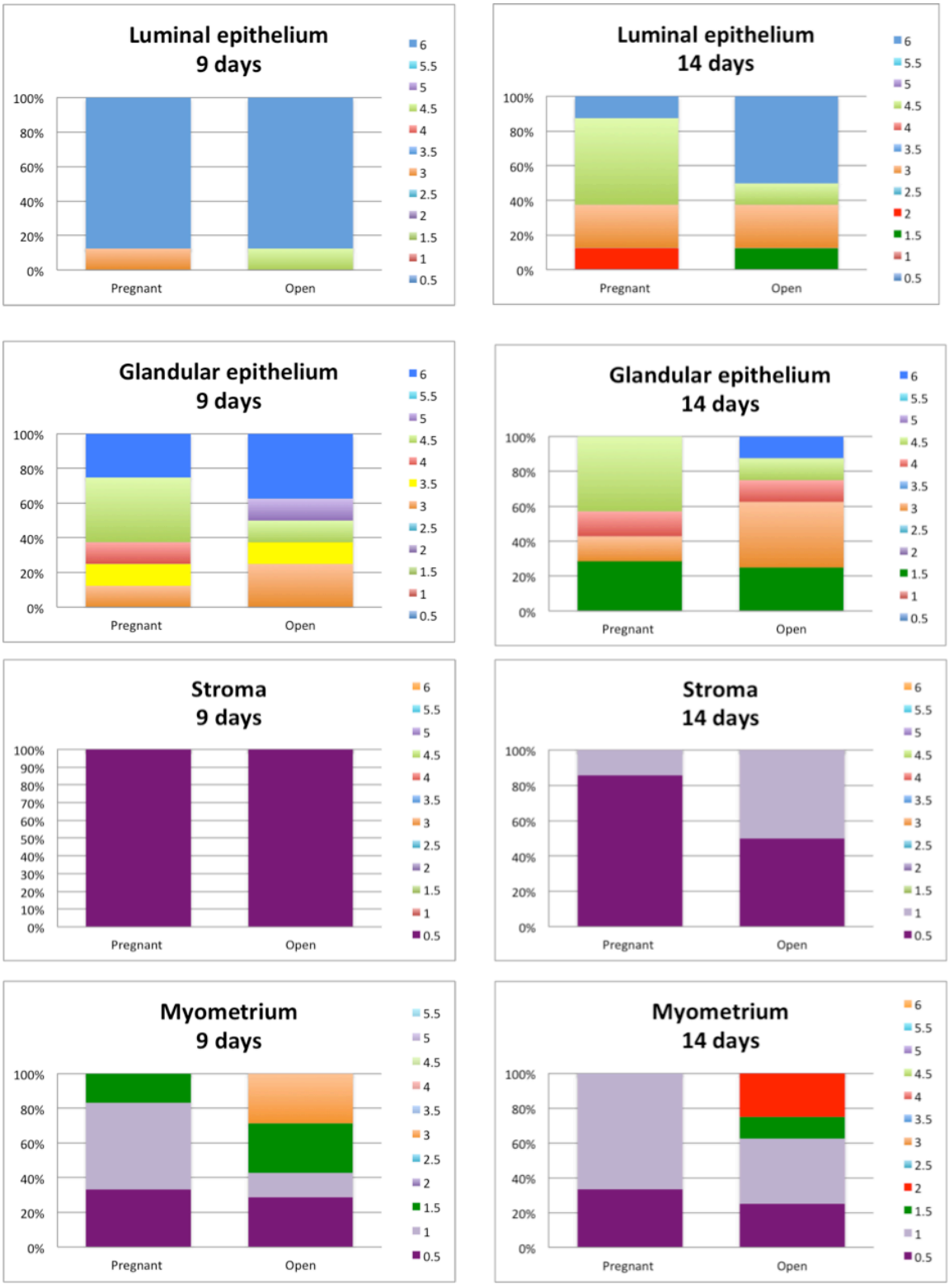

Figure 2.6 Experiment 3: Contingency graphs that show the immunoexpression of the oxytocin receptor in the different areas of the uterus at 9 and 14 days post-mating. The score represents the intensity and the percentage of cellular membranes immunoreactive for the oxytocin receptor.