THE EFFECT OF OVULATION-INDUCING FACTOR (OIF) IN BOVINE SEMINAL PLASMA ON OVARIAN FUNCTION IN CATTLE

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University of Saskatchewan

Saskatoon

by

Paula Tríbulo

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ABSTRACT

Three experiments were designed to gain an understanding of the role of ovulation-inducing factor (OIF) present in bovine seminal plasma. Within species, seminal plasma was pooled from 1 to 4 ejaculates per male (n=160 bulls, n=4 llamas in Experiments 1 and 2, and n=95 bulls in Experiment 3). The volume of seminal plasma used for treatment was adjusted to a total dose of 250 µg of OIF. Experiment 1 was done to verify the bioactivity of OIF in bovine seminal plasma. Mature female llamas were assigned randomly to be treated intramuscularly (i.m.) with either 10 ml of phosphate buffered saline (PBS, negative control, n=5), 50 µg GnRH (positive control, n=5), 6 ml of llama seminal plasma (n=6) or 12 ml of bull seminal plasma (n=6). Experiment 2 was done to determine the effect of OIF in bovine seminal plasma on LH-induced ovulation and luteal development. Beef heifers with a CL and a growing follicle ≥10 mm were given a luteolytic dose of prostaglandin followed by 25 mg pLH 12 h later. Heifers were assigned randomly to three groups and given 10 ml bovine seminal plasma i.m. 12 h after pLH treatment (n=10), bovine seminal plasma i.m. within 4 h after ovulation (n=9), or no further treatment (control, n=10).Experiment 3 was done to study the effect of OIF in bovine seminal plasma on LH release, ovulation and luteal development. Ovulation in beef heifers was synchronized using a protocol with progesterone and estradiol. Six days after ovulation, , when a mature CL and a dominant follicle of 11-13 mm diameter were expected to be present, heifers were assigned randomly to four groups (n=8 per group) using a 2-by-2 design and treated with either pLH or phosphate-buffered saline i.m., followed 12 h later by treatment with either 10 ml bovine seminal plasma or phosphate-buffered saline i.m.; i.e., LH+PBS, LH+SP, PBS+SP, and PBS+PBS groups. In all experiments, ovulation and CL development were monitored by transrectal ultrasonography. In Experiment 1, llamas were scanned daily from treatment to Day 6 after

treatment, while in the other two experiments ovulations were monitored every 4 h and CL development was monitored daily until the next ovulation. Ovulation rates were compared among groups by Fisher's exact test, and continuous data were compared among groups by ANOVA for repeated measures. Single point data were compared by ANOVA. In Experiment 1, ovulation was detected in 0/5, 4/5, 4/6, 4/6 in PBS, GnRH, llama seminal plasma, and bovine seminal plasma groups, respectively ($P<0.05$). No difference was detected among groups in luteal development. In Experiment 2,. All ovulations in the pre-ovulation treatment group occurred within a 4 h period, while the range for other groups was 22 h ($P<0.0001$). No difference was detected among groups in luteal development; however, plasma progesterone concentrations tended to be greater in the heifers treated with seminal plasma post-ovulation compared to the other two groups (treatment-by-day interaction, $P=0.1$). In Experiment 3, ovulations were detected in 5/8, 4/8, 0/7, 0/8 in pLH, pLH+BSP, BSP and control groups, respectively ($P<0.05$). Corpora lutea present at the time of treatment took longer to decrease significantly in size from the time they reached maximum size in heifers treated with seminal plasma ($p=0.04$), but, plasma progesterone concentrations did not differ among groups during this same period. Nevertheless, there was a more rapid increase in plasma progesterone concentration by 24 h after seminal plasma treatment than those not treated with seminal plasma ($P=0.03$). Results confirm the presence of bioactive OIF in bull seminal plasma and showed that bovine and llama seminal plasma have similar ovulatory and luteotrophic effects using a llama bioassay. Moreover, treatment of sexually mature heifers with OIF from bovine seminal plasma influenced the timing of ovulation and the duration of luteal function.

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DEDICATION

I dedicate this thesis to my parents María Manuela del Prado and Ricardo Tríbulo. They have always trusted me and encouraged me to follow my dreams even when that meant to be far away from home. Thank you for the values you seeded in me and for your unconditional love and support. I also dedicate this thesis to my husband Marcos Zenobi. He's the bearer of unconditional things, the best listener that I've ever met, and my best friend. Thanks for your patience and love! Lastly, I would like to dedicate this thesis to my son, Lautaro, who allowed me to work on this thesis until the day before he was born …

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LIST OF ABBREVIATIONS

°C	Celsius degrees
3β-OH-SDH	3β-hydroxysteroid dehydrogenase
µg	Micrograms
µM	Micrometers
MHz	Megahertz
ANOVA	Analysis of the variance
BMP-15	Bone morphogenetic protein 15
BSP	Bovine seminal plasma
cAMP	Cyclic adenosine monophosphate
CL	Corpus luteum
CIDR	Controlled internal drug releasing device
Cx-43	Connexin 43 protein
DF	Dominant follicle
eCG	Equine chorionic gonadotropin
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinases

FGF-8	Fibroblast growth factor-8
FSH	Follicle stimulating hormone
G	Grams
GDF-9	Growth differentiation factor
GnRH	Gonadotropin releasing hormone
HA	Hydroxy-apatite column
hCG	Human chorionic gonadotropin
h	Hours
i.m.	Intramuscular
IU	International units
kDa	Kilodalton
kg	Kilogram
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
LSD	Least significant difference
min	Minutes
mm	Millimetres

mg	Milligrams
ml	Millilitres
mRNA	Messenger ribonucleic acid
ng	Nanograms
NGF	β-nerve growth factor
OIF	Ovulation-inducing factor
P450scc	P450 side-chain cleavage enzyme
PBS	Phosphate buffered saline
PGE	Prostaglandin E
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
pLH	Porcine luteinizing hormone
SAS	Statistical analysis system
SEM	Standard error of the mean
SP	Seminal plasma
StAR	Steroidogenic acute regulatory protein
TrkA	NGF tyrosine kinase receptor
xg	times of centrifugal force

1 GENERAL INTRODUCTION

Mammals are classified as either induced-ovulators or spontaneous-ovulators, based on the hypothalamic stimulus responsible for eliciting GnRH release into the hypophyseal portal system and, in turn, triggering LH release from the pituitary and ultimately ovulation (Bakker and Baum, 2000).

In induced-ovulators (e.g., camelids, ferrets, cats, koalas), ovulation does not occur regularly. Genital somatosensory stimuli during mating were traditionally thought to be the trigger of the preovulatory LH surge (Bakker and Baum, 2000). As an example, ovulation occurred after mating in llamas (England et al., 1969), alpacas (San-Martin et al., 1968), and Old World camels (Shalash and Nawito, 1964). In addition, a luteal phase has been induced in koalas by copulation (Johnston 2000).

Conversely, in species that are spontaneous-ovulators (e.g., human, dogs, pigs, horse, goats, sheep, cattle), ovulation is a regular cyclic event. Preovulatory LH release is elicited after a certain threshold in systemic estradiol concentrations is surpassed (Chenault et al., 1975; Karsch, 1987), leading to a shift from a negative to a positive feedback effect of estradiol on gonadotropin secretion.

Due to the sporadic nature of the distribution of induced and spontaneous ovulators within the phylogenetic tree, no conclusion has been drawn regarding evolutionary trends (Asdell, 1946). However, it has been suggested that spontaneous-ovulators evolved from induced-ovulators (Conaway, 1971). Therefore, it is not surprising that spontaneous-ovulators may share some characteristics of the induced-ovulators as a mere vestige of evolution. Of special interest for the

present thesis work is the presence of an ovulation-inducing factor (OIF) in the seminal plasma of both groups of mammals.

The dogma of physical stimulation during coitus as a requisite for ovulation in these species arose many years ago with the conclusion of a classic study which stated that mounting with penile intromission was crucial to induce ovulation in alpacas (Fernandez-Baca et al., 1970). However, this well accepted dogma was later challenged with the introduction of the notion that a chemical substance present in ejaculates of Bactrian camels provoked ovulation in females of the same species (Chen et al., 1985), and that ovulation took place even after intramuscular injection of Bactrian camel seminal plasma (Pan et al., 1992). An endocrine response to copulation, presumably with semen emission, resulted ina rise in plasma LH concentration (Bravo et al., 1992) But did not determine wheter physical stimulation alone, semen emission or both were required to induce LH release. Stimulation of rat pituitary cells *in-vitro* with alpaca seminal plasma resulted in LH secretion (Paolicchi et al., 1999). The presence of an ovulation-inducing factor in seminal plasma of llama and alpaca that triggers a peak of circulating concentrations of LH and induces an ovulatory and luteotrophic response was reported (Adams et al., 2005). Similarly, the existence of OIF in rabbit seminal plasma has been recently documented (Silva et al., 2011).

An even more interesting discovery, however, was the presence of OIF in the seminal plasma of spontaneous-ovulators. It is now known that seminal plasma of horses, pigs, and cattle contain variable amounts of OIF (Ratto et al., 2006; Bogle et al., 2011). In addition, heifers mated to a vasectomized bull during the first 6-8 hours of behavioral estrus ovulated earlier compared to non-mated heifers (Marion et al., 1950). Furthermore, an effect of llama seminal plasma on

follicular dynamics in cattle has been documented (Tanco et al., 2012). The effect of seminal plasma on female reproductive physiology in pigs has also been reported (Signoret et al., 1972; Waberski et al., 1997; O'Leary et al., 2006).

Recently, the identity of OIF has been investigated revealing that its amino acid sequence had homology with human, porcine, bovine and murine sequences of ß-nerve growth factor (NGF; (Ratto et al., 2012). In this literature review, we will refer to NGF of seminal plasma origin as OIF, so as to distinguish it from NGF whose origins are other than those of the male reproductive tract.

Participation of NGF in ovarian function has been reported in mice (Dissen et al., 1996) and sheep (Barboni et al., 2002). However, the link between OIF present in seminal plasma and the role of NGF in ovarian function have not been established. The overall goal of the studies reported herein is to gain an understanding of the role of the OIF present in the bovine seminal plasma on the ovarian function of cattle.

1.1 Reproductive physiology in cattle

Follicular development during the bovine estrous cycle occurs in a wave pattern (Rajakoski, 1960; Pierson and Ginther, 1987), associated with recurrent periods of elevated circulating FSH concentration (Adams et al., 1992). Follicle stimulating hormone is responsible for the recruitment of a group (wave or cohort) of follicles that grow simultaneously (Adams et al., 1992). The cohort produces estrogen and inhibin, consequently, FSH begins to decline resulting in granulosa cell apopotosis, and loss of estrogen activity and gonadotropin receptors; i.e., atresia occurs in all but one "selected" follicle. The selected or dominant follicle (DF), acquires LH receptors and becomes LH-responsive. Therefore, the availability of LH determines whether the

DF regresses or ovulates. Selection of the DF takes place around three days after follicular wave emergence, when the largest follicle is around 8.5 mm in diameter in *Bos taurus* cattle (Ginther et al., 1997). Progesterone suppresses LH pulse frequency (Reviewed in Karsch, 1987; Adams et al., 1993); hence, the growing DF present at the time of luteolysis becomes the ovulatory follicle (Ginther et al., 1989a). Luteolysis is a programmed event that occurs around Day 16 or 19 in 2-wave and 3-wave estrous cycles, respectively (Ginther et al., 1989a; Ginther et al., 1989b; Knopf et al., 1989). The mechanism of luteolysis is mediated by progesterone secreted by the CL, as well as oxytocin and $PGF_{2\alpha}$ secreted by the uterus (Shirasuna et al., 2004; Ginther et al., 2007). Oxytocin receptors are expressed in the endometrium as a result of exposure to high levels of progesterone followed by estrogen. Production of $PGF_{2\alpha}$ is induced by the interaction of oxytocin and its receptors. Prostaglandins reach the CL through the utero-ovarian vascular counter-current diffusion system, causing luteolysis (Senger, 1997; Ginther et al., 2007). The corpus luteum in the cow can be detected 2 days after ovulation by ultrasonography and reaches its maximum size by Day 9 to 10 after ovulation (Kastelic et al., 1990). Plasma progesterone concentrations exceed 2 ng/ml by Day 4 or 5 and are maximal by Days 8 to 10 (Kastelic et al., 1990).

1.2 Reproductive physiology of camelids

Old World (Bactrian and Dromedary camels) and New World (llamas and alpacas) camelids are induced-ovulators. As mentioned previously, little is known about the mechanism of ovulation in these species, but in general, ovulation does not take place unless there is an external stimulus responsible for eliciting LH release from the pituitary gland. Llamas and alpacas have similar reproductive behaviour (San-Martin et al., 1968; England et al., 1971). The ovarian pattern may be divided into two main periods, the follicular phase and the luteal phase (Adams et al., 1989).

Follicular development occurs in a wave-like manner (Adams et al., 1990; Ratto et al., 2006), and is characterized by extended periods of sexual receptivity (San-Martin et al., 1968). A luteal phase occurs only if ovulation occurs resulting in a functional corpus luteum that secretes progesterone. In non-pregnant females, the luteal phase is 10-12 days long (Adams et al., 1990) and non-receptivity is the typical behaviour during this physiological status.

Follicles of at least 7 mm in diameter are capable of ovulating in female llamas and alpacas; smaller follicles and those regressing at the time of copulation will not ovulate (Adams et al., 1990; Vaughan et al., 2004). Although follicular growth has been associated with rising concentrations of circulating estradiol in llamas and alpacas, estradiol does not have a positive feedback on surge-release of LH (Bravo et al., 1991; Bravo et al., 1992) as occurs in spontaneous ovulators. Similarly, LH secretion was not induced after administration of exogenous estradiol, with or without progesterone, in rabbits and ferrets (Sawyer and Markee, 1959; Baum et al., 1990).

1.3 Ovulation

Mammalian ovulation is an exceptional biological phenomenon which implies the physical rupture of healthy tissue at the surfaces of the ovary and the release of an oocyte into the tubular female reproductive tract. The event begins when an ovulatory surge of LH stimulates a mature ovarian follicle. The reaction of LH with G-protein-coupled LH receptors on the plasma membranes of the theca and granulosa cells in the follicle leads to ovulation (Davis, 1994; Espey and Lipner, 1994; Richards, 1994; Richards et al., 1998; Richards et al., 2002).

The difference between induced and spontaneous ovulating species resides in the trigger for an increase in circulating LH concentrations. Much is known about spontaneously ovulating species

since it includes human, most laboratory species, and most domesticated species. Conversely, mechanisms involved in the ovulation cascade in induced-ovulators remain unclear.

1.3.1 Ovulation in cattle

In cattle, estradiol secreted by a dominant follicle is the factor responsible for eliciting the preovulatory surge of LH (Karsch, 1987). As a consequence of luteolysis, systemic progesterone concentrations decrease and LH pulsatility increases (Rahe et al., 1980). As a result of high LH pulse-frequency, the dominant follicle grows and secretes more estradiol that, after surpassing a certain threshold, will have a positive feedback effect on the hypothalamus. When circulating estradiol concentrations reach a critical threshold, a preovulatory LH surge is induced (Karsch, 1987).

1.3.2 Ovulation in camelids

The mechanisms implicated in elicitating the pre-ovulatory LH surge remain unclear. Stimulation of GnRH secretion as a consequence of coitus-associated stimuli has been reported in rabbits with a subsequent increase in systemic LH concentrations (Ramirez et al., 1986). In cats, however, one single mating did not consistently elicit LH release (Wildt et al., 1980). Three mating stimuli over an 8-h interval resulted in a rise in circulating LH concentrations and consequent ovulation in cats (Wildt et al., 1980). Similarly, a study carried out in llamas showed that systemic LH concentrations started to rise 15 minutes after mating, peaked at 2 hours, and returned to basal levels by 7 hours (Bravo et al., 1992). Although it was concluded that mating induces ovulation, , studies were not designed to determine the factors causing ovulation. The presence of an ovulation-inducing factor in the seminal plasma of induced-ovulators was first reported by researchers in China who concluded that a chemical stimulus was responsible for

causing ovulation (Chen et al., 1985). Ovulation occurred after intravaginal or intramuscular administration of seminal plasma in Bactrian camels (Chen et al., 1985; Xu et al., 1985; Pan et al., 1992). More recent studies have documented the existence of OIF in the seminal plasma of llamas, alpacas (Adams et al., 2005) and rabbits (Silva et al., 2011). Further, intramuscular administration of OIF purified from llama seminal plasma caused LH release and ovulation in llamas (Ratto et al., 2011). Results of this study confirmed the previously proposed hypothesis that OIF acts through a systemic pathway (Ratto et al., 2005). In addition, they confirmed that OIF was not a GnRH-like molecule based on its molecular mass (Ratto et al., 2011) and on the fact that immuno-neutralization of GnRH in OIF-stimulated pituitary cells did not suppress *in-vitro* LH secretion (Paolicchi et al., 1999). This hypothesis has been supported by the recent documentation of the identity of OIF; its amino acid sequence and structure are homologous with human, porcine, bovine and murine sequences of ß-NGF (Ratto et al., 2012).

1.4 Actions of the preovulatory LH surge on fully grown follicles

The role of LH in the final maturation of the preovulatory follicle involves several pathways that promote the initiation of oocyte maturation, cumulus cells expansion, differentiation of granulosa cells into progesterone-secreting cells (i.e. luteinization), and rupture of the follicular wall to allow ovulation (Amsterdam et al., 1975). Although LH receptors are present on both granulosa and theca cells (Xu et al., 1995), within the granulosa cells their distribution is confined to the mural granulosa cells. In addition, LH receptors are highly expressed in those layers closer to the basement membrane compared to those closer to the antrum (Peng et al., 1991). Consequently, LH needs second messengers to be able to affect the entire follicle and the oocyte. The effect of second messengers is facilitated by the intimate relationship among granulosa cells and cumulus cells and the oocyte through gap junctions (Lawrence et al., 1978). The two main second

messengers for LH are cAMP (Marsh, 1970; Cooke, 1999) and intracellular calcium (Kosugi et al., 1995). The first increases as a result of the adenyl cyclase enzyme stimulation (Marsh et al., 1974), while the latter is augmented through the phospholipase C enzyme pathway (Kosugi et al., 1995).

The multiple effects of LH are promoted in a strategic manner along the follicular cell layers (Park and Mayo, 1991) by, autocrine and paracrine pathways induced by the LH surge. Steroidogenesis is up-regulated through activation of StAR, P450scc (Strauss et al., 1999; Stocco, 2000) and 3β-ol-SDH (Gougeon, 1977), and it is inhibited by ERK (Seger et al., 2001). Oocyte maturation and resumption of meiosis is a consequence of the increase in cAMP. Luteinizing-hormone-induced EGF production is also involved in the process of oocyte maturation (Dekel and Sherizly, 1985; Goud et al., 1998; Prochazka et al., 2000). In addition, factors secreted by the oocyte modulate cumulus cell activity. Growth differentiation factor-9 (GDF-9), bone morphogenetic protein 15 (BMP-15) and fibroblast growth factor-8 (FGF-8) are defined as key paracrine agents originating in the oocyte (Dube et al., 1998; Su et al., 2009). These three key factors regulate the metabolic activity of cumulus cells to provide the oocyte with those products that it is not able to produce such as certain amino acids, glycosides and cholesterol (Su et al., 2008). Growth differentiation factor-9, BMP-15 and FGF-8 also modulate steroidogenesis and expansion of cumulus cells (Elvin et al., 1999; Yoshino et al., 2006). Cumulus expansion is also accomplished by the action of mural granulosa cells, in which, LH-induced prostaglandin (Lim et al., 1997; Hizaki et al., 1999) and EGF (Eppig, 1981; Eppig, 1991) production promotes cumulus expansion and hyaluronic acid synthesis (Downs and Longo, 1983). Follicular rupture is a consequence of collagenolysis and plasminogen activator, the latter is induced by LH through the prostaglandin pathway (Canipari et al., 1995). Nerve

growth factor has also been shown to be involved in this event by inhibiting communication through gap junctions among theca cells (Mayerhofer et al., 1996). Luteinizing hormone induces the phosphorylation of Cx-43, the primary component of gap junctions, hence the communication among granulosa cells is broken down. Oocyte-secreted factors, on the contrary, prevent collagenolysis within cumulus cells by inhibiting plasminogen activator in those cells (Elvin et al., 1999). Progesterone has been also implicated in the process of follicle wall rupture Intrafollicular injection of an inhibitor of synthesis of progesterone (isoxazol) prevented follicles from ovulation. This effect was reversed by systemic administration of progesterone, and by intrafollicular administration of collagenase (Murdoch et al., 1986). The role of progesterone in ovulation is also suggested by the upregulation of the expression of progesterone receptors by six hours after LH preovulatory surge (Cassar et al., 2002). Moreover, progesterone-receptor knocked out mice treated with LH showed luteinization of granulosa cells but not ovulation (Lydon et al., 1995; Robker et al., 2000).

1.5 Role of nerve growth factor and its receptor in ovulation

Nerve growth factor is synthesized in thecal cells of antral follicles in rats and sheep (Dissen et al., 1996; Mattioli et al., 1999). However, in human, pig, goat and cattle, it is produced in both granulosa and theca cells (Dissen et al., 2000; Ren et al., 2005; Seifer et al., 2006; Jana et al., 2011). In an experiment with rats, the LH preovulatory surge induced expression of Trk-A (NGF tyrosine kinase receptor) and production of NGF nine hours preceding ovulation (Dissen et al., 1996). In addition, pharmacological inhibition of NGF and blockage of Trk-A prevented ovulation in rats (Dissen et al., 1996). Similarly, the preovulatory LH surge stimulated an increase in NGF concentrations in follicular fluid in sheep (Barboni et al., 2002). Nerve growth factor has also been associated with the rupture of the follicular wall at ovulation through

inhibition of gap junctional communications between theca cells, (Mayerhofer et al., 1996) and proliferation of bovine theca cells (Dissen et al., 2000). The mechanism by which NGF breaks down cell-cell communication in the theca layer has been studied. Bovine thecal cells were cultured, and transfected with Trk-A expression vector to be able to evaluate its effect, regardless of LH action. They observed that cell-cell communication remained in those cultured cells via Connexin 43 protein (Cx43). However, after NGF treatment, they responded with a reduction in the number of gap junctions. These results suggest that NGF/Trk-A may be an important link in the process of follicular wall degradation (Mayerhofer et al., 1996). This finding, together with the reports that after NGF treatment, there is a proliferation of theca cells (Dissen et al., 2000) provide evidence that NGF is implicated in two crucial events leading to ovulation.

1.6 Role of NGF and its receptor in luteinization and luteal function

Nerve growth factor has been associated with steroid hormone production. The hypothesis that NGF may have an effect on steroid secretion arose in response to the finding of both NGF and Trk-A in human granulosa cells isolated from preovulatory follicles (Salas et al., 2006). In a study using human ovarian cells treated with NGF, a significant increase of FSH receptor mRNA levels was observed. Moreover, granulosa cells previously treated with NGF and subsequently treated with FSH released significantly larger amounts of estradiol. A decrease in progesterone production in these cells was also observed. Thus, it was concluded that NGF and the activation of its high affinity receptor are essential to maintain granulosa cell production of estradiol, and to prevent premature luteinization (Salas et al., 2006). In another experiment NGF/TrkA stimulated androstenedione and progesterone secretion in freshly plated, high density bovine thecal cell cultures. This study revealed that NGF also promotes prostaglandin E2 (PGE) release from bovine theca cells which is cyclooxigenase-2-independent (Dissen et al., 2000). Similar results

were obtained in rat ovaries; PGE production was attenuated after blocking Trk-A pharmacologically, or inhibiting NGF immunologically (Dissen et al., 1996). Based on these reports, it is evident that the role of NGF in steroidogenesis is controversial. While some groups found that LH induced NGF receptors (Trk-A) and an increase in NGF promoted progesterone and PGE synthesis, others suggest that Trk-A and NGF stimulate estradiol production, prevent premature luteinization, and reduce progesterone production. It is worthy of note that the latest findings were not associated with the preovulatory LH surge, providing a potential reason for this controversy. It is clear that LH stimulates NGF/Trk-A, but the different pathways followed by this system need to be further studied and related to time.

Nerve growth factor and its receptor Trk-A have been observed in luteal tissue in gilts (Jana et al., 2011), and goats (Ren et al., 2005). In cattle, in vitro treatment with NGF of luteal cells obtained from CL at different stages of the estrous cycle resulted in an acute release of progesterone (Miyamoto et al., 1992) providing evidence that NGF does affect progesterone secretion from luteal cells.

1.7 Ovulation-inducing factor (OIF)

Ovulation-inducing factor has been named after its function, since it causes the initiation of the ovulatory cascade in induced ovulator species (Chen et al., 1985; Ratto et al., 2006; Bogle et al., 2011). Since this substance is secreted into the seminal plasma it is believed that it is produced by the accessory glands of the male. To date, OIF has also been documented in the seminal plasma of bulls (Ratto et al., 2006), boars and stallions (Bogle et al., 2011) and rabbits (Silva et al., 2011). The fact that seminal plasma of these species induces ovulation in llamas led to the conclusion that OIF in seminal plasma is well conserved among species (Bogle et al., 2011).

1.7.1 Ovulation-inducing factor in camelids

Ovulation-inducing factor is a protein present in the seminal plasma of camelids (Pan et al., 2001; Li and Zhao, 2004; Ratto et al., 2011). Two laboratories reported the isolation of OIF from seminal plasma of Bactrian camel by anion-exchange chromatography. The first group of researchers reported that OIF consists of a peptide with 74 residues with GnRH-like bioactivity. The authors discarded the possibility of homology of OIF with LHRH, LH, hCG, eCG or $PGF_{2\alpha}$ due to the partial amino acid sequences and molecular weight (Pan et al., 2001). The second laboratory separated the seminal plasma of Bactrian camels obtaining six fractions. The bioactivity of two fractions was revealed by induction of ovulation in female camels after intramuscular treatment. The effect of these fractions on gonadotrophin secretion differs from that of seminal plasma treatment. The authors proposed that the different bioactive fractions may interact, and that interrupting this interaction may prevent the action of OIF (Li and Zhao, 2004). The biochemical isolation and purification of OIF in seminal plasma of llamas has recently been documented (Ratto et al., 2011). Three protein fractions were obtained by liquid chromatography, and their bioactivity assessed using a llama bioassay. Ovulation was detected in 0/10 and 2/10 llamas treated with Fraction A and B, respectively, and 10 of 11 treated with Fraction C (Ratto et al., 2011). Results documented that a 14 KDa protein elicited a preovulatory LH surge and induced ovulation and CL development in all treated llamas

Comparison of the effect of different routes of administration of OIF on ovarian function in alpacas led the authors to conclude that OIF acts through a systemic pathway (Ratto et al., 2005). Studies in llamas and alpacas revealed an increase in systemic LH concentration after intramuscular treatment with seminal plasma supporting the idea of a systemic effect of OIF (Adams et al., 2005; Bogle et al., 2011). A more recent study further supported this hypothesis

by showing that gonadotroph cells from the anterior pituitary gland release LH in response to *in vitro* OIF stimulation (Bogle et al., 2012). Furthermore, by assessing the LH response to OIF treatment of ovariectomized llamas with and without priming with estradiol, it was concluded that systemic concentrations of estradiol modulate OIF effect on LH release (Silva et al., 2012).

In addition to its key role in initiating the ovulatory cascade, OIF appears to have luteotrophic properties (Adams et al., 2005; Ratto et al., 2006; Tanco et al., 2011). Compared to GnRH treatment for the induction of ovulation in llamas, intramuscular administration of seminal plasma caused the CL to grow longer and reach a greater size (Adams et al., 2005). In the same study, plasma progesterone concentrations were twice as high in llamas treated with llama seminal plasma than those given GnRH. A more recent study concluded that treatment of llamas with pure OIF resulted in a dose-dependent effect on ovulation and luteal development; i.e. greater day-to-day CL diameter profile and higher systemic progesterone concentrations (Tanco et al., 2011). Similarly, a dose-related effect was observed after *in vitro* stimulation of llama pituitary cells with pure OIF from llama seminal plasma (Bogle et al., 2012).

The potency of OIF to elicit ovulation is high, considering that the amount of OIF contained in 1/60 of a llama ejaculate induced 90% of llamas to ovulate (Tanco et al., 2011). It is noteworthy, however, that a study in llamas and alpacas concluded that the concentration of OIF varies among males and among ejaculates within males (Bogle et al., 2011). Hence, it is important to consider that the conclusion that the concentration of OIF present in 1/60 of a llama ejaculate induced 90% llamas to ovulate was based on pooled llama seminal plasma, avoiding the effect of individual variations in seminal plasma composition.

1.7.2 Ovulation-inducing factor in cattle

The effect of seminal plasma on ovarian function in spontaneous ovulators has been documented (Marion et al., 1950; Signoret et al., 1972; Waberski, 1995) leading several research groups to investigate the existence of OIF in the seminal plasma of these species. The presence of OIF in bovine seminal plasma was first suggested in 2006 based on the capacity of bovine seminal plasma to induce ovulation in llamas after intramuscular injection (Ratto et al., 2006). Pure OIF from llama seminal plasma has been used as treatment in cattle to evaluate the effect of OIF on ovarian function (Tanco et al., 2012). Although pure llama OIF did not induce ovulation in prepubertal beef heifers, it was associated with a rise in systemic FSH concentrations and early follicular wave emergence. In sexually mature heifers, OIF treatment was also associated with modifications in follicular dynamics and luteotrophic effects (Tanco et al., 2012). Therefore, these pieces of evidence showing an effect of OIF in female reproductive function, led us to design a series of experiments to test the effect of OIF from bovine seminal plasma on ovarian function in cattle.

2 OBJECTIVES AND HYPOTHESES

The overall objective was to gain an understanding of the role of ovulation-inducing factor (OIF) present in bovine seminal plasma, and thereby contribute to our understanding of the ovulatory mechanism in cattle.

The specific objectives of the first study (Chapter 3) were to:

- Verify the bioactivity of ovulation-inducing factor in bovine seminal plasma.
- Compare the effect of bovine seminal plasma and llama seminal plasma on llamas when dose is adjusted according to OIF quantity.
- Assess the effect of bovine seminal plasma on LH-induced ovulation in heifers.
- Evaluate the effect of bovine seminal plasma on luteal function.

The hypotheses tested were:

- Bovine seminal plasma contains bioactive OIF.
- Bovine seminal plasma induces ovulation and CL development in llamas in a comparable manner as llama seminal plasma when OIF dose is adjusted to be similar.
- Ovulation-inducing factor in bovine seminal plasma enhances LH-induced ovulation and causes luteotrophic changes in the ovulatory follicle of heifers; it enhances form and function of the incipient CL.

The specific objectives of the second study (Chapter 4) were to:

- Test the capability of OIF in bovine seminal plasma to induce ovulation during the luteal phase in cattle.
- Investigate the relationship between bovine seminal plasma and LH on the synchrony of ovulations.
- Determine the effect of bovine seminal plasma on LH release.
- Evaluate the effect of bovine seminal plasma on the luteal phase.

The hypotheses tested were:

- Ovulation-inducing factor in bovine seminal plasma synchronizes LH-induced ovulations (Address objective 2).
- Ovulation-inducing factor in bovine seminal plasma induces ovulation by enhancing LH release (Address objectives 1 and 3).
- Ovulation-inducing factor in bovine seminal plasma enhances CL form and function (Address objective 4).

3 BIOACTIVITY OF OVULATION INDUCING FACTOR/NERVE GROWTH FACTOR (OIF/NGF) IN BOVINE SEMINAL PLASMA AND ITS EFFECTS ON OVARIAN FUNCTION IN CATTLE

3.1 Abstract

Two experiments were designed to gain an understanding of the role of ovulation-inducing factor (OIF) present in bovine seminal plasma. The objective of Experiment 1 was to test the hypothesis that bovine seminal plasma induces ovulation and CL development in llamas in a manner comparable to that of llama seminal plasma when the dose of seminal plasma is adjusted to OIF/NGF content. The objective of Experiment 2 was to determine the effect of bovine seminal plasma on the interval to ovulation, and luteal development in heifers. Within species, seminal plasma was pooled from 1 to 4 ejaculates per male (n=160 bulls, n=4 llamas). The volume of seminal plasma used for treatment was adjusted to a total dose of 250 μg of OIF/NGF. In Experiment 1, mature female llamas were assigned randomly to four groups and treated i.m. with either 10 ml of phosphate buffered saline (PBS, negative control, n=5), 50 μg GnRH (positive control, n=5), 6 ml of llama seminal plasma (n=6), or 12 ml of bull seminal plasma (n=6). Ovulation and CL development were monitored by transrectal ultrasonography. In Experiment 2, beef heifers were given a luteolytic dose of prostaglandin followed by 25 mg pLH 12 h later. Heifers were assigned randomly to three groups and given 12 ml bovine seminal plasma i.m. 12 h after pLH treatment (n=10), 12 ml bovine seminal plasma i.m. within 4 h after ovulation (n=9), or no further treatment (control, n=10). Ovulation was monitored by ultrasonography every 4 h and CL development was monitored daily until the next ovulation. Ovulation rates were compared among groups by Fisher's exact test, and continuous data were compared among groups by analyses of variance. In Experiment 1, ovulation was detected in 0/5,

4/5, 4/6, 4/6 in PBS, GnRH, llama seminal plasma, and bovine seminal plasma groups, respectively ($P<0.05$). Luteal development was not different among groups. In Experiment 2, the interval to ovulation was more synchronous (range: 4 h vs. 22 h; $P<0.0001$) in heifers treated with seminal plasma before ovulation compared to the other groups. Luteal development was not different among groups. However, plasma progesterone concentrations tended to be greater in the post-ovulation treatment group compared to other groups (treatment-by-day interaction, $P=0.1$). In summary, results confirmed the presence of bioactive OIF/NGF in bull seminal plasma and supported the hypothesis that bovine and llama seminal plasma have similar ovulatory and luteotrophic effects using a llama bioassay. Treatment with bovine seminal plasma resulted in greater synchrony of ovulation in heifers pre-treated with pLH. Plasma progesterone concentration tended to be higher in heifers given bovine seminal plasma within 4 h after ovulation, suggesting that bovine OIF/NGF is luteotrophic.

3.2 Introduction

The presence of an ovulation-inducing factor has been documented in the seminal plasma of induced ovulators such as llamas, alpacas (Ratto et al., 2006) and Bactrian camels (Pan et al., 2001). This substance is a protein that provokes ovulation by triggering LH release, and has luteotrophic properties in llamas and alpacas (Adams et al., 2005). The results of recent studies provide evidence for the presence of OIF in the seminal plasma of spontaneous ovulators such as cattle (Ratto et al., 2006), horses, and pigs (Bogle et al., 2011). In a previous study (Ratto et al., 2006), bovine seminal plasma induced ovulation in 26% (5/19) of llamas compared to 0% (0/19) in the placebo-treated group, but proportionately less than llamas treated with alpaca or llama seminal plasma (19/19, 100%). Treatments, however, were based on volume of seminal plasma; the actual dose of OIF was unknown. In a more recent study (Tanco et al., 2011), OIF from llama seminal plasma had a dose-dependent effect on ovulation rate, CL diameter and progesterone production in llamas. Recently, OIF in seminal plasma has been shown to be identical to βNGF (Ratto et al., 2012), and will be hereafter referred to as OIF/NGF.

The role of seminal plasma as the trigger of the ovulation event has been documented in some induced ovulators; i.e., camelids (Chen et al., 1985; Pan et al., 1992; Zhao et al., 1992; Adams et al., 2005) and koalas (Johnston et al., 2004). Although spontaneous ovulators do not require a copulatory stimulus to ovulate, the importance of seminal plasma and its components has been investigated. In pigs, intrauterine infusion of seminal plasma near the onset of estrus advanced ovulations (Waberski, 1995). In another study in pigs, no effect on ovulation was detected, but an increase in CL size and progesterone secretion was documented after intrauterine infusion of porcine seminal plasma (O'Leary et al., 2006). Although uterine exposure to seminal plasma in mice did not alter CL development or progesterone secretion in one study (Gangnuss et al.,

2004), intramuscular treatment of prepubertal mice with llama seminal plasma induced ovulation at a rate similar to treatment with GnRH (Bogle et al., 2011). In an early study in dairy cattle, ovulations occurred earlier in heifers mated to a vasectomized bull compared to those not mated (Marion et al., 1950). Authors of a more recent study concluded that intracervical deposition of seminal plasma improved pregnancy rates marginally in cows with compromised fertility (i.e., conception rates below 50%; (Odhiambo et al., 2009). Although pure llama OIF/NGF did not induce ovulation in prepubertal heifers, it was associated with a rise in systemic FSH concentrations and early follicular wave emergence (Tanco et al., 2012). In sexually mature heifers, OIF/NGF treatment was also associated with modifications in follicular dynamics and had luteotrophic effects (Tanco et al., 2012).

The objectives of the present study were to test the bioactivity of OIF/NGF present in bovine seminal plasma, and to study its effect on ovulation and luteal development in cattle. In Experiment 1, we tested the hypothesis that bovine seminal plasma induces ovulation and CL development in llamas in a manner similar to that of llama seminal plasma when the dose of seminal plasma is adjusted to the concentration of OIF/NGF. In Experiment 2, we tested the hypothesis that OIF/NGF in bovine seminal plasma enhances LH-induced ovulation and causes luteotrophic changes in the ovulatory follicle; i.e., enhances form and function of the incipient CL.

3.3 Materials and Methods

3.3.1 Experiment 1

3.3.1.1 Seminal plasma

Ejaculates were collected from four mature male llamas (5 to 7 years old) 2 to 3 times a week over a period of 2 months using an artificial vagina inserted into a wooden phantom (Lichtenwalner et al., 1996). Semen samples were processed according to procedures previously described (Adams et al, 2005). Briefly, ejaculates were diluted 1:1 (v:v) with phosphate buffered saline (PBS, Invitrogen, Grand Island, NY), drawn back-and-forth through an 18-guage needle attached to a 10 ml syringe to reduce viscosity, and centrifuged at 1500 x g for 30 minutes. The supernatant was transferred to a new tube and the pellet was discarded. A drop of the supernatant was evaluated by microscopy to confirm the absence of cells. If spermatozoa were detected, the sample was centrifuged and evaluated again until no spermatozoa were detected. Sperm-free seminal plasma was stored at -80ºC.

Ejaculates (2 to 4 ml each) were collected from 160 bulls by electro-ejaculation (Pulsator III; Lane Manufacturing, Denver, CO, USA) using a 75 mm in diameter rectal probe with three ventrally-oriented electrodes. Ejaculates collected on the same day (n = 10 to 50) were pooled and kept at 4ºC until transport to the laboratory. The pooled semen was centrifuged for 15 minutes at 500, 1000 and 1500 x g. After each centrifugation, the supernatant was transferred to a new tube, and the pellet was discarded. A drop of seminal plasma was examined by microscopy to confirm the absence of spermatozoa. If cells were detected, the sample was centrifuged again. The seminal plasma was stored at -80ºC. To obtain sufficient volume for treatments, pools of seminal plasma from different days were thawed and combined to form a

single large pool of bovine seminal plasma which was filtered through a 0.22 µ syringe filter (Millipore, Bradford, MA, USA). The filtered seminal plasma was stored frozen at -80ºC.

Total protein was estimated in two aliquots of each of the pooled llama and bovine seminal plasma by spectrometry (Bradford method, Bio-Rad Laboratories, Philadelphia, PA, USA). The concentration of OIF/NGF was estimated in two aliquots of each of the pooled llama and bovine seminal plasma using a double-antibody radio-immunoassay. A standard curve was made using known concentrations of purified OIF of 0, 1, 10, 25, 50, and 100 ng/ml in PBS. Purified OIF/NGF was iodinated with ^{125}I. The first antibody used in the double antibody radioimmunoassay was rabbit polyclonal against NGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The second antibody was goat anti rabbit. The minimum detectable limit of the assay was 10 ng/ml. Samples were analyzed in one assay and low- and high-reference samples (1 and 200 ng/ml) were distributed throughout the assay. The intra-assay coefficients of variation were 1.1% and 3.5% for low and high reference concentrations, respectively.

3.3.1.2 Animals, treatments, and ultrasonography

Mature, non-lactating female llamas (n=30), ≥4 years of age and weighing between 90 and 120 kg were used during May and June at the University of Saskatchewan, Canada (52^{o}N, 106^{o}W and 500 m above sea level). Llamas were given a single intramuscular dose of pLH (5 mg Armour standard; Lutropin- V, Bioniche Animal Health, Belleville, Ontario, Canada) to synchronize follicular wave emergence (Ratto et al., 2006). The ovaries were examined by transrectal ultrasonography using a 7.5 MHz linear-array transducer (MyLab5 VET, Canadian Veterinary Imaging, Georgetown, Ontario Canada) 12 days after LH treatment; i.e., enough time to allow CL regression in those that ovulated and for all to have developed a new mature

dominant follicle. Llamas with a follicle ≥8 mm were assigned randomly to four groups and given a single intramuscular dose of 1) 10 ml of phosphate buffered saline (PBS, negative control, n=5), 2) 50 µg GnRH (Fertiline, Vetoquinol N-A Inc., Lavaltrie, QC, Canada; positive control, n=5), 3) 6 ml of llama seminal plasma (n=6), or 4) 12 ml of bull seminal plasma (n=6). Selection of the dose of seminal plasma was based on previous studies in which 250 µg of purified OIF/NGF induced ovulation in 90% of llamas (Tanco et al., 2011). The ovaries were examined daily by transrectal ultrasonography from Day 0 (day of treatment) to Day 6 to detect ovulation and to monitor CL development (Adams et al., 1991). Ovulation was defined as the sudden disappearance of a large follicle (≥8 mm) from one examination to the next, and confirmed by the detection of a CL in subsequent examinations (Adams et al., 1989).

3.3.2 Experiment 2

3.3.2.1 Seminal plasma

The pooled bovine seminal plasma used for Experiment 1 was also used for Experiment 2.

3.3.2.2 Animals, treatments and ultrasonography

Aberdeen Angus cross-bred heifers, between 12 and 14 months of age and weighing between 315 and 422 Kg, were used during the month of June at the University of Saskatchewan Canada (52^{o}N, 106^{o}W and 500 m above sea level). Heifers were selected from a herd of 36 head based on detection of a CL by transrectal ultrasonography using 7.5 MHz linear-array transducer (MyLab5 VET, Canadian Veterinary Imaging, Georgetown, Ontario Canada). Selected heifers were fed alfalfa/grass hay and grain to gain approximately 1.3 Kg per day and had water ad libitum during the experimental period. When a CL and a growing follicle between 9 and 11 mm in diameter was detected, based on daily ultrasonographic examination on 3 consecutive days,

heifers were treated with a luteolytic dose of $PGF_{2\alpha}$ i.m. (25 mg Lutalyse; Pfizer Canada Inc.; Montreal, QC, Canada). Heifers were given 25 mg Armour standard pLH i.m. (Lutropin-V, Bioniche Animal Health, Belleville, Ontario, Canada) 12 h after $PGF_{2\alpha}$ treatment, and assigned randomly to three groups treated with 1) 250 μg OIF/NGF in bovine seminal plasma 12 hours after LH treatment (pre-ovulatory treatment n=10), 2) 250 μg of OIF/NGF in bovine seminal plasma ≤4 hours after ovulation (post-ovulatory treatment n=9), or 3) no further treatment (control n=10). Heifers were examined by transrectal ultrasonography every 4 hours beginning 22 h after pLH treatment until ovulation to determine the interval to ovulation. Transrectal ultrasonography was done once daily thereafter until the next ovulation to monitor luteal function.

3.3.2.3 Blood sampling and hormone assays

To measure plasma progesterone concentration, blood samples were taken by coccygeal venipuncture into heparinized tubes (Vacutainer Systems; Becton Dickinson, Franklin Lakes, NJ, USA) daily until the following ovulation. Plasma progesterone concentration was measured using a commercially available double-antibody radioimmunoassay kit (Coat-a-Count total progesterone; Diagnostic Products Corporation, Los Angeles, CA; (Adams et al., 1991). Samples were analyzed in one assay with intra-assay coefficients of variation of 4.4%, 3.6% and 2.8% for reference concentrations of 1.9, 3.6 and 16.6 ng/ml, respectively.

3.3.3 Statistical analyses

Ovulation rates were compared among groups by Fisher's exact test. Non-serial data (i.e., follicle size at treatment, maximum CL diameter, day of maximum CL diameter, and first day of CL detection) were compared among groups by one-way analyses of variance to determine the effect

of treatment. Serial data (CL diameter and plasma progesterone concentration) were compared among groups by analysis of variances for repeated measures to determine the effect of treatment over time. The covariance structure that best suited the data set for Experiment 1 was spatial covariance structure (power). In Experiment 2, the covariance structures that best suited the CL diameter and plasma progesterone profiles, respectively, were heterogeneous first order autoregressive and compound symmetry. Tukey's multiple comparison was used as a post-hoc test when a main effect or interaction was detected. Significance was assumed when the probability of values differing by chance alone was ≤ 0.05. Data were centralized to the day of treatment (Experiment 1) or ovulation (Experiment 2) for preparation of figures. In Experiment 2, to determine the effect of bovine seminal plasma treatment on ovulation, data from the control and post-ovulation treatment groups were combined to compare with the pre-ovulation treatment group. Although increasing the number of replicates in the control group decreases the variance, data were combined since at the moment of ovulation these groups were in identical conditions. Variances were heterogeneous between groups; hence, data was not compared statistically. The degree of ovulation synchrony was compared between the two groups by t-test of the absolute values of residuals in the interval to ovulation. All statistical analyses were made using SAS software (SAS, Statistical Analysis System Institute Inc., Cary, NC, USA).

3.4 Results

3.4.1 Experiment 1

Based on the concentration of OIF/NGF in pooled seminal plasma (Table 3.1), a volume of 12 ml of bovine seminal plasma and 6 ml of llama seminal plasma was used to obtain a treatment dose of 250 μg of OIF/NGF.

Table 3.1. Total protein and OIF/NGF concentrations in pooled bovine and llama seminal plasma (SP) used to determine the final volume of seminal plasma for treatment in Experiment 1.

Treatment	Total protein (mg/ml)	OIF/NGF (μg/ml)	Dilution	Dose (μg)	Volume (ml)
Pooled Bovine SP	75.2	20.5	0	250	12.2
Pooled Llama SP	1.7	80.0	1:1	250	6.3

The diameter of the largest follicle at the time of treatment did not differ among groups Table 3.2). The proportion of llamas that ovulated in response to treatment (i.e., within 2 days of treatment) was similar among the GnRH, llama seminal plasma and bull seminal plasma groups, all of which were higher than in the PBS group Table 3.2.

Table 3.2. Effect of intramuscular treatment with phosphate buffered saline (PBS, negative control), GnRH (positive control), or seminal plasma (llama SP or bull SP) on ovulation in female llamas (mean ± SEM).

End point	PBS (n=5)	GnRH (n=5)	Llama SP (n=6)	Bovine SP (n=6)
Follicle size at treatment (mm)	9.4 ± 0.67	9.9 ± 0.78	9.9 ± 0.78	9.3 ± 0.43
Llamas that ovulated	0/5[a] (0%)	4/5[b] (80%)	4/6[b] (66%)	4/6[b] (66%)

[ab] Within rows, values with different superscripts are different ($P<0.05$)

Of the llamas that ovulated, no differences were detected among groups in the diameter of the largest follicle at the time of treatment, day of first detection of the CL, maximum CL diameter, or day of maximum CL diameter (Table 3.3). Similarly, the day-to-day CL diameter profile did not differ among groups (Figure 3.1).

Table 3.3. Comparison of the effect of treatment with GnRH and seminal plasma (llama SP, bull SP) on CL development in llamas that ovulated in response to treatment (mean ± SEM; Day 0 = day of treatment).

End point*	GnRH (n=4)	Llama SP (n=4)	Bovine SP (n=4)
Follicle size at treatment (mm)	10.4 ± 0.80	8.9 ± 0.55	8.8 ± 0.48
Day of 1st detection of CL	2.2 ± 0.43	3.7 ± 0.75	2.2 ± 0.25
Maximum CL diameter (mm)	14.5 ± 2.58	11.1 ± 0.67	12.6 ± 1.28
Day of maximum CL diameter	5.2 ± 1.12	7.0 ± 0.71	5.2 ± 0.63

* No differences were detected among groups

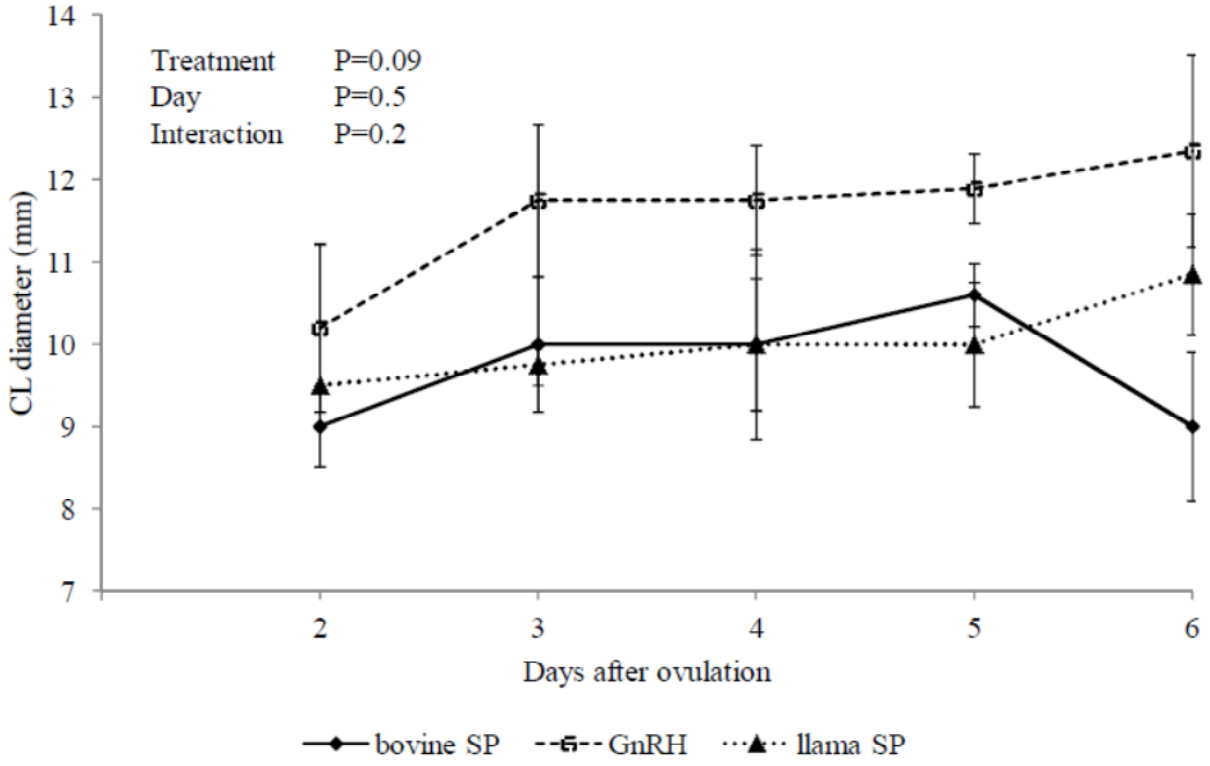

Figure 3.1. CL diameter in llamas that ovulated after treatment with GnRH (n=4), bovine seminal plasma (n=4), or llama seminal plasma (n=4). Day 0 = day of ovulation.

3.4.2 Experiment 2

The size of the pre-ovulatory follicle did not differ among groups, and all heifers ovulated (

Table 3.4.). All ovulations in the pre-ovulation treatment group occurred within a 4 h period, while the range for others was 22 h (

Table 3.4.). Greater synchrony in the pre-ovulation treatment group compared to others ($P<0.0001$) was reflected in analysis of the absolute values of the residuals of interval to ovulation (

Table 3.4.), and is illustrated in

Figure 3.2.

Table 3.4. Effect of intramuscular treatment with bovine seminal plasma on LH-induced ovulations in heifers (mean ± SEM). Comparison is made between heifers treated before ovulation (pre-ovulation) vs. others (i.e., those treated post-ovulation and control groups combined; Experiment 2).

End point	Pre-ovulation (n=10)	Others (n= 19)
Follicle size at PGF treatment (mm)	9.7 ± 0.23	9.7 ± 0.24
Follicle size at LH treatment (mm)	10.6 ± 0.24	10.9 ± 0.23
Follicle size before ovulation (mm)	10.9 ± 0.34	11.4 ± 0.35
Interval from LH to ovulation (h)	33.2 ± 0.53[x]	31.3 ± 1.02[y]
Range in interval to ovulation (h)	30 - 34	26 - 44
Synchrony of ovulations (absolute value of residuals)	1.9 ± 0.05 [a]	3.0 ± 0.75 [b]

[ab] Within rows, values with different superscripts are different (P<0.05)
xy within rows, values were not compared statistically since variances were heterogeneous.

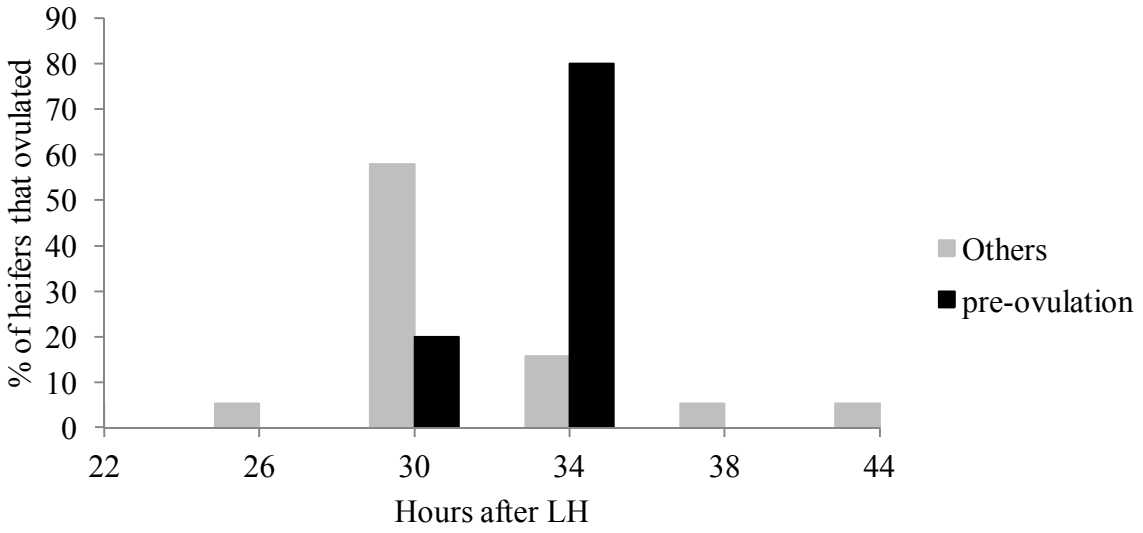

Figure 3.2. Distribution of ovulations after LH treatment in heifers treated with bovine seminal plasma pre-ovulation (n=10) and others (n=19, i.e., post-ovulation seminal plasma treatment and control groups combined; Experiment 2).

No difference was detected among groups in maximum CL diameter, the day of maximum CL diameter, or the day the CL was first detected (Table 3.5.). The day-to-day diameter profile of CL changed over time (P<.0001), but profiles did not differ among groups (

Figure 3.3). However, plasma progesterone concentrations tended to be greater in the post-ovulation treatment group compared to others (treatment effect, P=0.2; day effect, P<0.0001; treatment-by-day interaction, P=0.1; Figure 3.4).

Table 3.5. Effect of intramuscular treatment with bovine seminal plasma on CL development (mean ± SEM; Day 0 = day of ovulation)

End point*	Pre-ovulation (n=10)	Post-ovulation (n=9)	Control (n=10)
Maximum CL diameter (mm)	17.6 ± 0.88	18.6 ± 1.07	19.2 ± 1.22
Day of maximum CL diameter	7.0 ± 0.63	7.1 ± 1.01	6.0 ± 0.46
Day the CL was first detected	1.9 ± 0.28	2.1 ± 0.26	1.4 ± 0.24

* No differences were detected among groups

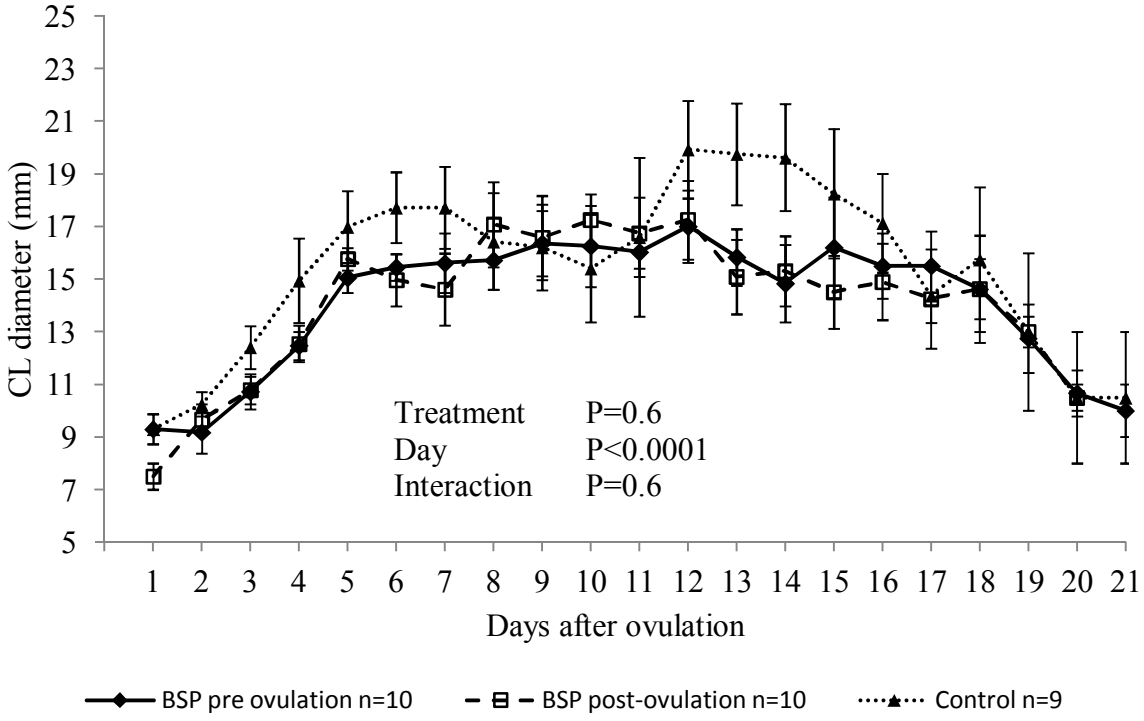

Figure 3.3. Effect of bovine seminal plasma on CL diameter (mean ± SEM) during one inter-ovulatory interval (Day 0 = ovulation) in cattle (Experiment 2).

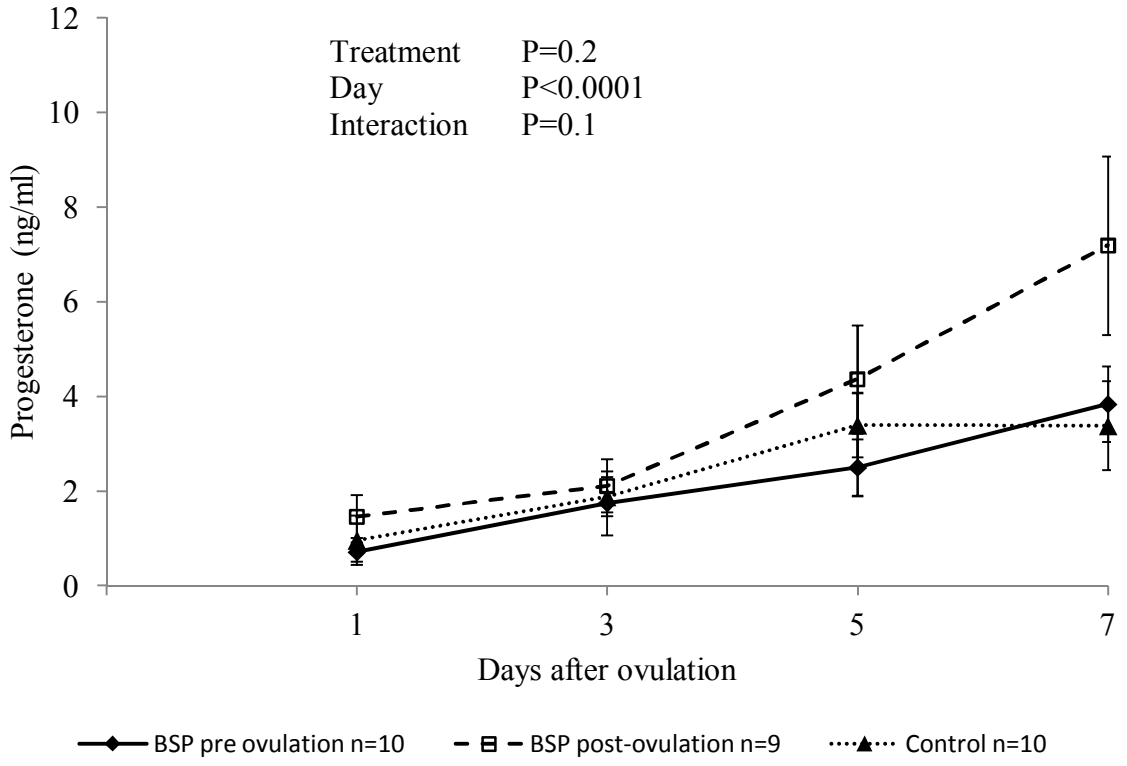

Figure 3.4. Plasma progesterone concentration during luteal development in heifers treated with bovine seminal plasma 12 hours after LH treatment (pre-ovulatory treatment), or ≤4 hours after ovulation (post-ovulatory treatment), compared to untreated controls (Experiment 2).

3.5 Discussion

Ovulation-inducing factor has been identified as critically important for ovulation to occur in species that are referred to as induced ovulators such as South American camelids (Adams et al., 2005; Ratto et al., 2005), challenging the notion that physical stimulation is required for the induction of ovulation (Fernandez-Baca et al., 1970). Interestingly, OIF/NGF is also present in the seminal plasma of spontaneous ovulators (Ratto et al., 2006; Bogle et al., 2011). While OIF/NGF would not appear to be necessary for the induction of ovulation in cattle, current results document bioactivity of OIF/NGF in bovine seminal plasma similar to that in llama seminal plasma (Experiment 1) and an effect of OIF/NGF in bovine seminal plasma on ovulation and luteal development in cattle (Experiment 2).

One of the most important findings in this study was the capability of OIF/NGF in bovine seminal plasma to synchronize ovulations. All ovulations in the pre-ovulation treatment group occurred within a 4 h period, while the range in the other groups was 22 h. A reported effect of NGF on bovine preovulatory follicles is to increase PGE and progesterone secretion from theca cells (Dissen et al., 2000). Both PGE and progesterone have been reported to be associated with follicular wall rupture (Murdoch et al., 1986). A possible explanation for more synchronous ovulations is that preovulatory follicles exposed to OIF/NGF in bovine seminal plasma accumulated PGE and progesterone, and when all conditions were met for ovulation to take place, rupture of the follicular wall occurred. Regardless, the capability of bovine seminal plasma to synchronize ovulation over such a narrow time interval is a promising tool for the improvement in the use of reproductive biotechnologies. For instance, artificial insemination, which is the first generation of modern reproductive biotechnologies (Thibier and Wagner, 2002), has been applied for more than sixty years and yet pregnancy rates seldom exceed 60%.

Moreover, artificial insemination near to the time of ovulation is critical for successful fertilization and embryo development with sex-sorted semen (Maxwell et al., 2004), since its in vivo fertilizing lifespan is significantly shortened because of capacitation-like changes which occur following the use of this technology (Watson, 1995).

Ovulation-inducing factor has been reported to enhance form and function of CL in llamas (Adams et al., 2005). In this regard, greater CL diameter and higher progesterone concentrations were also observed in cows treated with pure OIF/NGF from llama seminal plasma (Tanco et al., 2012). In the present study, OIF/NGF in bovine seminal plasma also had a luteotrophic effect on cattle. Although there was no apparent effect on CL development, systemic progesterone concentrations tended to increase more rapidly by Day 7 after treatment in the post-ovulation treatment group. This study was designed to minimize variations in LH exposure among groups. Therefore, the proposed hypothesis that the duration of the preovulatory LH surge is proportional to the degree of luteogenesis in llamas (Adams et al., 2005) does not seem to be applicable for cattle. Perhaps the luteotrophic effect of OIF/NGF in cattle is a consequence of theca cell proliferation caused by NGF stimulation (Dissen et al., 2000). Embryos reach the uterus between four and five days after fertilization (Wiebold, 1988). The microenvironment of the uterus is a key factor in determining embryo quality (Rizos et al., 2002), and low concentrations of progesterone have been shown to result in a suboptimal microenvironment where early embryonic development is not supported (Leroy 2008). In addition, an early rise in circulating progesterone concentrations was associated with enhanced conceptus development and size around the time of maternal recognition (Mann and Lamming, 2001).

The post-ovulatory group i.e., bovine seminal plasma treatment given within 4 h after ovulation, was included to evaluate the effect of OIF/NGF in bovine seminal plasma on the incipient CL. The increased plasma progesterone concentrations observed in the post-ovulation group provides support for this hypothesis and suggests that such a treatment may benefit the application of assisted reproductive technologies in cattle.

In the report of the biochemical isolation of OIF/NGF from llama seminal plasma (Ratto et al., 2011), the total protein concentration in llama seminal plasma was approximately 3.75 mg/ml, and the concentration of OIF/NGF was 0.98 mg/ml. However, the total protein concentration (1.7 mg/ml) and OIF/NGF concentration (80 μg/ml) in the pooled llama seminal plasma used in the present study were somewhat lower. The reason for such a disparity is not known, but may be attributed to biological variation in the amount of OIF/NGF in ejaculates within and among males (Bogle et al., 2011). Although OIF/NGF appears to be a robust protein (Ratto et al., 2010), the extended period of storage of the seminal plasma in the present study (i.e., >1 year) may also have been associated with protein degradation. Regardless, the OIF/NGF concentration in bovine seminal plasma was one-quarter that of the llama seminal plasma in the present study, and is consistent with the notion that the lower ovulatory response to bovine seminal plasma observed in the previous study (Ratto et al., 2006) was due to the dose of OIF/NGF given (i.e., only 2 ml of bovine seminal plasma).

In summary, results clearly document the existence of bioactive OIF/NGF in bovine seminal plasma and an effect of bovine seminal plasma on both ovulation and luteal function in cattle. While the effect of OIF/NGF in bovine seminal plasma on ovulation was revealed by a greater synchrony when treatment was given 12 h after LH treatment; its effect on luteal function was

evident when bovine seminal plasma was given within 4 h from ovulation by a tendency for increased circulating progesterone concentrations. This finding may have broad implications for the application of reproductive biotechnologies such as artificial insemination and embryo transfer in cattle.

3.6 Acknowledgements

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4 EFFECT OF BOVINE SEMINAL PLASMA ON OVARIAN FUNCTION IN HEIFERS

4.1 Abstract

The objective of the present study was to test the hypotheses that treatment with ovulation-inducing factor (OIF) from bovine seminal plasma will 1) induce LH release and ovulation in heifers during the luteal phase, 2) synchronize LH-induced ovulation, and 3) enhance CL form and function. Bovine seminal plasma was pooled from single ejaculates collected from 95 beef bulls. The concentration of OIF was measured by radio-immunoassay and 10 ml of seminal plasma were used for treatment; i.e., total dose of 250 µg of OIF. Ovulations were synchronized in thirty-two beef heifers by using an estradiol and progesterone protocol. Six days after ovulation (i.e., when a mature CL and a dominant follicle of 11-13 mm diameter were present), heifers were assigned randomly to four groups (n=8 per group) using a 2-by-2 design and treated i.m. with either 25 mg pLH or phosphate-buffered saline, followed 12 h later by an intramuscular treatment with either bovine seminal plasma or phosphate-buffered saline; i.e. LH+PBS, LH+SP, PBS+SP, and PBS+PBS. Ovulation was monitored by transrectal ultrasonography every 4 h and CL development was monitored daily until the next ovulation. Ovulation rates were compared among groups by Fisher's exact test, and continuous data were compared among groups by ANOVA for repeated measures. Single point data were compared by ANOVA. Ovulation was detected in 5/8, 4/8, 0/7, 0/8 in LH+PBS, LH+SP, PBS+SP, and PBS+PBS groups, respectively ($P<0.05$). The synchrony of ovulation tended to be greater in the LH+SP than in the LH+PBS group (absolute values of residuals 1.5±0.5 vs. 3.0±0.79; $P=0.1$). Plasma progesterone concentration increased more rapidly after seminal plasma treatment ($P=0.03$), but no difference was detected among groups in plasma progesterone concentration throughout the interovulatory

interval. Corpora lutea present at the time of treatment regressed later in heifers treated with bovine seminal plasma (P=0.04). Treatment with bovine seminal plasma did not induce LH release or ovulation in heifers during the luteal phase. In conclusion, results suggest that OIF/NGF in bovine seminal plasma synchronizes LH-induced ovulation, and support the notion that OIF/NGF in bovine seminal plasma is luteotrophic. The hypothesis that OIF/NGF in bovine seminal plasma induces LH release and ovulation in heifers during the luteal phase was not supported.

4.2 Introduction

Mammalian species have been categorized as either induced ovulators or spontaneous ovulators based on the stimulus responsible for eliciting GnRH release from the hypothalamus (Bakker and Baum, 2000). In both categories, ovulation is preceded by an LH surge. Somatosensory stimulation of the genitalia during mating is thought to trigger the preovulatory LH surge in induced ovulators (Bakker and Baum, 2000). Mechanical or electrical stimulation of the cervix induced ovulation in dromedary camels (Shalash and Nawito, 1964), and early studies in alpacas (San-Martin et al., 1968) led to the conclusion that mounting accompanied by intromission is required to provoke ovulation in these species (Fernandez-Baca et al., 1970). A similar conclusion was made in the domestic cat by the observation that repeated coitus was associated with an increased ovulation rate (Wildt et al., 1980). Mink are also induced ovulators (Hansson, 1947; Adams, 1981), but in addition to mating, brief contact with a male without intromission also elicited ovulation (Enders, 1952). Others, however, have reported that mere physical stimulation of the genitalia did not consistently induce ovulation in some species of induced ovulators such as the rabbit (Asdell, 1946; Elias et al., 1984), Bactrian and Dromedary camels (Asdell, 1946; Elias et al., 1984), and koala (Johnston et al., 2004). Furthermore, intravaginal, intrauterine or intramuscular treatment with seminal plasma caused LH release and ovulation in Bactrian camels (Chen et al., 1985; Xu et al., 1985), alpacas and llamas (Chen et al., 1985; Xu et al., 1985; Pan et al., 1992; Adams et al., 2005; Ratto et al., 2006).

Investigation of the composition of seminal plasma of Old and New World camelids has led to the conclusion that a potent molecule exists that is responsible for the stimulating hypophyseal release of LH (Pan et al., 2001; Li and Zhao, 2004; Adams et al., 2005; Ratto et al., 2006; Ratto et al., 2010). This molecule has been named ovulation-inducing factor (OIF). In addition to its

key role in initiating the ovulatory cascade, OIF appears to have luteotrophic properties (Adams et al., 2005; Ratto et al., 2006; Tanco et al., 2011). Compared to ovulations induced by treatment with GnRH, intramuscular treatment of llamas with seminal plasma induced the CL to grow longer, reach a greater size, and produce plasma progesterone concentrations that were twice as high (Adams et al., 2005). Results of a more recent study documented that treatment of llamas with pure OIF resulted in a dose-dependent effect on luteal development, revealed by greater day-to-day profiles of CL diameter and plasma progesterone concentrations (Tanco et al., 2011). The identity of OIF has recently been discovered; it is a protein with identical amino acid sequence and structure as ßNGF (Ratto et al., 2012), and will hereafter be referred to as OIF/NGF.

Conversely, in species that are spontaneous ovulators, the preovulatory LH release is elicited by a positive-feedback effect of rising circulating concentrations of estradiol, secreted primarily by the preovulatory follicle during the period of low circulating concentrations of progesterone. The normally negative feedback effect of estradiol on gonadotropin secretion shifts into a positive feedback after a certain threshold in systemic estradiol concentrations is surpassed (Chenault et al., 1975; Karsch, 1987). However, the presence of OIF/NGF has also been detected in the seminal plasma of spontaneous ovulators (i.e., horses, pigs, and cattle), suggesting a role in these species as well (Ratto et al., 2006; Bogle et al., 2011). In an initial study of the role of OIF/NGF in cattle, intramuscular treatment with purified OIF/NGF from llama seminal plasma failed to trigger LH release or induce ovulation in pre-pubertal heifers, but was associated with an increase in circulating concentrations of FSH and hastened follicular wave emergence (Tanco et al., 2012). Most recently, we have reported that treatment with bovine seminal plasma synchronized LH-induced ovulations in cattle (Tribulo et al., 2012b).

The objective of the present study was to test the hypotheses that treatment of beef heifers with OIF/NGF from bovine seminal plasma will 1) induce LH release and ovulation during the luteal phase in heifers, 2) enhance synchrony of ovulation induced by exogenous LH, and 3) enhance CL form and function.

4.3 Material and Methods

4.3.1 Seminal plasma

A single ejaculate was collected from 95 beef bulls over a period of 3 days by electro-ejaculation (Pulsator III; Lane Manufacturing, Denver, CO, USA) using a 75 mm in diameter rectal probe with three ventrally oriented electrodes. Ejaculates collected on the same day (n= 10 to 50) were pooled and kept at 4ºC until transport to the laboratory. The pooled semen was centrifuged for 15 minutes at 500, 1000 and 1500 x g. Between each centrifugation, the supernatant was transferred to a new tube and the pellet was discarded. A drop of seminal plasma was examined by microscopy to confirm the absence of spermatozoa. If cells were detected, the sample was centrifuged again. The seminal plasma was stored at -80ºC. To obtain sufficient volume for treatments, pools of seminal plasma from different days were thawed and combined to form a single large pool of seminal plasma which was then filtered through a 0.22 µM syringe filter (Millipore, Bradford, MA, USA). Aliquots of the filtered seminal plasma were stored at -80ºC until used.

The concentration of OIF/NGF was estimated in two aliquots of pooled bovine seminal plasma using a double-antibody radio-immunoassay. A standard curve was made using known concentrations of purified OIF from llama seminal plasma of 0, 1, 10, 25, 50, and 100 ng/ml in PBS. Purified OIF/NGF from llama seminal plasma was iodinated with I^{125}. The first antibody

used in the double antibody radioimmunoassay was rabbit polyclonal anti-NGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The second antibody was sheep anti-rabbit immunoglobulin (NIDDK-bLH4; Rawlings et al., 1984; Evans et al., 1994). The minimum detectable limit of the assay was 10 ng/ml. Samples were analyzed in one assay and low- and high-reference samples (1 and 200 ng/ml) were distributed throughout the assay. The intra-assay coefficients of variation were 6.1% and 13.8% for low and high reference concentrations, respectively. Before treatment, aliquots of seminal plasma were thawed and sodium penicillin (Pharmaceutical Partners of Canada Inc., Richmond Hill, ON, Canada) was added to a final concentration of 10,000 IU/ml.

4.3.2 Animals, treatments and ultrasonography

Thirty-two Aberdeen Angus cross-bred heifers, 16 to 18 months old and weighing 400 to 450 Kg, were used during October and November at the University of Saskatchewan, Canada (52ºN, 106ºW and 500 m above sea level). Heifers were maintained in paddocks and fed alfalfa/grass hay and grain to gain approximately 1.3 Kg per day, and had water ad libitum during the experimental period. Ovulation was synchronized among heifers with a protocol involving the use of an intramuscular administration of 2.5 mg estradiol-17β and 50 mg of progesterone in sesame oil and intravaginal placement of a controlled internal drug-releasing device impregnated with 1.38 g of progesterone (CIDR, Pfizer Canada Inc., Montreal, QC, Canada). Seven days later, the CIDR was removed and a luteolytic dose of prostaglandin was given i.m. (25 mg Lutalyse; Pfizer Canada Inc.; Montreal, QC, Canada), followed by 1 mg estradiol benzoate in sesame oil i.m. the next day. Ovulations were expected two days after estradiol benzoate treatment (Ross et al., 2004). Six days after ovulation; i.e., when a mature CL and a dominant follicle of 11-13 mm diameter were expected to be present (Ginther et al., 1989c), heifers were

assigned randomly to four groups (n=8 per group) using a 2-by-2 design (Figure 4.1): intramuscular treatment with either pLH (25 mg Armour standard; Lutropin- V, Bioniche Animal Health, Belleville, Ontario, Canada) or phosphate-buffered saline, followed 12 h later by intramuscular treatment with either bovine seminal plasma or phosphate-buffered saline. The treatment volume of bovine seminal plasma (10 ml) was based on a dose of 250 µg of OIF/NGF; i.e., the dose that induced ovulation in 66 to 90% of treated llamas (Tribulo et al., 2012a). The experiment was conducted in two replicates (n=4 per group per replicate) one week apart.

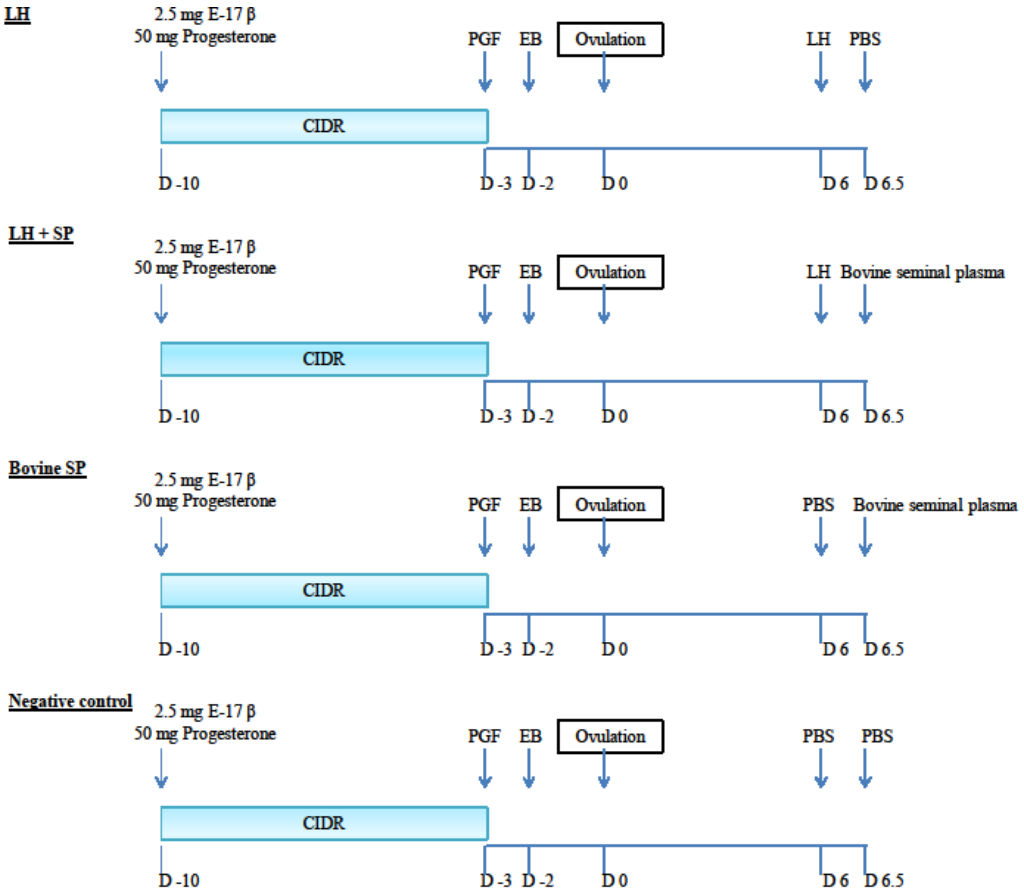

Figure 4.1. Experimental protocol. Heifers in the four treatment groups were synchronized with the same protocol; differences reside in the last two treatments given on Days 6 (D 6) and 12 h later (D 6.5).

The ovaries were examined by transrectal ultrasonography using a 7.5 MHz linear-array transducer (MyLab5 VET, Canadian Veterinary Imaging, Georgetown, Ontario Canada) six days after estradiol benzoate-induced ovulation to record follicular size at the time of LH treatment. Ovulation was expected to occur at 24-30 h after LH treatment (Ree et al., 2009); hence, the ovaries were examined ultrasonically every 4 h beginning 22 h after LH/PBS treatment for 24-hour or until ovulation to determine the interval from treatment to ovulation. Heifers that did not ovulate within 24 h were examined every 12 h thereafter for three days or until ovulation,

whichever occurred first. Transrectal ultrasonography was done daily thereafter until the next ovulation to monitor luteal and follicular dynamics. Ovulation was defined as the sudden disappearance of a large follicle (≥10 mm) from one examination to the next, and was confirmed by the detection of a CL in subsequent examinations (Adams et al., 1989).

4.3.3 Blood samples and hormone assays

Blood samples were collected daily by coccygeal venipuncture into heparinized tubes (Vacutainer Systems; Becton Dickinson, Franklin Lakes, NJ, USA) from the day of bovine seminal plasma/PBS treatment on day 6 of the estrus cycle until the next ovulation for measurement of plasma progesterone concentration. In heifers that were not given exogenous LH, blood samples were collected via jugular catheter into heparinized tubes every 10 minutes for 8 h to assess LH pulsatility. The first sample was collected immediately before treatment with either bovine seminal plasma or phosphate buffered saline. Within one hour of collection, blood samples were centrifuged at 3000 x g for 15 minutes, and the plasma was frozen at -20ºC.

Plasma progesterone concentration was measured using a commercially available double-antibody radioimmunoassay kit (Coat-a-Count total progesterone; Diagnostic Products Corporation, Los Angeles, CA; (Adams et al., 1991). Samples were analyzed in one assay with intra-assay coefficients of variation of 6.0%, 15.1% and 2.8% for reference concentrations of 1.9, 3.6 and 16.6 ng/ml, respectively. Plasma LH concentrations were measured using a double-antibody radioimmunoassay (NIDDK-bLH4; Rawlings et al., 1984; Evans et al., 1994). The minimum detectable limit of the assay was 0.1 ng. The standard curve was made using known LH concentrations of 0, 0.062 (80% ligand labeled LH), 0.125, 0.25, 0.5, 1, 2, 4, and 8 ng/ml (20% ligand labeled). Low and high references were distributed throughout the assay every 50

samples. Samples were analyzed in three consecutive assays. The intra-assay coefficients of variation for the low reference plasma LH concentration were 3.1%, 6.6% and 8.0% for the first, second and third assays, respectively. The intra-assay coefficients of variation for the high reference plasma LH concentration were 6.8% 9.3% and 10.7% for the first, second and third assays, respectively. Inter-assay coefficients of variation were 8% and 12.9% for low and high reference concentrations, respectively.

4.3.4 Statistical analyses

Data are expressed as mean ± SEM, unless otherwise indicated. No replicate effect was detected for any end-point; hence, data from replicates were combined for further analysis of treatment effects. Ovulation rates were compared among the 4 treatment groups by chi-square analysis. Single-point measurements (e.g., follicle diameter at time of LH treatment, luteal tissue area at the time of treatment, number of follicular waves, interval from ovulation to first follicular wave emergence, etc.) were compared among groups by one-way analyses of variance using PROC MIXED in SAS (Statistical Analysis System Institute Inc., Cary, NC, USA). Residuals of number of follicular wave and inter-ovulatory interval data did not follow a normal distribution; hence, the data were transformed to their logarithm before comparison. Similarly, residuals of data for interval from bovine seminal plasma treatment to follicular wave emergence were not normally distributed. This data set was transformed to its co-sine before analyses. The interval from LH treatment to ovulation was analyzed by t-test for heifers in the two groups that were given exogenous LH (i.e., no ovulations in remaining two groups). To evaluate the degree of synchrony of ovulations, absolute values of residuals were compared between the two groups by t-test. Serial data (e.g., luteal tissue area profiles, plasma progesterone profiles) were compared among groups by analyses of variance for repeated measures to determine the main effects of

treatment and day, and their interactions. Tukey's multiple comparison test was used when a main effect of treatment or interaction was detected. A p-value of ≤ 0.05 was considered significant.

Corpora lutea present on Day 6 are referred to as existing CL, while newly forming CL as a consequence of LH-induced ovulations are referred to as incipient CL. The luteal area was calculated from orthogonal diameter measurements of the largest cross-sectional ultrasound image of the CL using the formula for an ellipse (1/2 height x 1/2 width x π). The area of a central cavity, if present, was subtracted from total area of the gland to estimate the area of luteal tissue (Ginther et al., 1989c). Luteal area was compared among groups using data for existing and incipient CL separately, as well as combined. For analysis of the regressing phase of the CL, data were centralized to the day of maximum luteal tissue area. All heifers were included in analyses of progesterone concentrations after treatment, regardless of whether ovulation was induced by treatment on Day 6. In retrospect, the magnitude of the change in plasma progesterone concentration 24 hours after treatment was compared among groups by one-way analyses of variance using PROC MIXED in SAS (Statistical Analysis System Institute Inc., Cary, NC, USA).

4.4 Results

An existing CL was detected on Day 6 in all but one heifer; data from this heifer were excluded from the experiment; i.e., n=7 for the SP group. The diameter of the preovulatory follicle at the time of treatment with LH/saline (Day 6) was not different among groups (Table 4.1.). Ovulation was detected only in heifers in the two groups that were given LH, and the interval to ovulation was not different between these groups (Table 4.1.). The synchrony of ovulation tended to be

greater in the LH+SP group than in the LH+PBS group (absolute values of residuals: 1.5 ± 0.5 vs. 3.0 ± 0.79; P=0.1).

Table 4.1. Effect of bovine seminal plasma (SP) on ovulation in cattle (mean ± SEM). Heifers were treated on Day 6 (Day 0 = ovulation) with LH or phosphate buffered saline (PBS) i.m., followed 12 hours later by SP or PBS.

End point	LH+ SP	LH+PBS	PBS + SP	PBS + PBS
Follicle diameter on day of treatment	12.9 ± 1.15	13.1 ± 0.55	14.4 ± 0.81	13.1 ± 0.52
Number of heifers that ovulated	4/8[a]	5/8[a]	0/7[b]	0/8[b]
Interval from treatment to ovulation (h)	33.0 ± 1.00	32.4 ± 1.60	-	-

[ab] Values with different superscripts are different (P<0.05)

To examine the effect of seminal plasma on follicular dynamics, only data from heifers that were given SP or PBS alone were analyzed. The inter-ovulatory interval was composed of 2 follicular waves in all but one heifer; this heifer was in the negative control (PBS) group and had 3 waves. No differences were detected between groups in the inter-ovulatory interval, the interval from treatment to new wave emergence, or the maximum diameter of the dominant follicle of the first wave (

Table 4.2). Plasma LH concentration was similar in SP and negative control (PBS) groups for all evaluated parameters (

Table 4.2).

Table 4.2. Effect of bovine seminal plasma (SP) on follicular wave dynamics and LH release in heifers (mean ± SEM). Analyses include only heifers treated on Day 6 (Day 0 = ovulation) with phosphate-buffered saline followed 12 hours later with either SP or PBS; i.e., excluding heifers in the LH treatment groups (mean ± SEM).

End point*	PBS + SP (n=7)	PBS + PBS (n=8)
Inter-ovulatory interval (days)	19.9 ± 0.34	19.8 ± 0.37
Interval from treatment to new wave emergence (days)	2.3 ± 0.29	2.4 ± 0.18
Maximum diameter of dominant follicle (mm)	14.8 ± 0.44	14.9 ± 0.38
Mean LH concentration (ng/ml)	0.1 ± 0.01	0.2 ± 0.03
Basal LH concentration (ng/ml)	0.1 ± 0.01	0.1 ± 0.02
Number of LH pulses in 8 h period	3.6 ± 0.72	4.7 ± 0.84
LH pulse-frequency (pulses per h)	0.4 ± 0.09	0.9 ± 0.25
LH amplitude (ng/ml)	0.3 ± 0.04	0.3 ± 0.03

* No significance difference between groups

Luteal area increased, then decreased during the interovulatory interval ($P<0.0001$), but profiles did not differ among groups when all CL (existing and incipient) or only existing CL were included in the analysis (Figure 4.2). However, a day-by-group interaction ($P=0.01$) in the profiles of the incipient CL was attributed to a lesser maximum diameter, but greater persistence in the LH+SP group than in the LH+PBS group (Figure 4.2c).

On an individual animal basis, maximum luteal area, the day on which maximum luteal area was attained, and the onset of regression did not differ among groups for the existing CL (Table 4.3). Incipient CL did not differ in maximum luteal area, and the day when the maximum luteal area was observed, but tended to initiate regression later in the LH+SP group than in the LH+PBS group (Table 4.4).

In heifers that were not given exogenous LH (i.e., to determine an effect of seminal plasma alone), CL area profiles during the luteal regressing phase were greater in the SP group than in the negative control group (P=0.04; Figure 4.3). Plasma progesterone concentration, however, did not differ between groups (Table 4.5).

Table 4.3. Developmental characteristics (mean ± SEM) of the CL existing at the time of treatment in heifers given LH or phosphate buffered-saline (PBS) on Day 6 (Day 0 = ovulation) followed 12 hours later with bovine seminal plasma or PBS.

End point*	LH + SP (n=8)	LH + PBS (n=8)	PBS + SP (n=7)	PBS + PBS (n=8)
Inter-ovulatory interval (days)	21.9 ± 0.79	19.4 ± 1.21	19.8 ± 0.63	19.7 ± 0.47
Luteal area on Day 6 (mm^2)	339.2 ± 25.70	358.9 ± 31.80	339.1 ± 40.60	368.2 ± 33.70
Maximum luteal area (mm^2)	433.1 ± 45.80	453.0 ± 40.90	432.6 ± 37.90	375.2 ± 16.38
Day of maximum luteal area	9.1 ± 0.29	10.0 ± 0.58	10.0 ± 0.75	10.6 ± 1.10
Day of onset of CL regression	16.0 ± 1.04	15.8 ± 1.00	15.9 ± 0.26	15.9 ± 0.61

* No significant difference among groups

Table 4.4. Characteristics (mean ± SEM) of the newly forming (incipient) CL in heifers as a consequence of ovulation induced by LH treatment on Day 6 (Day 0 = day) followed 12 hours later with bovine seminal plasma (SP) or phosphate-buffered saline (PBS).

End point	LH + SP (n=4)	LH + PBS (n=5)
First day incipient CL detected	10.5 ± 0.29	10.6 ± 0.24
Maximum area of incipient CL (mm^2)	296.6 ± 30.73	346.4 ± 24.68
Day of maximum area of incipient CL	13.8 ± 0.48	14.0 ± 0.32
Day of onset of regression of incipient CL	17.5 ± 1.19[a]	15.4 ± 0.51[b]

[ab] Values with different superscripts tended to be different (P=0.1)

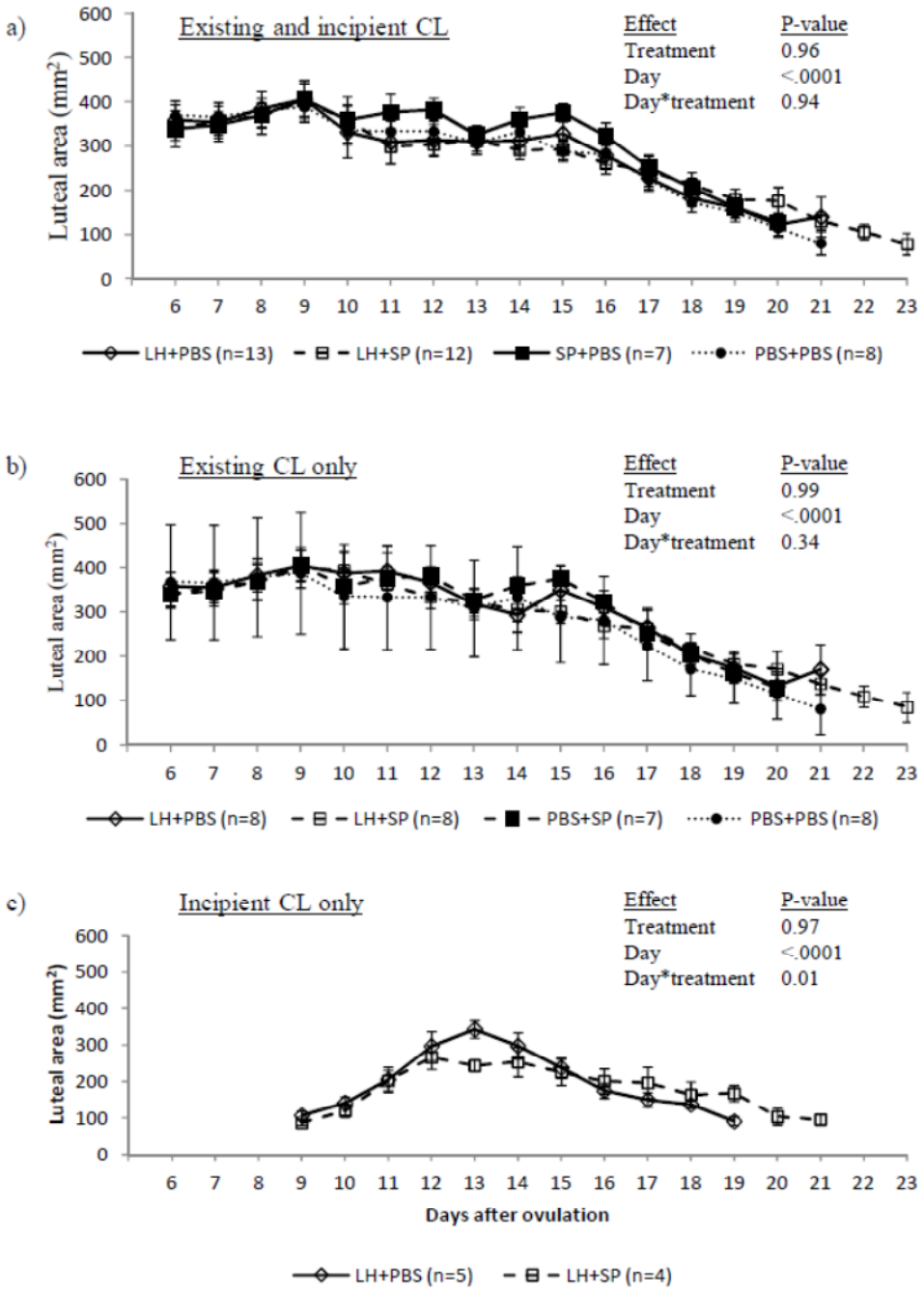

Figure 4.2. Effect of bovine seminal plasma (SP) on CL development in heifers. Heifers were treated on Day 6 (Day 0 = ovulation) with LH or phosphate buffered saline (PBS) followed 12 hours later with bovine SP or PBS. Profiles (mean±SEM) include existing and incipient CL (a), existing CL only (b) or incipient CL only (c).

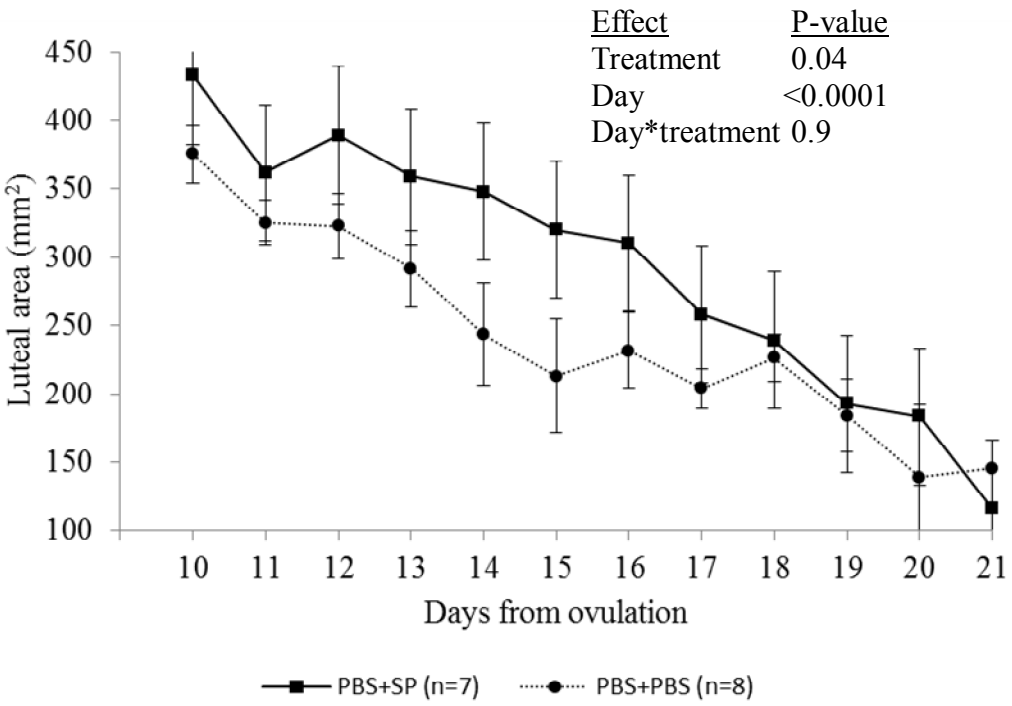

Figure 4.3. Effect of bovine seminal plasma on luteal regression. CL area profiles during the luteal regressing phase of heifers that were not given exogenous LH (i.e. Existing CL) but were treated with either bovine seminal plasma (PBS+SP) or phosphate buffered saline (PBS+PBS). CL profiles are represented from the mean day of maximum luteal area (i.e., Day 10) to ten days later.

Table 4.5. Effect of bovine seminal plasma on luteal regression of the existing CL. Mean (± SEM) plasma progesterone concentration (ng/ml) during the regressing luteal phase of heifers that were not given exogenous LH but were treated with either bovine seminal plasma (PBS+SP) or phosphate buffered saline (PBS+PBS).

Days of progesterone concentration determination*	PBS + SP (n=7)	PBS + PBS (n=8)
Day of existing CL maximum luteal area	5.4 ± 0.43	6.1 ± 0.80
Five days after existing CL max. luteal area	5.5 ± 1.77	6.4 ± 1.77
Ten days after existing CL max. luteal area	0.1 ± 0.06	0.2 ± 0.08

*No significant difference between groups

Plasma progesterone concentrations changed over days ($P<0.0001$), but profiles did not differ among treatment groups during the first three days after treatment with bovine seminal plasma (Figure 4.4) There was no significant effect for LH treatment and interaction between LH and bovine seminal plasma treatments. However, there was a significant effect of treatment with bovine seminal plasma in that progesterone concentrations increased within 24 h after bovine seminal plasma treatment ($P=0.03$; Table 4.6).

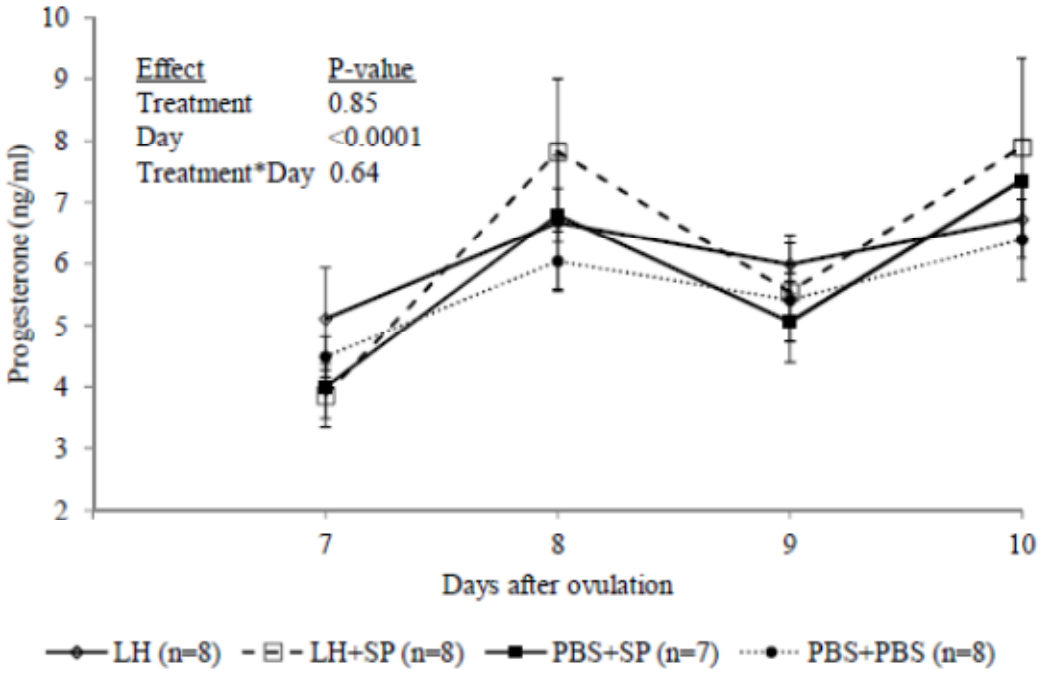

Figure 4.4. Plasma progesterone concentration per group regardless of ovulation due to different treatments (i.e., including existing and incipient CL) along days after ovulation. Heifers were treated on Day 6 (Day 0 = ovulation) with LH or phosphate buffered saline (PBS), followed 12 hours later by bovine seminal plasma (SP) or PBS. Plasma progesterone concentrations for Day 7 are before treatment with either bovine seminal plasma (SP) or phosphate buffered saline (PBS).

Table 4.6. Effect of bovine seminal plasma on plasma progesterone concentration (ng/ml; mean ± SEM) in heifers treated on Day 6 (Day 0 = ovulation) with LH or phosphate buffered saline (PBS) followed 12 hours later with bovine SP or PBS.

Day of progesterone concentration determination	LH		PBS	
	SP	PBS	SP	PBS
Day 7 (ng/ml)	3.9 ± 0.51	5.1 ± 0.83	4.0 ± 0.33	4.5 ± 0.39
Day 8 (ng/ml)	7.8 ± 1.19	6.7 ± 1.08	6.8 ± 0.49	6.0 ± 0.76
Percent change (%)	102.3[a]	30.6[b]	70.1[a]	34.5[b]

[ab] Percent change with different superscripts are different ($P<0.05$)

4.5 Discussion

Ovulation-inducing factor (OIF) is a protein that has been detected in the seminal plasma of both induced and spontaneously ovulating species (Adams et al., 2005; Ratto et al., 2006). In camelids (induced ovulators), OIF induced ovulation by triggering LH release (Adams et al., 2005). The function of OIF in cattle (spontaneous ovulators), however, remains unclear. Bovine seminal plasma induced ovulation in llamas (Ratto et al., 2006), and synchronized LH-induced ovulations in cattle (Experiment 2). In the present study, ovulations occurred only when exogenous LH was given, indicating that 250 µg OIF in bovine seminal plasma by itself will not induce ovulation during the luteal phase in cattle. However, ovulations in the group of heifers treated with bovine seminal plasma tended to be more synchronous than in those heifers treated only with LH, consistent with results of an earlier study (Experiment 2). Similar to the earlier study, all ovulations in the LH+SP group occurred within a four-hour period. The lack of significant

difference in the synchrony of ovulation may be due to the small number of animals included in the analysis; i.e. LH n=5; LH+SP n=4.

Ovulation-inducing factor enhanced form and function of CL in llamas (Adams et al., 2005). Luteotrophic effect of OIF/NGF has also been documented in cattle (Tanco et al., 2012). In the present study, the most interesting finding in this regard was the rapid increase in circulating progesterone concentration within 24 h after bovine seminal plasma treatment. Due to the brief time for this effect, we suspect that bovine seminal plasma has an acute effect on progesterone release. In agreement with our hypothesis, *in-vitro* stimulation of bovine lutein cells with NGF resulted in an acute release of progesterone (Miyamoto et al., 1992). Another indication of luteotrophic effect of OIF/NGF in bovine seminal plasma is the difference in CL profiles when analyzed from maximum luteal area until luteolysis; the interval from maximum size to a significant decrease in size of CL present at the time of treatment (i.e., Existing CL) was longer. Lastly, incipient CL profiles were smaller but more persistent in heifers treated with bovine seminal plasma. In addition, the onset of incipient CL regression in these heifers, tended to be later than in the other group. Taken together, bovine seminal plasma resulted in delayed regression of CL. In the present study, OIF/NGF did not trigger LH release as has been reported in llamas (Adams et al., 2005). Therefore, the proposed hypothesis that the luteotrophic effect of OIF/NGF is proportional to the LH surge (Adams et al., 2005) was not supported in cattle. We speculate that in cattle the luteotrophic effect of seminal plasma is a consequence of a local rather than a systemic effect. Contrary to previously published data (Tanco et al., 2012), OIF/NGF in bovine seminal plasma did not result in CL that grew longer and produced higher progesterone concentrations in the current study. Knowing that OIF/NGF has a dose-dependent effect on luteal development and function (Tanco et al., 2011), one possible explanation to such a

disparity is the different dose used in these studies i.e., 1 mg pure OIF versus 250 µg of OIF in bovine seminal plasma.

Ovulation-inducing factor provokes LH release in llamas and alpacas (Adams et al., 2005). We set out to determine if OIF/NGF in bovine seminal plasma has the same effect on cattle. Patterns of LH did not differ between groups and were consistent with those expected for Day 7 of the oestrus cycle in cattle (Rahe et al., 1980). It is important to note that all data reported on the effect of OIF on LH release in llamas has been collected under conditions of no systemic progesterone. Patterns of LH release vary over the oestrous cycle in cattle due to different levels of progesterone (Rahe et al., 1980). Furthermore, the magnitude of the GnRH-induced LH surge in cattle varies according to circulating progesterone concentrations (Colazo et al., 2008; Dias et al., 2010). Hence, it would be worth investigating the effect of OIF/NGF from bovine seminal plasma on the preovulatory LH release (surge) in cattle.

In summary, results support our hypotheses that OIF/NGF from bovine seminal plasma synchronizes LH-induced ovulation, and enhances CL form and function in heifers. However, results fail to support the hypothesis that OIF/NGF from bovine seminal plasma induces LH release and ovulation in heifers during the luteal phase.

5 GENERAL DISCUSSION

The presence of an ovulation-inducing factor in the seminal plasma of induced ovulatory species such as llamas and alpacas has been clearly documented (Adams et al., 2005; Ratto et al., 2006) challenging the dogma that mating stimuli were essential for ovulation to occur. Recently the identity of this potent factor has been shown to be identical to ßNGF (Ratto et al., 2012). Interestingly, the existence of OIF/NGF in the seminal plasma of spontaneous ovulators, has also been reported recently (Bogle et al., 2011). Based on the known effects of OIF/NGF on llamas (Adams et al., 2005) and studies in cattle (Tanco et al., 2012), it was hypothesized that OIF/NGF in bovine seminal plasma affects ovulation and luteal development and function in cattle.

Two studies were conducted to determine the effects of OIF/NGF in bovine seminal plasma on female cattle. We examined 1) the bioactivity of OIF/NGF in bovine seminal plasma (Chapter 3; Experiment 1); 2) The effect of OIF/NGF in bovine seminal plasma on LH-induced ovulations and luteal development in sexually mature heifers (Chapter 3; Experiment 2); and 3) The effect of OIF/NGF in bovine seminal plasma on LH release, ovulation and luteal development in sexually mature heifers (Chapter 4).

Although purification of OIF/NGF from bovine seminal plasma and its subsequent use for treatments would have been ideal for the study of the effects of OIF/NGF in cattle, the research studies reported in this thesis involved the use of raw bovine seminal plasma due to its availability. Hence, we recognize the limitations in the interpretation of our results.

The differences in the proportion of ovulations induced by bovine seminal plasma relative to llama seminal plasma when female llamas were given 2 ml of each treatment (i.e., OIF /NGF dose was unknown; Ratto et al., 2006), and the fact that OIF/NGF had a dose-dependent effect in

the induction of LH release and ovulation in female llamas (Tanco et al., 2011) were the rationale for Experiment 1 in this thesis. The treatment volume of bovine seminal plasma used for the first experiment and all other studies reported in this thesis, was based on a dose of 250 μg of OIF/NGF i.e., the dose that induced ovulation in 90% of treated llamas (Tribulo et al., 2012a). The bioactivity of OIF/NGF in bovine seminal plasma was clearly similar to that in llama seminal plasma. Moreover, results supported our hypothesis that bovine seminal plasma induces ovulation and CL development in llamas in a manner comparable to llama seminal plasma when dose is adjusted to a similar amount of OIF/NGF.

In llamas, OIF/NGF caused LH release (Adams et al., 2005) indicating that its effect on ovulation and luteal function is through a systemic pathway. Current knowledge in this regard is controversial in cattle. While *in vitro* stimulation of bovine pituitary cells with OIF/NGF resulted in LH release (Bogle et al., 2012), no LH increase was evident in vivo (Tanco et al., 2012). In addition, *in vitro* stimulation of theca cells with LH induced Trk-A expression after 8 hours (Dissen et al., 1996). To assess the effect of OIF/NGF in bovine seminal plasma on ovulation in cattle, ovulations were induced with exogenous LH. Results did not support our hypothesis that OIF/NGF in bovine seminal plasma induces ovulation in cattle by itself. However, bovine seminal plasma treatment prior to ovulation had a synchronizing effect so that all ovulations occurred within a 4-hour period (Chapter 3; Experiment 2). Interestingly, these results are consistent with those observed in the subsequent trial where the hypothesis that bovine seminal plasma induces ovulation was tested. In order to avoid confusion with spontaneous ovulations, heifers were treated on Day 6 of the estrous cycle, i.e., luteal phase. Results did not support our hypothesis. We recognize that assessing the capability of bovine seminal plasma to induce ovulation during luteal phase limits the interpretation of results since the endocrine environment

is different from that of heifers and llamas in the follicular phase. Circulating progesterone concentrations have been shown to suppress GnRH-induced increase of systemic LH (Colazo et al., 2008) and the effect was compensated for by higher doses of GnRH (Giordano et al., 2012). Similar interactions among hormones may be required to further investigate the effect of bovine seminal plasma on cattle. The contribution of OIF/NGF to follicular wall rupture has been suggested, since both pharmacological blockade of Trk-A signaling and immunoneutralization of NGF actions in vivo significantly reduced the number of ovulations in rats (Mayerhofer et al., 1996). Moreover, *in vitro* stimulation of bovine theca cells with NGF resulted in an increase in PGE and progesterone secretion(Dissen et al., 2000). These hormones are involved in follicular wall rupture (Lydon et al., 1995; Robker et al., 2000). Contrary to the systemic effect of OIF/NGF reported for llamas, alpacas and Bactrian camels (Adams et al., 2005; Ratto et al., 2005 and Li et al. 2002.), we believe that the synchronization of ovulations in cattle associated with treatment with bovine seminal plasma is due to a local action. Perhaps OIF/NGF from bovine seminal plasma is absorbed from the vagina in the cow and goes directly to the ovary affecting the dominant follicle, acting synergistically with endogenous NGF and LH.

Data reported in this thesis further support the notion that OIF/NGF has a luteotrophic effect in cattle, and provide evidence of an effect on ovulation as well. However, OIF/NGF seems to have a different pathway than that reported in induced ovulators.

The luteotrophic effect of OIF/NGF in induced ovulators has been shown to be associated with a higher and longer preovulatory LH surge (Adams et al., 2005). In cattle, OIF/NGF treatment on Days 3 and 6 after ovulation resulted in a greater CL profile. In addition, concentrations of circulating progesterone were increased by OIF/NGF treatment on Days 6 and 9 after ovulation

in cattle (Tanco et al., 2012). Results of research reported in this thesis support the notion that OIF/NGF is luteotrophic in cattle. However, treatment of sexually mature heifers with bovine seminal plasma on Day 6 after ovulation did not reveal the same luteal response reported previously by Tanco et al. (2012). It is important to bear in mind that OIF/NGF has a dose-dependent effect (Tanco et al., 2011). Therefore, it is not rare to obtain different results when varying the dose of OIF/NGF used for treatments i.e., 250 μg vs. 1 mg. The most interesting finding related to the luteotrophic effect of OIF/NGF in bovine seminal plasma on cattle, was the rapid and sharp increase in circulating progesterone concentrations within 24 hours after treatment (Chapter 4). Due to the brief time of this effect, we suspect that bovine seminal plasma had an acute effect on progesterone release. In this regard (and supportive of our hypothesis), in-vitro stimulation of bovine lutein cells with NGF resulted in an acute release of progesterone (Miyamoto et al., 1992). An increase in systemic progesterone concentration was also observed when heifers were treated with bovine seminal plasma during estrus (Chapter 3; Experiment 2). However, higher levels of progesterone were observed by Day 7 in the group of animals treated within four hours of ovulation. In this case, the luteotrophic effect was not as acute as mentioned above. We speculate that the rise in progesterone was a consequence of the documented effect of NGF on theca cell proliferation (Mayerhofer et al., 1996).

Further studies are needed to elucidate the role of OIF/NGF in bovine seminal plasma on cattle, and its pathways in affecting ovarian function. Research using pure OIF/NGF from bovine seminal plasma and involving histology of the preovulatory follicle, and CL will help to provide an understanding of the action of OIF/NGF in bovine seminal plasma. Moreover, the inclusion of different administration route would be interesting; as well as treatments given at different stages of the estrus cycle. There are published data documenting that OIF/NGF is involved in the

ovulatory event, however, in vivo experiments are imperative to link those effects with the existence of this protein in the seminal plasma.

6 GENERAL CONCLUSIONS

- OIF/NGF is present in bovine seminal plasma
- Bioactivity of OIF/NGF in bovine seminal plasma is similar to that of OIF/NGF in llama seminal plasma on a μg basis
- Treatment with bovine seminal plasma containing 250 μg OIF/NGF synchronized LH-induced ovulations over a four-hour period in cattle
- Treatment with bovine seminal plasma containing 250 μg OIF/NGF did not induce ovulation during the luteal phase in cattle
- Treatment with bovine seminal plasma containing 250 μg OIF/NGF did not induce LH release during the luteal phase in cattle
- Treatment with bovine seminal plasma containing 250 μg OIF/NGF was luteotrophic in cattle
- Treatment with bovine seminal plasma containing 250 μg OIF/NGF within four hours after ovulation results in higher circulating concentrations of progesterone by Day 7 after ovulation
- Treatment with bovine seminal plasma containing 250 μg OIF/NGF induced a rapid and sharp increase in systemic progesterone concentration within 24 hours
- Treatment with bovine seminal plasma containing 250 μg OIF/NGF did not affect the appearance of either the existing or the incipient CL
- Treatment with bovine seminal plasma containing 250 μg OIF/NGF seemed to resulted in a slower regression of existing CL.

7 FUTURE STUDIES

The findings reported in this thesis provide knowledge that contributes to further understanding of the role of OIF/NGF in bovine seminal plasma on female cattle reproductive physiology. However, the main limitation of these results is that bovine seminal plasma was used for treatments instead of pure OIF/NGF. In order to conclude that the observed effects of bovine seminal plasma are due to OIF/NGF, it is imperative to perform similar studies but utilizing pure OIF/NGF.

It is interesting that OIF/NGF, the protein responsible for eliciting ovulation in induced ovulatory species, is present in spontaneous ovulators. The association between OIF/NGF and ovulation in spontaneous ovulators has been already established since pharmacological blockade of Trk-A signaling and immunoneutralization of OIF/NGF actions in vivo significantly reduced the number of ovulations in rats. Moreover, the preovulatory LH surge induced Trk-A expression in rats nine hours prior to ovulation. From our results, we speculate that synchronization of ovulations in cattle associated with bovine seminal plasma treatment is due to a local action. Therefore, it would be worth studying the role of OIF/NGF and Trk-A on ovulation, and their relationship with the preovulatory surge of LH.

Although our results led us to think that in cattle OIF/NGF does not follow a systemic pathway as it does in llamas, all data reported on the effect of OIF/NGF on LH release in llamas has been collected under conditions of no systemic progesterone. Patterns of LH release vary over the oestrous cycle in cattle due to different levels of progesterone. Furthermore, the magnitude of the GnRH-induced LH surge in cattle varies according to circulating progesterone concentrations. Hence, it would be worth investigating the effect of OIF/NGF from bovine seminal plasma on

the preovulatory LH surge in cattle. This approach will imitate the physiological conditions at which OIF/NGF in bovine seminal plasma is deposited in the female. In addition, intrauterine treatment with OIF/NGF would be of great value to evaluate the role of OIF/NGF in bovine seminal plasma on female cattle reproductive physiology.

Besides research addressed to further understand the importance of OIF/NGF in cattle physiology, investigations need to be performed to find applications of OIF/NGF in cattle reproductive technologies. For instance, knowing that OIF/NGF has a luteotrophic effect on cattle, it would be worth evaluating whether or not that effect is reflected on pregnancy rates after artificial insemination where seminal plasma deposition is minute.

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