

**THE RABBIT AS AN ANIMAL MODEL FOR THE STUDY OF
OVULATION-INDUCING FACTOR**

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By

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“Believe in yourself and all that you are. Know that there is something inside you that is greater than any obstacle.” – Christian D. Larson

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ABSTRACT

Ovulation-inducing factor (OIF) is a seminal plasma protein that has been found in the ejaculate of different species. Evidence suggests that OIF and its function may be conserved among species. Our general objective was to develop a rabbit model for the study of OIF in seminal plasma. In the first study, we conducted two experiments to validate the use of ultrasound biomicroscopy as a method for assessing ovarian structures in rabbits, and to develop a method that permits serial noninvasive repetitive ultrasound evaluation of ovarian structures *in vivo* in rabbits. In Experiment 1, the number and size of follicles ≥ 0.6 mm and corpora lutea (CL) detected by ultrasound biomicroscopy (UBM) *ex situ* were correlated with those detected by histology in each pair of ovaries from 4 female New Zealand White rabbits (5-5.5 months old) given an ovulation-inducing treatment. In Experiment 2, we translocated the ovaries of female New Zealand white rabbits (n=12; 5 months old) to a subcutaneous position in order to develop a method that permits serial UBM evaluation of ovarian structures *in vivo* in rabbits. Results showed strong correlations ($P < 0.05$) between UBM and histology in all the ovarian variables evaluated in Experiment 1, and also showed that ovarian structures are easily identified using UBM *in vivo* in the rabbits submitted to the surgical approach performed in Experiment 2. In the second study, we conducted two experiments to test the hypothesis that llama and rabbit seminal plasma elicits a surge of LH release and is responsible for inducing ovulation in rabbits. In Experiment 1, we compared the effect of an intramuscular administration of saline, GnRH, llama or rabbit seminal plasma in female New Zealand White rabbits (n=4-6 per group, 5.5 months old) that were group-housed. In Experiment 2, we compared the effects of the same treatments in rabbits (n=5-

7 per group, 5.5 months old) that were individually caged. Ovulation and CL formation occurred in most rabbits regardless of the treatment given when animals were group housed, while rabbits given similar treatment but individually housed did not ovulate. In the GnRH group, a surge in plasma LH concentrations was observed in all the rabbits, followed by CL formation and an increase in plasma progesterone concentrations. In summary, we developed a rabbit model for the study of OIF, where ovarian structures can be evaluated *in vivo* by UBM. Results did not support the hypothesis that OIF in seminal plasma elicits ovulation in rabbits. Further studies are needed to determine the effects of dose and route of administration of seminal plasma in rabbits.

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DEDICATION

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LIST OF ABBREVIATIONS

CL	Corpus luteum
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
hCG	Human chorionic gonadotropin
h	Hours
µg	Microgram
kDa	Kilodalton
kg	Kilogram
LH	Luteinizing hormone
LH-RH	Luteinizing hormone-releasing hormone
PMSG	Pregnant mare serum gonadotropin
PGF	Prostaglandin F _{2α}
ml	Milliliter
min	Minute
MHz	Megahertz
µm	Micrometer
mm	Millimeter
OIF	Ovulation-inducing factor
PBS	Phosphate buffered saline
COC	Cumulus-oocyte complex
SAS	Statistical analysis system
SEM	Standard error of the mean
PDS	Polydioxanone
vs	Versus

1.0. GENERAL INTRODUCTION

Ovulation-inducing factor (OIF) is a protein found in the ejaculate of camelids and other species (Chen *et al.*, 1985; Zhao *et al.*, 2001; Adams *et al.*, 2005; Ratto *et al.*, 2005; 2006b; 2010; Bogle *et al.*, 2011). This protein has been isolated from the seminal plasma of llamas and alpacas and has been shown to have a potent ovulatory effect when given to females of these species (Ratto *et al.*, 2010, 2011; Tanco *et al.*, 2011). South American camelids are induced ovulators; i.e., they ovulate only after an external stimulus is given. Since ovulation is a pivotal event for successful reproduction in mammals, the discovery of OIF has broad implications and the development of models for studying the effects of OIF is important. In addition, OIF may also be applied in the development of products that can be used for research or therapeutic purposes involved with ovulation induction.

Using female camelids as animal models, investigators documented that a single intramuscular administration of camelid seminal plasma or purified OIF elicits a preovulatory surge of LH and induces high percentages of ovulation in llamas and alpacas (Adams *et al.*, 2005; Ratto *et al.*, 2005; 2011; Bogle *et al.*, 2011). However, the mechanism of action and biological pathways of OIF are poorly understood. The use of large animals as models for the study of OIF (i.e., camelids) carries the disadvantages of their large size and weight, cost of maintenance, genetic variability, and practical issues regarding evaluation of post-mortem tissues.

Given the need for increasing knowledge about OIF, new animal models are required. When considering animal models for the study of OIF we need to ask whether we can truly determine if ovulation occurred as a consequence of the treatment given. In this regard,

spontaneous ovulators (i.e. no external stimulus is required to initiate ovulation) such as mice are not the most appropriate model to investigate about OIF. Conversely, rabbits are a promising animal model because they are induced ovulators - ovulation occurs only after an external stimulus is given. Unlike camelids, the genetic uniformity in laboratory rabbits will facilitate the reproducibility of experimental results. Furthermore, rabbits are small, docile, easy to handle, readily available, and relatively inexpensive to maintain. Unlike mice, some experimental procedures are more easily performed in rabbits; e.g., ultrasonography and serial blood sampling. Moreover, smaller animals require a lower absolute treatment dose.

There is growing evidence that the biological activity of OIF is conserved in the other mammals (e.g. bovine, equine, porcine; Ratto *et al.*, 2006b; Bogle *et al.*, 2011), and questions associated with the role of OIF in the seminal plasma of induced ovulators like the rabbit arise. Is the biological activity of OIF conserved in seminal plasma of rabbits? Is the OIF in the seminal plasma of llamas able to induce ovulation in rabbits? Is a single administration of seminal plasma enough to induce ovulation in rabbits? Can ovulation be easily evaluated in live rabbits? These are some of the questions that can help us to decide whether or not the rabbit is a suitable animal model for the study of OIF in the seminal plasma.

The following literature review provides general information about the reproductive physiology in rabbits, and is focused on the event of ovulation in rabbits and other induced ovulators, and our knowledge of the role of seminal plasma and/or purified OIF on induction of ovulation.

1.1. Reproductive physiology of the female rabbit

Mammalian species are classified as spontaneous or induced ovulators (Conaway, 1971). Spontaneous ovulators (e.g., cattle, pigs, sheep, humans) display a cyclical increase of estradiol which triggers GnRH release from the hypothalamus, LH release from the pituitary, and ultimately ovulation regardless of mating activity (Wettemann *et al.*, 1972; Kelly *et al.*, 1988; Brooks and McNeilly, 1994). Induced ovulators (e.g., rabbits, camelids, cats), however, require stimulation associated with mating to provoke GnRH release and then the surge of LH which is necessary to elicit ovulation (Bakker, 2000).

Female rabbits are classified as induced or reflex ovulators because ovulation takes place after mating (Heape *et al.*, 1905; Friedman *et al.*, 1929; Spies *et al.*, 1997; Harkness *et al.*, 2010); hence, rabbits do not have a regular estrous cycle (Harkness *et al.*, 2010) as in other domestic species (e.g., cattle). Rabbits display periods of sexual receptivity and non receptivity. Investigators have reported that the period of receptivity lasted about 5 to 6 days (Myers and Poole, 1962; Stein and Walshaw, 1996) or 7 to 10 days (Harkness *et al.*, 2010) in the absence of mating. This period was followed by 1 to 2 days (Harkness *et al.*, 2010) or 4 days (Alvariño, 1993) of non receptivity. Sexual receptivity, or the willingness of the female rabbit to allow mating (Tsiligianni *et al.*, 2004), involved the adoption of a lordosis posture (Beyer *et al.*, 2007). In female rabbits, lordosis is usually displayed in response to male pelvic thrusting (Moralí *et al.*, 2003; cited in Beyer *et al.*, 2007). In females that are willing to accept mating, it is more likely to observe an enlarged reddish-purple vulva as a result of high concentrations of estrogens; however, some females accepted mating when the vulva was small and pale (Beyer *et al.*, 2007). The color of the vulva has been considered useful to estimate receptivity at the time of artificial

insemination (Leyún *et al.*, 1982; Torres *et al.*, 1984; Roca *et al.*, 1986; Abdel-Ghaffar and Agag, 1994; Morura *et al.*, 2003; Vicente *et al.*, 2008), but some authors reported no clear relationship between this external sign and subsequent occurrence of ovulation or pregnancy (Adams, 1983). The display of chin-scent marking (“chinning”, “a scent marking behavior in which the female rubs the undersurface of her chin on objects in order to deposit scent gland secretions”) has also been observed in some receptive female rabbits. In a study was reported that post-mating administration of estradiol prevented the decline in chinning and receptivity (Hoffman *et al.*, 2010).

Sexual behavior in female rabbits has been studied during pregnancy, pseudopregnancy, and post partum. Some pregnant rabbits accepted mating, but release of gonadotropins and ovulation did not occur (Mills and Gerardot, 1984). Sexual behavior in pregnant rabbits was markedly reduced compared to that in non pregnant rabbits and ended by the final third of pregnancy (Beyer and Rivaud, 1969; Stoufflet and Caillol, 1988). Likewise, sexual behavior or receptivity was reduced in pseudopregnant rabbits but increased again by the end of the pseudopregnant period when progesterone concentration returned to basal levels (Caillol *et al.*, 1983). During the post partum period, rabbits were found to be highly receptive on the day and the following day of parturition and after weaning (Day 28 of lactation), when they were able to ovulate (Beyer and Rivaud, 1969). Receptivity also has been found to vary depending on parity (i.e., primipara are less receptive than nullipara) and stage of lactation (i.e., lactating are less receptive than non-lactating) and receptivity may also influence reproductive performance (reviewed by Theau-Clément, 2007).

In general, female rabbits reach puberty (i.e., the onset of sexual receptivity and ovulation) at around 14 weeks of age (Hulot *et al.*, 1982; Diaz *et al.*, 1988, Rommers *et al.*, 2001), although the age at first mating depends upon the breed of rabbit (Harkness *et al.*, 2010). In the Californian and New Zealand breeds, the females are sexually receptive and achieve high reproductive performance (ovulation and in litter size) by 17-20 weeks of age (Hulot *et al.*, 1982; Gosálvez *et al.*, 1994; Rommers *et al.*, 2001). Different feeding systems (*ad libitum* vs restrictive) will affect reproductive performance in rabbits (Rommers *et al.*, 2001), and some researchers recommended that breeding begin when the female rabbit reaches about 75% of its adult weight (Gosálvez *et al.*, 1994). Better litter size was observed in rabbits with higher body weights (i.e., fed *ad libitum*) at first insemination (Rommers *et al.*, 2002). In modern management schemes, however, young does are not inseminated at a fixed body weight but at a fixed age (Rommers *et al.*, 2002).

Ovarian function in rabbits is not well understood. Unlike South American camelids (i.e., induced ovulating species in which follicular wave activity has been well documented), the pattern of follicle development in rabbits has not been established. Unlike most other mammals, the formation, activation and development of ovarian follicles occurred entirely postnatally in rabbits, with primordial follicle assembly presumably completed between 2 and 4 weeks of age (Hutt *et al.*, 2006). With the onset of puberty, follicles reached ovulatory status and were related with sexual behavior. For instance, some authors have indicated that follicles with a diameter >1.8 mm were present only in receptive females (Lefèvre and Caillol, 1979). However, there is no clear understanding of the relationship between follicle diameter and ovulatory capability in rabbits. Preovulatory follicles were also referred as those >800 to 900 um in diameter (Kranzfelder *et al.*, 1984). Others have reported that preovulatory follicles are those that are >1.5

mm or > 2 mm in diameter (Parkes, 1931; Hunzicker-Dunn *et al.*, 1979; Marongiu and Gulinati, 2008). A continuous growth and regression of follicles appears to occur in receptive rabbits and investigators in an early study suggested that follicles able to ovulate remain for about 7 to 10 days and then regress (Hill and White, 1933). Follicular development is thought to be supported by the growth of 5 to 10 follicles on each ovary at any one time. Once follicles reach an ovulatory size, they secrete estrogens in increasing amounts and rabbits show sexual receptivity for a period of time. When those follicles degenerate, secretions of estrogen decline and female rabbits become non receptive (Harcourt-Brown, 2002). Likewise, results of others have shown that receptive rabbits had more large follicles and a higher concentration of estradiol in the follicular fluid than those of non receptive rabbits (Lefèvre and Caillol, 1979). Additionally, the ability to ovulate in response to mating was suggested to differ between receptive (accepted mating) and non receptive rabbits (refused mating and were subjected to assisted mating); and 12 h post-mating, ovulations were detected in 4/6 vs 0/4 rabbits, respectively (Boumahdi-Merad *et al.*, 2011). Findings from others (Hulot *et al.*, 1988) have suggested that failure to ovulate in rabbits may be caused by a lack of discharge of LH rather than to a lack of mature follicles in the ovaries.

Pseudopregnancy occurs after a sterile mating or after hormonal induction of ovulation in rabbits and lasts about 16-18 days (Scott and Rennie, 1970; Kelley and Brinkley, 1971; Dharmarajan *et al.*, 1988; Harcourt-Brown, 2002). An average of 8 to 10 corpora lutea developed after induction of ovulation in rabbits mated with vasectomised males (Browning *et al.*, 1980) or after intravenous administration of hCG (Hulot *et al.*, 1988). During development of the corpora lutea, the theca interna takes part in the active growth of each CL, while the theca externa takes part in the vascularisation and forms the sheath of the fully-grown CL (Deanesly, 1930). In

addition, LH causes the differentiation of granulosa cells to luteal cells (Labib *et al.*, 1978). The growth and subsequent regression of CL resulted primarily from changes in the volume rather than changes in the number of individual luteal cells (Dharmarajan *et al.*, 1988). In one study (Kelley and Brinkley, 1971), corpora lutea reached maximum size by Day 9 (Day 0 = Day of mating), started to regress by Day 15, and were significantly reduced in size by Day 21. In another study (Dharmarajan *et al.*, 1988a), CL volume decreased after Day 11. The progesterone concentrations were found to be high (13.4ng/ml) on Day 10 and low (1.5ng/ml) by Day 18 (Miller and Keyes, 1976). Similar results (Browning *et al.*, 1980) documented high progesterone concentrations on Days 10-12, followed by a steady decline till less than 1 ng/ml on Days 16-18. By using an ovarian *in vivo* perfusion method, results (Dharmarajan *et al.*, 1988b) showed a significant rise in progesterone secretion from Day 1 to Day 11, a decline by Day 18. Interestingly, when pseudopregnant rabbits were hysterectomized on Day 1 and treated with estradiol, the progesterone concentrations remained elevated (around 11 ng/ml) at least until Day 28 (last day of observation) (Miller and Keyes, 1976). In addition, when pseudopregnant rabbits were hysterectomized on Day 4 and treated with LH on Day 9, life span of corpora lutea was prolonged to Day 24 (Kelley and Brinkley, 1971). Results of the latter studies suggested that the uterus has a role in limiting the life span of the CL in pseudopregnant rabbits (Kelley and Brinkley, 1971; Miller and Keyes, 1976). Prostaglandin F_{2α} secretion by the uterine endometrium has been suggested to have a direct luteolytic effect on the rabbit CL (Scott and Rennie, 1970; Koering, 1974).

In pregnant rabbits, the maximum mass of CL was reached on Days 16 to 20 and then declined gradually to Day 28, near term (Abdul-Karim and Bruce, 1973). The progressive reduction in luteal cell numbers was related to the gradual decline in mass of CL, but a clear

structural regression of CL (i.e., reduction in surface area of capillaries per CL, decline in CL blood flow) was observed by Day 28-30 (Dharmarajan *et al.*, 1989). In an early study, investigators reported that serum progesterone concentrations from CL declined after Day 16 (Hilliard *et al.*, 1968) in pregnant rabbits. Results of later studies showed that highest values of serum progesterone concentrations were reached on Days 10-12 and were similar to those found during pseudopregnancy, then progesterone declined slightly but remained elevated through Day 28 (Browning *et al.*, 1980). Similar results were described by others (Egea del Prado *et al.*, 1984), who found maximum progesterone concentrations by Days 11 to 13 (10.8-11.8 ng/ml), followed by a slightly decrease but elevated progesterone until Day 25 to 28 (7.15 ng/ml). Maintenance of pregnancy (30 to 32 days) in rabbits requires progesterone production by CL (Greewald and Rothchild, 1968; Rashwan *et al.*, 2003), and the maintenance of the CL function is dependent on the presence of the conceptus and estradiol (Greep, 1941; Holt, 1989). A critical period for the CL appeared to be days 5 to 6 after mating, during which presence of estradiol is essential for continued luteal development and secretion of progesterone (Miller and Keyes, 1978).

1.2. Mechanism of ovulation in rabbits

Ovulation is induced by copulation in rabbits (Heape *et al.*, 1905; Friedman *et al.*, 1929; Spies *et al.*, 1997; Harkness *et al.*, 2010). In studies involving direct and histological examinations of the ovaries at different times post-mating, ovulation was detected 10 to 13 h after mating (Walton and Hammond, 1928; Fee and Parkes, 1929). A neuro-endocrine reflex is thought to provoke a surge of LH within 1-2 h post-mating (Hilliard *et al.*, 1974; Jones *et al.*, 1976; Meunier *et al.*, 1983; Spies *et al.*, 1997). Authors of an early study (Hammond and Asdell,

1926) reported that artificial insemination followed by sterile mating resulted in higher percentages of conception than after artificial insemination alone (20/22 [91%] vs 1/28 [4%]). There is, however, no clear evidence to support that the mechanical stimulus of the penis or the presence of semen in the vagina initiates ovulation (Walton and Hammond, 1928). Results of another study showed that repetitive intravenous or intraperitoneal administration of rabbit spermatozoa or fresh testicular extracts into female rabbits delayed the return of successful fertility after mating, because animals failed to become pregnant during 6 to 25 weeks even after being repeatedly mated (Pommerenke, 1928). This may imply that antibodies directed against spermatozoa antigens have been produced in those rabbits, which then can cause infertility. While after repetitive intravenous administration of rabbit seminal plasma, fertility was not altered (Weil and Roberts, 1965; Menge, 1971). The primary trigger for inducing ovulation in rabbits is not well understood, but the pituitary gland plays a major role in the occurrence of ovulation; its surgical removal within 1 h after copulation inhibited ovulation (Fee and Parkes, 1929), while tissue pituitary extracts produced ovulation in hypophysectomized rabbits (Hill and Parkes, 1931).

Evidence has suggested that copulation in rabbits induces activation of GnRH cells in the hypothalamus and results in a GnRH surge and then in a LH surge (Spies *et al.*, 1997; Blaustein and Ershine, 2002). An investigator indicated that hypothalamus may influence the activity of the pituitary by a humoral mechanism (Harris, 1948). However, some others showed that electrical stimulation of central nervous system (cerebral and spinal) induced ovulation in rabbits (Marshall and Verney, 1936), and surgical transection of the pituitary stalk (connection between pituitary and hypothalamus) prevented ovulation after mating-related stimulation in rabbits

(Brooks and Lambert, 1939). Likewise, vaginal stimulation in rabbits has been reported to increase electrical activity in hypothalamic neurons (Vincent *et al.*, 1970).

In rabbits, ovulation can also be induced by exogenous administration of hormones (Harkness *et al.*, 2010). The occurrence of ovulation by direct examination of the ovaries was detected between 10 and 14 h after intravenous administration of LH (Harper, 1961) or LH-RH (Kanematsu *et al.*, 1974; Carlson and Perrin, 1979). Ovulation was also detected after intravenous administration of hCG (Kobayashi *et al.*, 1981; Molina *et al.*, 1987) and intramuscular administration of GnRH (Rodríguez *et al.*, 1988). In commercial rabbit production systems, GnRH has been used after insemination to stimulate ovulation, given intramuscularly or intravaginally added to the seminal dose (Ondruška *et al.*, 2008; Quintela *et al.*, 2008).

1.3. Ovulation in other induced ovulators

In other induced ovulators, such as the discussed in this review, the mechanism underlying the ovulatory process remains unclear. As in rabbits, there is evidence that a release of LH occurs after copulation or administration of exogenous gonadotropin hormones.

In South American camelids (i.e., llamas, alpacas), ovulation was detected 26 h after mating by post-mortem examination (San Martín *et al.*, 1968) and at 30 h after mating by laparoscopic examination (Sumar *et al.*, 1993). Using ultrasonography, ovulation was detected 1-3 days (mean, 1.8 days, Adams *et al.*, 1989; mean, 2 days, Adams *et al.*, 1990, 1991), or 30.0 ± 0.5 h after mating (Mean \pm SEM, Ratto *et al.*, 2006a). Ovulation was also detected 24-30 h after intramuscular administration of hCG, LH or GnRH (San Martín *et al.*, 1968; Huanca *et al.*, 2001; Ratto *et al.*, 2006a). Plasma LH concentrations increased after mating, reaching maximum

concentrations (3-8 ng/ml) after 2 h, then decreasing and returning to basal levels (0.96 ng/ml) after 7 h (Bravo *et al.*, 1992). Similarly, a rise in plasma LH concentration has also been documented after administration of GnRH (Bravo *et al.*, 1992); maximum concentrations were observed at 1.4 ± 0.2 h, began to decrease at 5 h, and returned to basal levels by 5.5 h (Mean \pm SEM, Adams *et al.*, 2005). After mating, if ovulation occurs, a corpus luteum (CL) develops and produces progesterone (England *et al.*, 1969; Fernandez-Baca *et al.*, 1970a; Adams *et al.*, 1990, 1991; Vaughan *et al.*, 2003; Ratto *et al.*, 2006a). When a functional CL is present this period is referred as luteal phase (Adams *et al.*, 1991). Maximum plasma progesterone concentrations for non pregnant females occurred on Days 8 to 9 (Day 0 = Day of mating) (Adams *et al.*, 1991; Aba *et al.*, 1995; Ratto *et al.*, 2006a), followed by a sharp decrease starting between Days 12 to 13 (Fernandez-Baca *et al.*, 1970a; Ratto *et al.*, 2006a), and reached a nadir on Days 14 to 15 (Fernandez-Baca *et al.*, 1970a; Adams *et al.*, 1991). In pregnant llamas, a transient decrease in plasma progesterone concentration occurred between Days 10 and 12, then increased and remained above 2 ng/ml after Day 12 (Adams *et al.*, 1991). Progesterone produced by CL is necessary for the maintenance of pregnancy (11 months) in camelids (San Martin *et al.*, 1968; Sumar *et al.*, 1988; Leon *et al.*, 1990) and was found to remain elevated during the whole pregnancy (Leon *et al.*, 1990; Aba *et al.*, 1995). By the time of parturition, luteolysis occurred and progesterone concentrations declined dramatically (Bravo *et al.*, 1991b). Five days after parturition, females were receptive to males and ovulation and fertilization was achieved (Sumar *et al.*, 1972), but greater percentages of successful ovulation were obtained 20 days after parturition (Bravo *et al.*, 1995). Studies related to ovarian follicular dynamics have shown a wave-like pattern of follicular development in llamas (Adams *et al.*, 1990; Bravo *et al.*, 1990; Chaves *et al.*, 2002) and alpacas (Bravo and Sumar, 1989; Vaughan *et al.*, 2004). Some authors

(Adams *et al.*, 1990) have suggested that a growing or regressing follicle of at least 6 mm was expected to be found at any given time due to the occurrence of successive follicular waves in camelids. Growing follicles ≥ 6 mm had the ability to ovulate (Adams *et al.*, 1990) while those regressing lost that ability (Bravo *et al.*, 1991a). Ovulatory capability depends of the ovarian follicular size at the time of mating (Adams *et al.*, 1990; Bravo *et al.*, 1991a); release of LH surge occurred but did not elicit ovulation in alpacas and llamas unless a growing or mature follicle ≥ 6 mm in diameter was present (Bravo *et al.*, 1991a).

In Old World camels (ie., dromedary camel, Bactrian camel), ovulation was detected by rectal palpation 36-48 h after mating (Xu *et al.*, 1985), or after insemination (Chen *et al.*, 1985). Intramuscular administration of LH, hCG and LH-RH also was shown to induce ovulation (Chen *et al.*, 1985). An increase in LH concentrations was found within 1 h of mating and reached maximum concentrations 2-4 h later, and decreased 6-8 h after mating (Xu *et al.*, 1985; Marie and Anouassi, 1986). The pattern of plasma progesterone concentrations (Xu *et al.*, 1985; Marie and Anouassi, 1986, 1987; Skidmore *et al.*, 1996) was reported to be similar to the one described above in South American camelids, although the duration of the luteal phase in non pregnant females was indicated to be shorter.

In ferrets, ovulation occurred 30-40 h after mating and after sufficient LH release (3- to 4-fold elevation compared to basal levels) (Hammond and Walton, 1934; Carroll *et al.*, 1985). Failure to ovulate was found when copulation did not lead to an increase in LH, which was suggested to be due to incomplete coitus (Carroll *et al.*, 1985); considering that copulation in ferrets lasts on average 1-2 h (Hammond and Walton, 1934; reviewed by Lindeberg, 2008). Although, vagino-cervical stimulation even after 1 min of copulation induced an LH surge (Carroll *et al.*, 1985). The lifespan of CL was reported to be 40-42 days, regardless of whether

fertilization occurred (Chang and Yanagimachi, 1963) and progesterone concentrations were detected to decline progressively between days 24 and 42 (Blatchley and Donovan, 1976). Pregnancy lasted about 41 days (reviewed by Lindeberg, 2008).

In cats, a linear relationship was found between the number of sexual contacts, the magnitude and duration of LH release, and the occurrence of successful ovulation (Concannon *et al.*, 1980). After a single mating in late estrus, ovulation occurred but conception rates were low (25%) (Glover *et al.*, 1985). Whereas repeated matings in mid-estrus were related to high plasma LH concentrations, which stimulated ovulation and provided high (> 80%) conception rates (Tsutsui *et al.*, 2009). In general, LH increases were detected within 5 to 10 min after coitus. The LH surge occurred within 1 h of coitus (Johnson and Gay, 1981; Tsutsui *et al.*, 2009), and remained higher than basal levels for about 8-16 h (Banks and Stabenfeldt, 1982; Shille *et al.*, 1983). If fertilization does not occur, the luteal phase of non pregnant cats has been indicated to be half as long as pregnancy (Brown *et al.*, 1994). Pregnancy lasted about 62 to 71 days (Root *et al.*, 1995).

In koalas, a full copulatory stimulus from the male has been shown to be required for ovulation, formation of CL and secretion of progesterone (Johnston *et al.* 2000a). The full copulation was thought traditionally to induce ovulation due to the neural excitation of the female urogenital system, but later studies found evidence that this ovulation-induction may be related to a component of the semen (Johnston *et al.*, 2004). Mechanical stimulation of the urogenital sinus with a glass rod failed to induce a luteal phase (Johnston *et al.*, 2004). As in other species, elevated LH concentrations were observed after mating (about 24–32 h) (Johnston *et al.*, 2004). The duration of the luteal phase and the concentration and profiles of fecal

progesterone (term used in recognition that the assay system may overestimate the absolute value of progesterone in the samples) during pregnancy were found to be similar to those during non pregnancy, with high progesterone concentrations for 36 to 38 days (Kusuda *et al.*, 2009). Pregnancy lasted about 34 to 49 days (Johnston *et al.* 2000b).

1.4. Effect of seminal plasma on ovulation in induced ovulators

Seminal plasma is the fluid and noncellular component of the semen (reviewed by Leibfried-Rutledge *et al.*, 1997). This fluid is produced mainly by the male accessory sex glands and the epididymis (Mann, 1964; Foxcroft *et al.*, 2008). It contains a variety of components such as amino acid, lipids, fatty acids, peptides and proteins (reviewed by Jonakova *et al.*, 2010), as well as other factors such as prostaglandins, proteases, immunoglobulins (Alexander and Anderson, 1987 cited by Croy and Whitelaw, 1998) and cytokines (reviewed by Hedger and Hales, 2006). The contribution of accessory glands to seminal plasma is variable from specie to specie (reviewed by Leibfried-Rutledge *et al.*, 1997).

Seminal plasma components have been thought to play a role in the maintenance, function and transportation of spermatozoa (Mann, 1964; Lindholmer, 1974; Jones and López, 2006; Pesch *et al.*, 2006). However, , investigators have revealed that seminal plasma also has a role in female reproductive function in both spontaneous ovulators (e.g., pig; reviewed by Maxwell and Johnson, 1999; O’Leary *et al.*, 2006; Robertson *et al.*, 2007) and induced ovulators (e.g., camelids; Adams *et al.*, 2005).

Early investigators demonstrated that ovulation (detected by rectal palpation) in female Bactrian camels without any physical contact with males was induced by camel or bovine

seminal plasma when given intravaginally (Chen *et al.*, 1985). They suggested that seminal plasma in this species contains an ovulation-inducing factor (OIF). Additionally, ovulation occurred after intramuscular administration of camel seminal plasma, but the methodology or data analysis was not presented in the study. In another study (Xu *et al.*, 1985), intravaginal administration of bactrian camel semen in female bactrians resulted in a peak in plasma LH concentrations at 4 h and increased plasma progesterone concentrations at 3 days. A fraction (“L3”) isolated from camel seminal plasma caused LH release from *in vitro* rat pituitary tissue culture and *in vivo* after intramuscular administration in female camels (Zhao *et al.*, 2001). Pan *et al.* (2001) reported that purified OIF from seminal plasma of camels was a novel hormone different from LH-RH, LH, hCG, PMSG, and PGF, according to the analysis of amino acid components and the partial amino acid sequence of aminated N-terminus. In addition, when “L3” from seminal plasma of camels was fractionated into four subfractions, “F3-3” still stimulated the *in vivo* release of LH; however, later attempts to further purify OIF from “F3-3” were not successful (Li and Zhao, 2004).

In South American camelids, earlier investigators evaluated ovulation by direct inspection of the ovaries and by flushing of the oviducts (ova collection), and concluded that mating accompanied by penile intromission was necessary for induction of ovulation (Fernandez-Baca *et al.*, 1970b). In a later study (Rios *et al.*, 1985) using laparoscopic examination of the ovaries, ovulation occurred in alpacas after intravaginal insemination of alpaca and bovine semen (53% and 67%, respectively). A lower ovulation rate (8%), however, was reported after intravaginal insemination of alpaca seminal plasma vs alpaca or bovine semen. It is plausible that the disparity in the ovulation rates after administration of seminal plasma and semen of alpaca may be explained by the samples used (e.g., pool samples vs

individual samples), differences in the insemination procedure (e.g., degree of vaginal stimulation) or other factors. The induction of ovulation in alpacas by insemination in the study by Rios (1985) led to a proposition that semen in this species may contain an ovulation-inducing factor (OIF), similar to Bactrian camels. In addition, intravaginal insemination of alpaca semen stimulated ovulation in 6/10 alpacas and 5/8 llamas. (Sumar, 1994), and progesterone secretion occurred after insemination, albeit for a period 1-2 days shorter than that after mating. Another study investigated the effect of alpaca seminal plasma on LH release using a bioassay of rat pituitary cells, and documented that a stimulating effect of seminal plasma could be mediated by a factor chemically different from GnRH (Paolicchi *et al.* 1999). The findings of further studies reinforced the existence of a potent ovulatory factor in the seminal plasma in camelids. An intramuscular administration of seminal plasma of alpacas and llamas was clearly documented to elicit 30 h after a surge in circulating concentrations of LH, and to induce high percentages of ovulation as detected by transrectal ultrasonography in 13/14 alpacas and 6/6 llamas (Adams *et al.*, 2005). In the same study, intrauterine administration of seminal plasma without curettage did not induce ovulation. In a follow-up study on the effects of seminal plasma in alpacas (Ratto *et al.*, 2005), higher ovulation rate occurred when females were treated with an intramuscular administration of seminal plasma compared to intrauterine administration of seminal plasma without curettage (93% vs 41%). An intermediate ovulation rate occurred when females were treated with endometrial curettage following intrauterine administration of seminal plasma (67%), while no ovulation occurred in alpacas treated with endometrial curettage following intrauterine administration of saline (negative control group) (Ratto *et al.*, 2005). The disparity between the effects of intrauterine administration of seminal plasma in noncurettaged alpacas on ovulation (41% vs 0%, respectively) in the Ratto (2005) and Adams (2005) studies may be

attributed to the dose of seminal plasma used (i.e., 2 ml vs 1 ml) and/or site of deposition of seminal plasma which may have disrupted the endometrial mucosa. In general, (Ratto *et al.*, 2005) concluded that OIF in seminal plasma effects ovulation via a systemic rather than a local route, curettage disrupted the endometrial mucosa and facilitated the absorption of OIF into the circulatory system, and ovulation was not associated with physical stimulation of the genital tract alone. The effects of different doses of purified OIF from llama seminal plasma given intramuscularly to llamas was investigated, and was concluded that the effects of OIF on ovulation as well as the development and function of corpus luteum are dose-dependent (Tanco *et al.*, 2011). Purified OIF from llama seminal plasma was also evaluated using an *in vitro* bioassay of llama and bovine pituitary cells (Bogle, 2009). Results revealed a dose-related effect on LH release from the llama pituitary cell, documenting that OIF stimulated LH release directly from the pituitary in camelids (Bogle, 2009). Conversely, a recent study has suggested that the preovulatory LH surge induced by OIF is mediated primarily by a direct or indirect effect of OIF on hypothalamic GnRH neurons since OIF did not induce ovulation in llamas pre-treated with a GnRH antagonist (cetrorelix; Silva *et al.*, 2011).

OIF in llama seminal plasma has been identified as a protein molecule with a molecular mass of approximately 14-30 kDa (Ratto *et al.*, 2010, Ratto *et al.*, 2011), which is able to elicit a preovulatory LH surge followed by ovulation and CL formation in llamas. It is speculated that the 14 kDa protein identified in the latter study may be a part of a larger protein complex or represented a bioactive prohormone. Future research is required to confirm the structure of OIF in order to fully understand its function and underlying mechanism of action.

In koalas, elevated progesterone concentrations were detected when koala semen was inseminated with and without subsequent urogenital stimulation with a glass rod (7/9 vs 4/9, respectively) (Johnston *et al.*, 2004). Unlike seminal plasma, the physical stimulation with a glass rod alone did not elicit an LH surge or effect ovulation, nor was it associated with a subsequent rise in plasma progesterone concentration. Authors of this study suggested that koala semen contains a factor or factors that induce the development of a functional CL (luteal phase). The authors speculated, however, that the glass rod stimulation method used in their study probably did not mimic the stimulation provoked by the koala penis. With regard to this, they suggested that their findings should not be used to conclude that ovulation is not associated with physical stimulation of the genital tract in the koala.

In rabbits, few studies about the effect of semen on female function have been reported. (Walton and Hammond, 1928) suggested that ovulation is not initiated by the deposition of semen in the vagina. (Pommerenke, 1928) indicated that repetitive intravenous or intraperitoneal administration of rabbit spermatozoa or fresh testicular extracts into female rabbits may delay the return of successful fertile mating, and females failing to become pregnant during 6 to 25 weeks even after repetitive mating. This may imply that antisperm antibodies were produced in those female rabbits, a condition that can decrease fertility (Naz *et al.*, 1986). When seminal plasma was administered, fertility was not altered (Weil and Roberts, 1965). No studies have been reported on the effects of seminal plasma on ovulation in rabbits, but (Silva *et al.*, 2010) demonstrated that OIF is present in the seminal plasma of rabbits.

Evidence for the presence of OIF in seminal plasma was also found in spontaneous ovulators, including cattle, horses and pigs, suggesting that OIF may be a conserved molecule

among species (Ratto *et al.*, 2005, 2006b; Bogle *et al.*, 2011). Interestingly, there is some evidence that OIF affects ovarian function in spontaneous ovulators such as mice (Bogle *et al.*, 2011) and cattle (Van Steelandt *et al.*, 2008).

1.5. Ultrasonographic assessment of the effect of seminal plasma on induced ovulators

In camelids, transrectal ultrasonography has been successfully used to detect and confirm ovulation in live females treated with seminal plasma and/or purified OIF (Adams *et al.*, 2005; Ratto *et al.*, 2005, 2010, 2011; Bogle *et al.*, 2011; Tanco *et al.*, 2011; Silva *et al.*, 2011). Ultrasonic examinations have been carried out using a real-time, B-mode scanner equipped with either a 5.0 or 7.5 MHz linear-array transducer. Ovulation in llamas and alpacas was defined ultrasonographically as the sudden disappearance of a large (≥ 7 mm) follicle from one day to the next, and confirmed by the subsequent detection of a corpus luteum (Adams *et al.*, 1989; Adams *et al.*, 1991; Vaughan *et al.*, 2003). A corpus luteum was characteristically circular and hypoechogenic relative to the surrounding tissues, often with an indistinct horizontal white (echogenic) area traversing the center of the corpus luteum (Adams *et al.*, 1989).

While transrectal and transcutaneous ultrasonography (5.0 or 7.5 MHz) have been used successfully to assess ovarian structures in large animals, ovarian structures could not be reliably imaged by conventional ultrasonography in small animals (Kahn *et al.*, 1992). To date, the resolution of conventional ultrasonography is inadequate for the study of structures ≤ 2 mm; i.e., ovarian structures in small laboratory species (Pallares and Gonzalez-Bulnes, 2008). In rabbits, however, the use of ultrasonography with a 7.5 MHz linear-array transducer was described for the examination of ovarian structures in female rabbits prior to and after intravenous

administration of hCG (Marongiu and Gulinati, 2008). The authors referred to the ultrasound images of follicles as well-defined anechogenic circular areas, and the CL as hyperechogenic structures (Marongiu and Gulinati, 2008). To our knowledge, there are no reports about ultrasonographic characterization of ovarian dynamics in rabbits, or for assessment of response to treatment with seminal plasma and/or OIF.

The advancement of ultrasound technologies has permitted the development of high-frequency ultrasound systems for imaging structures <1 mm (<http://www.visualsonics.com>). This technology is often referred as ultrasound biomicroscopy (UBM) and involves a single crystal to emit a sound wave with frequencies of 20–70 MHz, providing resolution to 30 μm (<http://www.visualsonics.com>). The UBM characteristics have enabled *in vivo* study of embryogenesis (Turnbull and Foster, 2002) and ovarian follicular dynamics in mice (Jaiswal *et al.*, 2009). The use of UBM to examine ovarian structures in mice (Pallares and Gonzalez-Bulnes, 2008; Mircea *et al.*, 2009) has offered several advantages over traditional methods used previously. Methods of a terminal or invasive nature (e.g., histology or laparoscopy) involve laborious and time-consuming procedures and limit the number of examinations possible in the same animal. Studies of ovarian function in rabbits using UBM have not been reported.

2. 0. OBJECTIVES & HYPOTHESIS

The general objective of this research project was to develop a rabbit model for the study of ovulation-inducing-factor (OIF).

The specific objectives were:

Objective 1: To validate ultrasound biomicroscopy (UBM) as a tool for assessing ovarian follicles and corpora lutea in rabbits (Chapter 3)

Objective 2: To determine if surgical translocation of the ovaries to a subcutaneous position will permit serial *in vivo* transcutaneous imaging by UBM (Chapter 3).

Objective 3: To test the hypothesis that llama and rabbit seminal plasma elicits a surge of LH release and is responsible for inducing ovulation in rabbits (Chapter 4).

3.0. SURGICAL TRANSLOCATION AND ULTRASOUND BIOMICROSCOPY OF THE OVARIES IN RABBITS

3.1. Abstract

Two experiments were designed to determine the efficacy of ultrasound biomicroscopy (UBM) for assessing ovarian structures in rabbits. Experiment 1 was designed to validate the use of UBM as a method for assessing ovarian structures in rabbits. Four female New Zealand White rabbits (5-5.5 months old) were given ovulation-inducing treatments. The ovaries were examined *ex situ* by UBM using a 25 MHz oscillating sector transducer, and then ovaries were processed for histology. Follicles ≥ 0.6 mm and corpora lutea (CL) were counted and measured by scrolling through the UBM cine-loops and the serial micrographs. Pairwise correlations between variables were examined by Pearson's correlation coefficient (r). Results show strong correlations ($P < 0.05$) between UBM and histology in the number (mean \pm SEM) of follicles ≥ 0.6 mm (17.3 ± 2.3 vs 19.0 ± 1.6 , respectively; $r = 0.96$; $P = 0.040$), and follicle diameter (1.1 ± 0.05 vs 1.1 ± 0.03 mm, respectively; $r = 0.96$; $P = 0.035$), as well as in the number of CL (8.5 ± 2.9 vs 8.8 ± 3.0 , respectively; $r = 0.99$; $P = 0.003$) and the diameter of CL (2.1 ± 0.7 vs 1.8 ± 0.6 mm, respectively; $r = 0.99$; $P < 0.001$). Experiment 2 was conducted to develop a method that permits serial evaluation of ovarian structures *in vivo* in rabbits, involving surgical translocation of the ovaries to a subcutaneous position. Twelve female New Zealand white rabbits (5 months old) were successfully submitted to surgery, without intra- or post-operative complications. Results show that by using serial UBM *in vivo*, ovarian structures were easily and consistently identified in the rabbits submitted to the surgical approach. The mean number of follicles ≥ 0.6 mm and the mean diameter of the largest 7 follicles per pair of ovaries were similar in three successive daily examinations. Follicles >2 mm in diameter were detected in 3/12 rabbits on the first and second

days, and in 5/12 on the third day. Rabbits were not treated with ovulation-inducing agents, but one or more corpora lutea were detected consistently in 2 of the 12 rabbits, and suggested the occurrence of spontaneous ovulation. There were no apparent effects of surgical translocation on ovarian function. Interestingly, rabbit cumulus-oocyte complexes (COC) were clearly visualized using UBM *ex situ* and *in vivo*. In conclusion, results document the efficacy of UBM as a method for assessing rabbit ovaries *ex situ* and *in vivo*.

3.2. Introduction

Rabbits have been used as a research model for human and other mammals in a variety of areas (e.g., physiology, toxicology, biomedical research, placentation) (Kaplan and Timmons, 1979, Foote and Carney, 2000, reviewed by Brewer, 2006, Bősze and Houdebine, 2006; Carter, 2007) because they are medium size, readily accessible, easy to handle, and easy to maintain (Manning *et al.*, 1994). Study of ovarian function in the rabbit has been based on findings at post-mortem examination and histology (Deanesly, 1930; Armstrong *et al.*, 1978; Kranzfelder *et al.*, 1989; Agrawal and Jose, 2011), laparotomy (Marshall and Verney, 1939; Kauffman *et al.*, 1998; Agrawal and Jose, 2011), laparoscopy (Blasco *et al.*, 1994; Argente *et al.*, 2010), and based on endocrinological findings (Miller and Keyes, 1978; Labib *et al.*, 1978; Caillol *et al.*, 1983; Mills and Gerardot, 1984). The terminal or invasive methods, however, provide little or no opportunity for serial examination and restrict progress in our understanding of follicle development and ovulation in rabbits.

The advent of real-time B-mode ultrasonography resulted in rapid advancement of our understanding of ovarian follicular and luteal dynamics in every species in which it has been used, including cattle (Pierson and Ginther, 1984; Adams and Pierson, 1995), horse (Ginther and

Pierson, 1984), camelids (Adams *et al.*, 1989), sheep (Ravindra *et al.*, 1994) and humans (Queenan *et al.*, 1980; Pierson *et al.*, 1990). Conventional ultrasonography, using frequencies of 5-7.5 MHz, has been used widely for ovarian studies in large species, but resolution is inadequate for detailed study of small structures ≤ 2 mm; i.e., for visualization of ovarian structures in small laboratory species (Pallares and Gonzalez-Bulnes, 2008). Based on a report in which conventional ultrasonography (using a 7.5 MHz transducer) was described as a method of evaluating follicular dynamics in rabbits (Marongiu and Gulinati, 2008), we initiated preliminary studies to examine the effects of ovulation-inducing treatments in rabbits *in vivo* using conventional transabdominal ultrasonography with a 12 MHz transducer (Cervantes *et al.*, unpublished data). The images obtained, however, were inadequate for resolving dynamic changes in follicular development or for evaluating ovarian response to treatments in the rabbits.

High frequency ultrasound systems (ultrasound biomicroscopy) have provided the technological basis for imaging tissue at the cellular level (Czarnota *et al.*, 1997). Ultrasound biomicroscopy (UBM), using frequencies of 20-55 MHz, has enabled the *in vivo* study of embryogenesis in mice (Turnbull and Foster, 2002), as well as ovarian follicular dynamics at the level of the theca / granulosa cell layer, and visualization of cumulus-oocyte complex in mice (Singh *et al.*, 2003) and in humans ovaries *in vitro* (Baerwald *et al.*, 2009). Recent studies documented the feasibility of UBM for imaging ovarian structures in mice (Pallares and Gonzalez-Bulnes, 2008; Mircea *et al.*, 2009). In one study, serial *in vivo* UBM was used to characterize ovarian dynamics in mice using a transcutaneous approach; however, a similar approach was not successful in rats (Jaiswal *et al.*, 2009). In addition, UBM may have better resolution than conventional ultrasonography to distinguish more clearly ovarian structures in

rabbits, as reported in humans (Baerwald *et al.*, 2009). To date, studies of ovarian function in rabbits using UBM have not been reported.

The objectives of the present study were to validate the use of ultrasound biomicroscopy as a method for assessing ovarian structures in rabbits, and to develop a method that permits serial noninvasive evaluation of ovarian structures *in vivo* in rabbits.

3.3. Materials and methods

New Zealand White rabbits were housed under controlled environmental conditions (20.2 ± 2°C, 14:10-h light:dark cycle) and were allowed *ad libitum* access to food and water. The experimental protocol was approved by the University of Saskatchewan's Animal Research Ethics Board, in accordance with guidelines of the Canadian Council on Animal Care.

3.3.1. Experiment 1

The experiment was conducted to validate the use of ultrasound biomicroscopy (UBM) as a method for assessing follicles and corpora lutea in rabbit ovaries. Four female rabbits (5-5.5 months old) were given 20 µg of GnRH (Fertagyl; Intervet Canada Ltd., Whitby, Ontario, Canada) intramuscularly as an ovulation-inducing treatment. The day of treatment was designated Day 0. Rabbits were euthanized on Day 8 by an overdose of sodium pentobarbital, and the ovaries were removed for evaluation *ex situ*. Immediately following removal, each ovary was imaged in a saline bath using a high-resolution ultrasound biomicroscope (UBM; Vevo 660 Imaging System™, Visual Sonics Inc., Toronto, Ontario, Canada) equipped with a 25 MHz oscillating sector transducer (RMV 704; Fig. 3.1). The measured lateral resolution of the

transducer was 75 μm , the axial resolution was 40 μm , and the depth range of imaging was 1.5 to 10 mm with a focal length of 6 mm (<http://www.visualsonics.com>). The saline bath was placed on a manually operated mechanical stage and moved through two horizontal axes (forward-to-back and side-to-side). The transducer was placed in a stationary holder and the plane of the beam was oriented transverse to the long axis of the ovary. The stage was moved until the ovary was visualized from one end to the other, and images were recorded in cine-loops (300 frames in 10 seconds). Following UBM imaging, the ovaries were cleaned of adhering connective tissue and fat, fixed in 10% formaldehyde, and stored at room temperature until histological processing.

Individual follicles ≥ 0.6 mm and corpora lutea were identified, counted and measured by scrolling through the digital UBM cine-loops frame-by-frame. Two orthogonal diameter measurements were taken from images that contained the greatest cross-sectional area of the structure in question. The overall diameter (mm) of the structure was taken as the average of the two measurements.

Standard histological procedures were used to prepare ovarian sections. In brief, the ovaries were dehydrated, embedded in paraffin wax, serially sectioned at 10 μm thicknesses, dewaxed, hydrated, and stained with Masson's trichrome. Stained slides were examined with conventional light microscopy, and digital photomicrographs of every 3rd section (i.e., at 30 μm intervals) of each ovary were taken. Photomicrographs were sequentially loaded into Image J NIH Software System (National Institutes of Health, Bethesda, Maryland, USA), and individual follicles ≥ 0.6 mm and corpora lutea were identified, counted and measured by scrolling through the serial digital images. The procedure to measure the structures of interest was similar that

described for assessing UBM images. All UBM and histological measurements and counts were performed by a single observer.

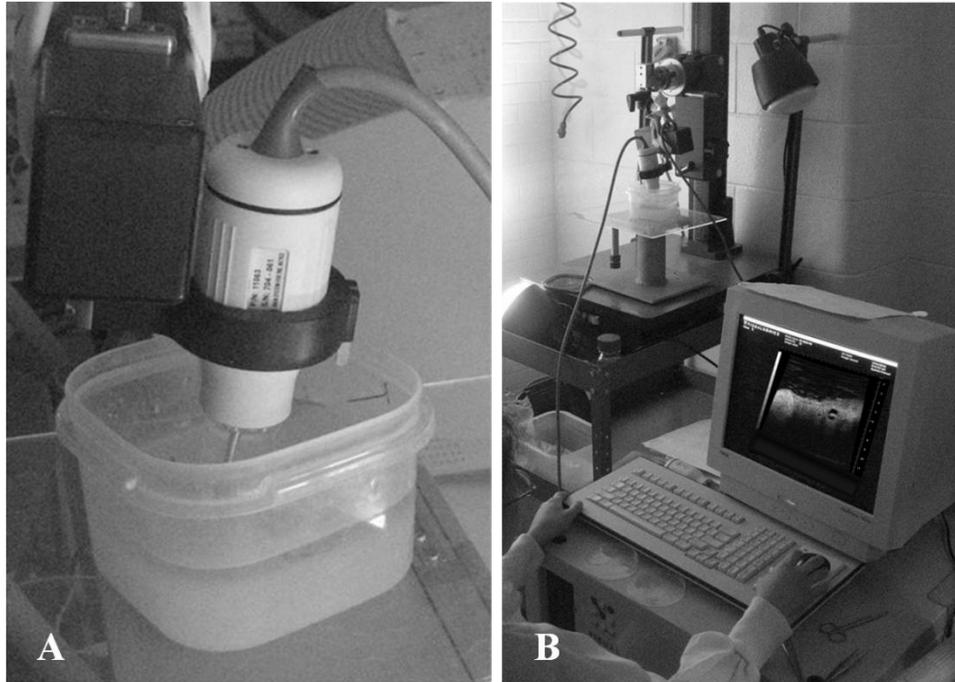


Figure 3.1. Ultrasound imaging of rabbit ovaries *ex situ* by ultrasound biomicroscopy (UBM) using a 25 MHz transducer. **(A)** The saline bath containing the ovary was placed on a manually operated mechanical stage with the transducer on a stationary holder. **(B)** Ovarian imaging was recorded in UBM cine-loops.

3.3.2. Experiment 2

Attempts were made in Experiment 1 to assess ovarian structures *in vivo* by transabdominal UBM, but the acoustic impedance of the body wall prevented acquisition of high-quality ovarian images. Therefore, a novel approach to enable *in vivo* imaging by UBM was developed in Experiment 2, which involved surgical translocation of the ovaries to a subcutaneous position.

Twelve 5-month-old female rabbits were housed in individual cages under controlled conditions, as described in Experiment 1. Sedation was induced by intramuscular administration of a combination of Butorphanol (0.5 mg/kg, Torbugesic, Wyeth Animal Health, Guelph, Ontario, Canada), Medetomidine (70 ug/kg, Domitor, Pfizer Animal Health, Kirkland, Quebec), and Ketamine (3.5 mg/kg, Vetalar, Bioniche, Belleville, Ontario, Canada), and the left and right lateral abdominal walls were shaved. Rabbits were positioned in the lateral recumbency position, anesthetized with isoflurane (Isoflo, Abbott Laboratories, LH, St. Laurent, Quebec, Canada) and given supplemental oxygen via a facemask. Lack of muscle tone and loss of ear pinch reflex were used as indicators of anesthetic depth. An ophthalmic gel (Refresh lacri-lube, Allergan Inc, Markham, Ontario, Canada) was applied to the eyes to prevent corneal desiccation. The surgical area was prepared for aseptic surgery with multiple scrubs of chloroxylenol 2% (Vetoquinol Inc., Lavaltrie, Quebec, Canada), and was draped using sterile technique. The heart and respiratory rates were monitored during the anesthetic period.

A lateral flank approach to each ovary was used. A vertical skin incision (2-3 cm long) was made 2 cm cranial to the anticipated site of muscle incision so that the sutured skin-wound would not directly overlie the translocated ovary. The muscle incision was done approximately half way between the last rib and the cranial border of the thigh. The external abdominal oblique muscle was incised and a grid-approach (blunt dissection) was used to go through the internal oblique and transversus muscles. The peritoneum was lifted with thumb forceps and incised carefully to give access to the peritoneal cavity. The ovary was exteriorized gently on the respective side, without interrupting its blood supply (i.e., ovarian attachments remained intact). The mesovarium was transfixed to the muscle layers of the abdominal wall at cranial and caudal poles of the ovary using an interrupted vertical mattress suture of 3-0 polydioxanone (PDS;

Ethicon, Markham, Ontario, Canada) such that the ovary remained superficial to the external abdominal oblique muscle (Fig. 3.2). An additional interrupted vertical mattress suture was placed carefully beneath the middle of the ovary to ensure that the ovary remained superficial to the external abdominal oblique muscle, taking care not to strangulate the ovarian blood supply. The muscle layers beyond the transfixation points were closed with simple interrupted sutures using 3-0 PDS. The subcutaneous layer was closed with simple continuous sutures of 3-0 PDS. The skin incision was closed using an interrupted cruciate pattern with 4-0 Novafil (Tyco Healthcare group, St. Laurent, Quebec, Canada) (Fig. 3.2). The surgical procedure was performed without intra- or immediate post-operative complications.

After surgery, topical antibacterial ointment (Furacin, Vetoquinol, Joliette, Quebec, Canada) and gauze pad (Primapore, Smith & Nephew, China) were applied to the incision site. The rabbits were returned to their cages, kept warm, and monitored until they recovered from anesthesia. Postoperative analgesia consisted of buprenorphine (30 ug/kg im, Vetergesic Multidose, Alstoe Animal Health Inc., Whitby, Ontario, Canada) given 12 and 24 h after surgery, and meloxicam (0.2 mg/kg sc, Metacam; Boehringer Ingelheim, Burlington, Ontario, Canada) given daily for 3 days after surgery. Delayed wound healing was observed in two rabbits, but both healed progressively over time; all other rabbits recovered uneventfully post surgery.

Daily transcutaneous ultrasound biomicroscopic examinations of the ovaries were initiated when wound closure was complete and the sutures were removed (≥ 2 weeks after surgery). Before ultrasound examination, both flanks of the rabbit were shaved; i.e., an area of about 8×8 cm on each side. A towel was wrapped around the forelimbs and the head for physical restraint during the scanning period. Rabbits were placed in lateral recumbency and

remained calm without the need for sedation or anesthesia. Acoustic gel (Eco Gel 200™, Eco-Med Pharmaceutical Inc., Mississauga, Ontario, Canada) was applied to the skin over the area to be scanned. The same UBM equipment was used as in Experiment 1 (Fig. 3.3). The transducer was moved gently over the skin surface, and ovarian images were recorded in cine-loops, as described in Experiment 1. Evaluations were performed for three consecutive days to determine consistency in detection of ovarian structures (i.e., CL and follicles ≥ 0.6 mm), to detect changes in follicular size, and to evaluate ovarian functionality.

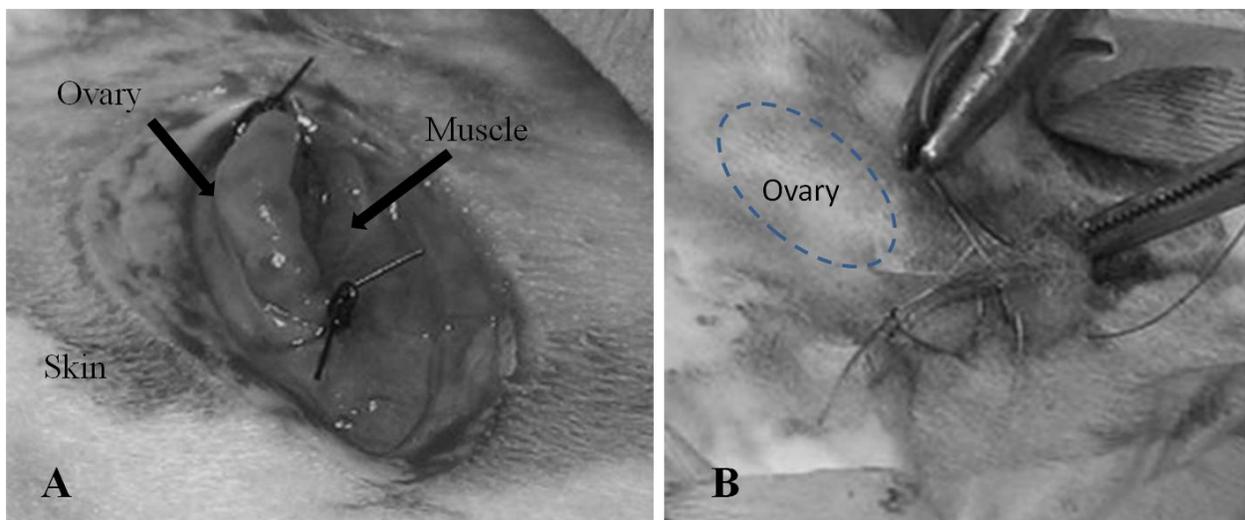


Figure 3.2. Surgical translocation of the ovary to a subcutaneous position in rabbits. **(A)** The ovary was transfixed to the superficial surface of the muscle layers of the abdominal wall. **(B)** The skin incision was closed such that the suture line was not directly over the translocated ovary.



Figure 3.3. Ultrasound imaging of rabbit ovaries by transcutaneous ultrasound biomicroscopy *in vivo* using a 25 MHz transducer. The head and forelimbs of the rabbit were wrapped in a towel for physical restraint; sedation was not necessary.

3.3.3. Statistical analysis

In Experiment 1, Pearson's correlation coefficient (r) was used to assess the pairwise correlation between UBM and histological data. Values are expressed as mean \pm SEM. Data in Experiment 2 were analyzed by one-way ANOVA.

3.4. Results

3.4.1. Experiment 1

Follicles ≥ 0.6 mm and corpora lutea were clearly distinguished from the ovarian stroma by UBM and histology (Fig. 3.4). By UBM, antral ovarian follicles were identified as spherical

anechoic structures, varying in diameter from 0.6 to 1.7 mm. Corpora lutea were identified as relatively well circumscribed spherical hyperechoic structures, varying in diameter from 2.2 to 3.1 mm. The presence and location of ovarian structures were confirmed by histological examination of the corresponding ovaries (Fig. 3.4).

The number and size of follicles ≥ 0.6 mm and corpora lutea per rabbit (i.e., per pair of ovaries) were recorded using both UBM and histology and all endpoints were positively and strongly correlated (Table 3.1).

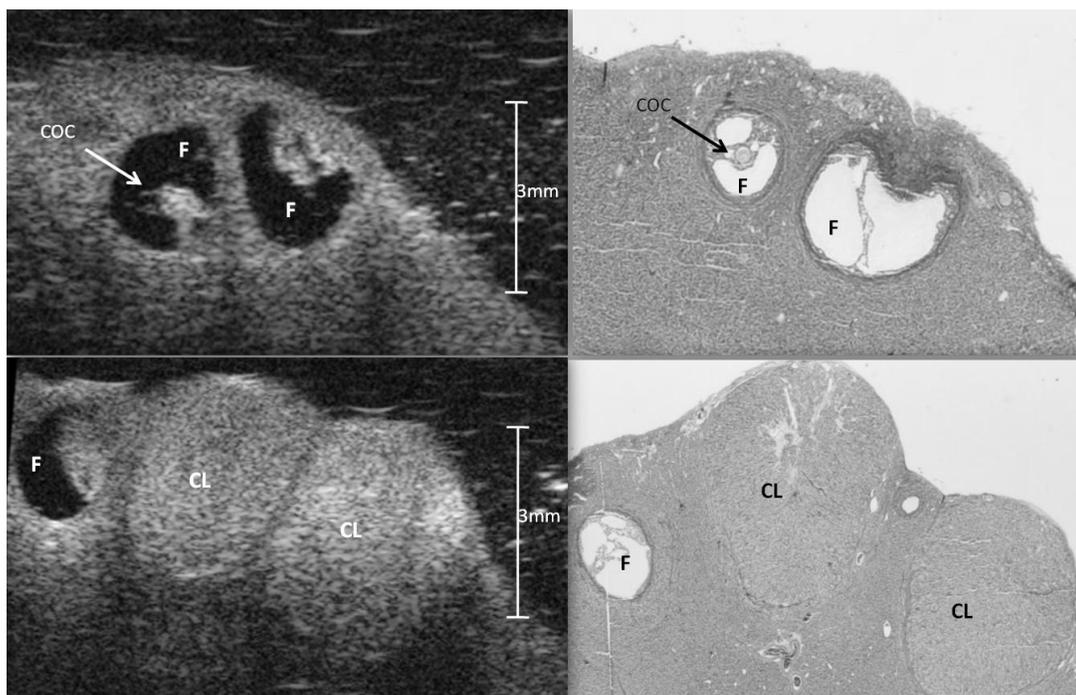


Figure 3.4. Representative paired images of single sections of rabbit ovaries showing antral follicles (F) and corpora lutea (CL) by ultrasound biomicroscopy *ex situ* (left) and histology (right). Note cumulus-oocyte complexes (COC) within the follicular antrum.

Table 3.1. Correlation between *ex situ* ultrasound biomicroscopy (UBM) and histology of rabbit ovarian structures (n = 4 rabbits [4 pairs of ovaries]; mean \pm SEM; r = Pearson correlation coefficient; Experiment 1).

Endpoint	UBM	Histology	r	P value
Number of follicles ≥ 0.6 mm	17.3 \pm 2.3	19.0 \pm 1.6	r = 0.96	P = 0.040
Diameter of follicles ≥ 0.6 mm	1.1 \pm 0.05	1.1 \pm 0.03	r = 0.96	P = 0.035
Number of CL	8.5 \pm 2.9	8.8 \pm 3.0	r = 0.99	P = 0.003
Diameter of CL (mm)	2.1 \pm 0.7	1.8 \pm 0.6	r = 0.99	P < 0.001

3.4.2. Experiment 2

Ovarian imaging was performed about 2 weeks after surgery. Well circumscribed, spherical, anechoic structures were recognized as antral follicles ranging in diameter from 0.6 to 3.1 mm, and were easily distinguished in 9 of 12 rabbits using transcutaneous UBM *in vivo* (Fig. 3.5). A second surgery was performed to determine the cause of our inability to obtain clear ovarian images of one ovary in three rabbits. In two rabbits, the affected ovary appeared to be embedded in a thin layer of connective tissue, while in the third, a thick layer of fatty tissue encapsulated the ovary. The connective tissue and fat were easily removed and ovarian imaging improved markedly in all three of the rabbits. In a fourth rabbit, not submitted to corrective surgery, only a portion of one ovary was imaged by UBM apparently because the ovary was partially buried within the abdominal muscle layers.

The number of follicles ≥ 0.6 mm per pair of ovaries ranged from 7 to 18. The mean number of follicles ≥ 0.6 mm and the mean diameter of the largest 7 follicles per pair of ovaries

were similar in three successive daily examinations (Table 3.2). However, follicles >2 mm in diameter were detected in 3/12 rabbits on the first and second days, and in 5/12 on the third day. One or more corpora lutea were detected in 2/12 rabbits during each of three daily examinations; i.e., the same 2 rabbits each day (Table 3.2, Fig. 3.5).

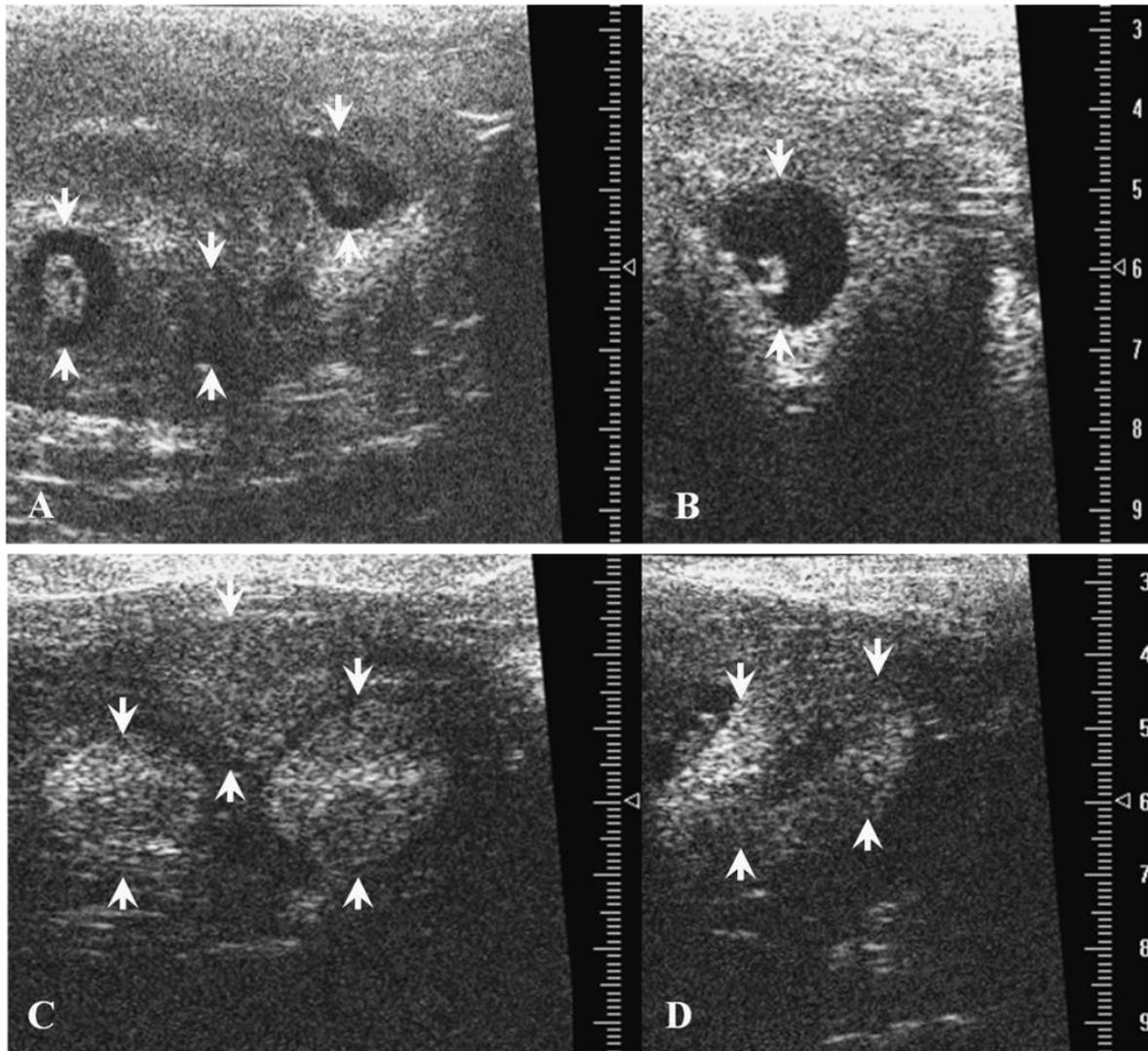


Figure 3.5. Rabbit ovaries imaged *in vivo* by transcutaneous ultrasound biomicroscopy showing follicles (arrowheads; A, B) and corpora lutea (arrowheads; C, D). Numbers on the scales to the right are in millimetres, with 0.1 mm increments

Table 3.2. Number and size (mean \pm SEM) of ovarian structures detected per rabbit by transcutaneous ultrasound biomicroscopy (UBM) *in vivo* on 3 consecutive days beginning at least two weeks after surgical ovarian translocation (n= number of rabbits; Experiment 2).

Endpoint	Day 1	Day 2	Day 3
Number of follicles \geq 0.6 mm	11.0 \pm 0.82 (n = 12)	11.8 \pm 0.85 (n = 12)	12.3 \pm 0.75 (n = 12)
Diameter of the largest follicle	1.8 \pm 0.10 (n = 12)	1.9 \pm 0.12 (n = 12)	2.0 \pm 0.13 (n = 12)
Mean diameter of the 7 largest follicles	1.5 \pm 0.09 (n = 12)	1.6 \pm 0.09 (n = 12)	1.6 \pm 0.08 (n = 12)
Proportion of rabbits with a CL	2/12	2/12	2/12
Number of CL per rabbit	3.1 \pm 1.00 (n = 2)	3.5 \pm 1.50 (n = 2)	3.5 \pm 1.50 (n = 2)
Diameter of CL (mm)	2.6 \pm 0.11 (n = 2)	2.5 \pm 0.01 (n = 2)	2.4 \pm 0.13 (n = 2)

Changes in the number or size of the ovarian follicles were observed in all the 12 animals. In addition, the presence of CL was distinguished in two animals. These findings may indicate that there was no apparent effect of the surgical procedure on ovarian function (i.e., necrosis, atrophy, lack of ovarian structures).

Two months after surgery, ovarian structures were clearly discernible by UBM in 11 of 24 translocated ovaries. Imaging was still possible in the remaining 13 ovaries by re-directing the UBM beam in different planes around the ovary or by gently pulling or stretching the skin and subcutaneous tissue of the rabbit from the underlying translocated ovary. Ovarian follicles \geq 0.6 mm were detected in the ovaries of all rabbits. Image deterioration was attributed to attenuation of sound waves associated with increased skin thickness and growth of subcutaneous fat and connective tissue around the ovary, assessed at necropsy.

3.5. Discussion

The present study documented the efficacy of UBM for assessing ovarian follicles and corpora lutea in rabbits. After *in vitro* (Experiment 1) or *in vivo* examinations (Experiment 2), ovarian structures were clearly differentiated from ovarian stroma by using UBM. Results in Experiment 1 demonstrated that UBM is a feasible and reliable technique to examine follicles and corpora lutea in rabbits. The use of UBM provided similar data to histological analysis of the same samples, thus validating UBM as a tool to quantify and measure follicles ≥ 0.6 mm and CL in *ex situ* rabbit ovaries (Table 3.1). These results agree with those reported in mice (Pallares and Gonzalez-Bulnes, 2008; Mircea *et al.*, 2009) who found agreement among counts of follicles and corpora lutea by comparing UBM and histology. Therefore, we propose the use of UBM for the examination of serial ovarian imaging considering that evaluation of each ovary *ex situ*, required on average, 2 hr by UBM compared to 30 hr by histology. Histological assessment of ovarian structures has been used as an accurate technique for reproductive studies in small species (Kranzfelder *et al.*, 1989; Singh and Gouraya, 1993; Myers *et al.*, 2004; Agrawal and Jose, 2011). However, histology is a time-consuming method, which commonly is based on one time examination of the ovaries and that requires procedures of terminal nature.

The use of UBM has also been shown to be a useful tool for *in vivo* evaluation of ovarian structures in mice (Jaiswal *et al.*, 2009). However, *in vivo* imaging of ovarian structures by transabdominal UBM failed in rabbits (preliminary results), similar to results reported in rats (Jaiswal *et al.*, 2009), due to the low penetration of high-frequency sound waves and to the high impedance of the body wall. Given these findings, it was thought that the translocation of ovaries to a more superficial position without altering blood supply would favor the penetration of the

high-frequency transducer, facilitating ovarian imaging. In sheep, it was reported that ovarian function can be preserved after relocation of the ovary to the vagina (Dierschke and Williams, 1970); while in monkeys, the transposition of the ovary from the pelvis into the vaginal fornix did not affect severely ovarian function and most ovaries were functional for 2–5 months, despite some evidence of infection (Iffy *et al.*, 1978). In addition, based on evaluations of size and morphological characteristics (e.g., such as growth of follicles and vascularisation of ovarian tissue), survival of the grafts of mice, rabbits, and bovine under the kidney capsules of athymic nude mice was reported (Hosoe *et al.*, 2008).

In Experiment 2, we demonstrated that the translocation of ovaries to a subcutaneous position as a previous step to UBM examination enabled the imaging the ovarian structures in live rabbits. The feasibility of the surgical approach without major complications and apparent maintenance of ovarian function was important for preliminary *in vivo* examination of the rabbit ovaries. This is especially important for studies of normal ovarian function and studies oriented to investigate ovarian response to particular treatments. An additional benefit of UBM is that it does not harm the rabbits and there is no need to apply anesthesia or sedatives. Until now, rabbit ovaries have been mainly examined at a single time in a terminal way (e.g., histology) or in a serial manner using an invasive method (e.g., laparoscopy). To our knowledge, this is the first study that reports the use of real-time ultrasound biomicroscopy to identify the number of follicles and corpora lutea present in the ovaries of rabbits submitted to one time ovarian translocation. Therefore, UBM provides a non-terminal alternative for noninvasive repetitive examination on the ovaries of rabbits once their ovaries have been translocated. During the serial post-surgical UBM examinations, the detection of ovarian structures (i.e., CL and follicles ≥ 0.6

mm) was consistent, and the variations in size and numbers in all ovaries indicated that there was no apparent effect on ovarian functionality.

The mean number of CL detected per rabbit after the ovulation-inducing treatment in Experiment 1 (Table 3.1) was similar to that reported in previous rabbit studies based on post mortem, laparoscopic or laparotomy examination (Scott and Rennie, 1970; Rodriguez *et al.*, 1988; Hulot *et al.*, 1988). Detection of CL in non-treated rabbits in Experiment 2 (Table 3.2) was not expected as rabbits are induced ovulators; however, CL were only detected in 2/12 rabbits and in low numbers (i.e., 2 and 5 per rabbit) and represent spontaneous ovulation by unknown factors. The ability to distinguish follicles ≥ 0.6 mm using UBM is an important finding. Investigators have reported preovulatory follicular diameters ranging from >800 μm to >2 mm (Parkes, 1931; Hunzicker-Dunn *et al.*, 1979; Kranzfelder *et al.*, 1984; Marongiu and Gulinati, 2008), but the size at which follicles gain ovulatory capability in rabbits is not known. Evaluation of ovarian status prior to treatment is therefore, critical in any experimental design in which determination of ovulatory response is required (i.e, preovulatory follicular size ≥ 7 mm in llamas, Bravo *et al.*, 1991).

Rabbits are accessible, easy to handle and relatively inexpensive to maintain. In addition, these animals have been used successfully to address several reproductive research questions in the past without the use of serial *in vivo* imaging technology. Thus, at the present the possibility to include UBM in rabbit studies gives major advantages for the evaluation of ovarian function and ovulation, and also for the evaluation of diverse ovulatory inducing-treatments; allowing determining their effects in a non-terminal way. In addition, this approach could reduce the

number of research animals because allow serial ovarian examinations of same individuals during one and subsequent studies.

In summary, results of the present study validate UBM as a valuable tool to determine/quantify the number and size of follicles ≥ 0.6 mm and corpora lutea on the ovaries of rabbits. This is a considerable advance because histological methods are labor intensive and require much more time to analyze. Ovarian imaging by UBM is relatively fast and accurate. Translocation of the ovaries to a subcutaneous position without altering blood supply is safe and with no apparent effect on ovarian function. With this method, *in vivo* transcutaneous UBM assessment of the ovaries is facilitated in rabbits and ovarian images are obtained without deterioration for at least 2 months. Therefore, consecutive UBM might be helpful to efficiently monitor the ovaries to assess the ovarian response to treatments, will be particularly advantageous in ovulation induction follow-up experiments, and can likely be applied to the study of ovarian dynamics in rabbits.

4.0. EFFECT OF SEMINAL PLASMA ON OVULATION IN RABBITS

4.1. Abstract

Ovulation-inducing factor (OIF) is a protein found in the seminal plasma of different species. This protein has a potent ovulatory effect in female camelids. Evidence suggests that biological activity of OIF may be conserved among species. Although OIF has been identified in seminal plasma of rabbits, there are no reports about the role of seminal plasma on ovulation in rabbits. Two experiments were conducted to test the hypothesis that llama and rabbit seminal plasma elicits a surge release of LH and is responsible for inducing ovulation in rabbits. In Experiment 1, mature female New Zealand White rabbits were group-housed and treated intramuscularly with saline, GnRH, or llama or rabbit seminal plasma (n = 4 to 6 per group). The day of treatment was designated Day 0. Rabbits were euthanized on Day 8 to evaluate ovarian response by ultrasound biomicroscopy *ex situ*. Ovulation was detected in >80% of rabbits in all groups. There were no differences among groups in the proportion of rabbits that ovulated or in the number and size of corpora lutea in the ovaries 8 days after treatment. Group housing of female rabbits was associated with a high incidence of ovulation and confounded the detection of an ovulation-inducing effect of seminal plasma. In Experiment 2, mature female New Zealand White rabbits were caged individually, and were treated as in Experiment 1 (n = 5 to 7 per group). The ovarian response was evaluated *in vivo* by transcutaneous ultrasound biomicroscopy. Ovulation was detected only in rabbits given GnRH (5/5, 100%; P < 0.05) and the number (mean \pm SEM) of corpora lutea was 7.0 ± 0.50 per rabbit. Increases in plasma concentrations of LH and progesterone, and the formation of corpora lutea were observed only in the GnRH-treated group.

In conclusion, no clear evidence was found to support the hypothesis that seminal plasma elicits a surge of LH release and stimulates ovulation in rabbits.

4.2. Introduction

In rabbits, coitus has been reported to be the main factor to induce ovulation (Spies *et al.*, 1997). Authors of an early study (Hammond and Asdell, 1926) reported that artificial insemination followed by sterile mating resulted in higher percentages of conception than after artificial insemination alone (20/22 [91%] vs 1/28 [4%]). There is, however, no clear evidence to support that the mechanical stimulus of the penis or the presence of semen in the vagina initiates ovulation (Walton and Hammond, 1928). Although the primary trigger for inducing ovulation in rabbits is not well understood, it is clear that stimulation of the pituitary gland (Fee and Parkes, 1929; Hill and Parkes, 1931) results in a rise in circulating luteinizing hormone (LH) concentration after coitus (Brambell and Parkes, 1932; Spies *et al.*, 1997) to cause ovulation.

In alpacas and llamas, results of more recent studies have challenged the traditional idea that physical stimulation of the genital tract during coitus triggers ovulation in these species and have shown that seminal plasma plays a role in the induction of ovulation (Adams *et al.*, 2005, Ratto *et al.*, 2005). Intramuscular administration of seminal plasma resulted in a surge release of LH followed by ovulation (Adams *et al.*, 2005; Ratto *et al.*, 2005; 2011). Results of studies in other species have also shown that administration of seminal plasma of bactrian camels (Chen *et al.*, 1985; Zhao *et al.*, 2001) and koalas (Johnston *et al.*, 2004) induces ovulation in their corresponding females. All of these studies suggested the presence of a factor in the seminal plasma which induces ovulation in species considered to be induced ovulators.

Ovulation-inducing factor (OIF) is a seminal plasma protein that has been found in the ejaculates of alpacas and llamas (Adams *et al.*, 2005; Ratto *et al.*, 2005; 2006b; 2010). This protein was initially isolated from llama seminal plasma as a molecule with a molecular mass of about 30 kDa (Ratto *et al.*, 2010). However, the same researchers reported more recently that OIF is a 14 kDa protein molecule responsible for eliciting a preovulatory LH surge and ovulation when administered in llamas (Ratto *et al.*, 2011). Additionally, administration of purified OIF in llamas influenced ovulation and corpus luteum (CL) form and function in a dose-dependent manner (Tanco *et al.*, 2011). OIF has been detected in the ejaculates of bactrian camels, cattle, horses and pigs (Chen *et al.*, 1985; Zhao *et al.*, 2001; Ratto *et al.*, 2006b; Bogle *et al.*, 2011). Furthermore, biological activity of OIF has been reported in seminal plasma from different species by using the llama as an *in vivo* ovulation bioassay, and has suggested that OIF molecule and its functions may be conserved among species (Ratto *et al.*, 2006b; Bogle *et al.*, 2011).

Studies in llamas and alpacas confirmed that ovulatory capacity depends on follicular maturity at the time of mating (Bravo *et al.*, 1991); that is, animals with an ovarian follicle <6 mm or with follicle that has entered the regressing phase did not ovulate. This supports the notion that the effectiveness of an ovulatory treatment depends of the presence of a follicle of ovulatory size at the time of treatment. The lack of suitable methods for monitoring the ovaries in live rabbits has provided little or no opportunity for assessing the size of follicles in the ovaries at the time of treatment. Indeed, ovarian characteristics are usually evaluated only post treatment either after using terminal (Deanesly, 1930; Guraya *et al.*, 1968; Kranzfelder *et al.*, 1989) or invasive procedures (Marshall and Verney, 1936; Kauffman *et al.*, 1998; Agrawal and Jose, 2011). High frequency (i.e. 25 MHz), high resolution (<100 μ m) ultrasound biomicroscopy (UBM; Jaiswal *et al.*, 2009) has been developed as a tool for imaging the ovaries of small

species (e.g. mice) *in vivo*. . Furthermore, UBM has recently been developed and validated as a feasible and reliable tool to examine ovarian structures in rabbits (Cervantes *et al.*, 2011).

Conventional ultrasonography (i.e., 5 to 7.5 MHz) has been useful for detecting ovulation in llamas and alpacas treated with seminal plasma and/or purified OIF (Adams *et al.*, 2005; Ratto *et al.*, 2005, 2010, 2011; Bogle *et al.*, 2011; Tanco *et al.*, 2011; Silva *et al.*, 2011). Ultrasonography using higher frequencies (e.g. > 10 MHz) may be useful for study of ovulation induction in rabbit in which ovarian structures are smaller and require better resolution. Development of an *in vivo* UBM approach offers the important advantage of allowing serial examination compared to an *ex vivo* UBM approach, thus permitting an opportunity to evaluate different treatments in the same animal.

The presence of OIF in the seminal plasma of rabbits has been reported recently (Silva *et al.*, 2010); however, the role of OIF in female rabbits has not been determined. The objective of the present study was to test the hypothesis that llama and rabbit seminal plasma elicits a surge release of LH and is responsible for inducing ovulation in rabbits. Experiments were designed to determine the effects of seminal plasma (llama or rabbit), GnRH, and saline on group-housed rabbits *ex vivo* (Experiment 1), and on individually housed rabbits *in vivo* (Experiment 2).

4.3. Materials and Methods

New Zealand White rabbits were housed under controlled environmental conditions (20.2 ± 2°C, 14:10-h light:dark cycle) and were allowed *ad libitum* access to food and water. The experimental protocol was approved by the University of Saskatchewan's Animal Research Ethics Board and procedures were done in accordance with guidelines of the Canadian Council

on Animal Care.

4.3.1. Experiment 1.

Llama semen was collected from five adult males once a week for 3 months by artificial vagina (Huanca and Adams, 2007). Ejaculates were diluted 1:1 (v:v) with phosphate buffered saline (PBS, Invitrogen, Grand Island, New York, USA), drawn back-and-forth through an 18-gauge needle attached to a 10 ml syringe as previously described (Adams *et al.*, 2005). Seminal plasma was separated from the cellular components of the ejaculate by double centrifugation at 1500 x g (Sorvall Legend RT, Mandel Scientific Co Ltd, Guelph, Ontario, Canada) for 30 min, and sperm-free samples were stored at -80°C. Upon thawing, the diluted seminal plasma samples from all 5 males were pooled. Rabbit semen was collected from two adult males twice a day, two times a week for 4 months by artificial vagina (adapted from Naughton *et al.*, 2003). An interval of 10-15 min between the first and the second ejaculates per day was permitted. Undiluted semen was centrifuged as described above and seminal plasma was separated from sperm and gel fraction of the semen and was stored at -80°C. Upon thawing, the seminal plasma samples from both males were pooled. For both species, 1% penicillin/streptomycin (v/v; 10 000 units/ml penicillin and 10 mg/ml streptomycin; Sigma-Aldrich) was added to the pooled semen.

Mature female rabbits (5.5 months old; n=22) were housed together in a single room. An acclimation period of 7 days was allowed before ovarian examinations were initiated. Imaging of the ovaries was performed daily using transabdominal ultrasonography (MyLab™ Five, Esaote, Ajax, Ontario, Canada) with a 12 MHz linear-array probe to ensure the presence of follicles ≥ 1.5 mm in diameter prior to treatment, large follicle diameter detected in all the rabbits. Before ultrasound examination, both flanks of the rabbit were shaved. A towel was wrapped around the

forelimbs and the head of the rabbit for restraint during examination. Rabbits were placed in lateral recumbency and remained calm without the need for sedation or anesthesia (Cervantes *et al.*, Chapter 3). Acoustic gel (Eco Gel 200™, Eco-Med Pharmaceutical Inc., Mississauga, Ontario, Canada) was applied to the skin over the area to be scanned. Rabbits were divided randomly into four groups and given an intramuscular dose of: (1) 1.5 ml of saline (negative control), (2) 0.02 mg GnRH (Fertagyl, Intervet, City, Province, Canada; positive control), (3) 2 ml of llama seminal plasma (1:1 dilution with PBS) or (4) 1.5 ml of rabbit seminal plasma (undiluted). The day of treatment was designated Day 0. Injection sites were monitored post-treatment. No swelling or redness was noted at the injection site in the rabbits, although a transient limp was clinically apparent in animals administered with rabbit seminal plasma. All animals were normal within 30 min after treatment.

On Day 8, females were euthanized with an overdose of sodium pentobarbital, and the ovaries were removed for evaluation *ex situ*. Each ovary was imaged in a saline bath using a high-resolution ultrasound biomicroscope (UBM; Vevo 660 Imaging System, Visual Sonics Inc., Toronto, Ontario, Canada) equipped with a 25 MHz oscillating sector transducer (RMV 704), as described previously (Cervantes *et al.*, 2011). Ovarian images were recorded in cine-loops for further analysis. Individual corpora lutea were identified, counted and measured by scrolling through the digital UBM cine-loops frame-by-frame as described in Chapter 3 (Experiment 1).

4.3.2. Experiment 2.

Given the unexpected results of group-housed rabbits in Experiment 1, female rabbits (5.5 months old, n=13) were housed individually in stainless steel cages for Experiment 2. Surgical translocation of the ovaries to a subcutaneous position in the flank region was

performed to permit *in vivo* ovarian imaging by transcutaneous UBM, as previously described (Chapter 3). Transcutaneous UBM (Vevo 660; 25 MHz probe) of the ovaries was done daily for at least four days before treatment to confirm the presence of follicles ≥ 1.8 mm in diameter (i.e., sufficiently large to respond to an ovulatory stimulus; Parkes, 1931), and for 8 days after treatment to detect ovulation and the development of corpora lutea. Rabbits were restrained and prepared for ultrasound examination as in Experiment 1. Follicles were more clearly distinguished *in vivo* by UBM compared to conventional ultrasonography used in Experiment 1, and greater precision was possible in assessing follicle diameter before treatment. When 6 to 10 ovarian follicles ≥ 1.8 mm were detected, each rabbit was assigned randomly to one of four groups (n= 5 to 7 per group) and given an intramuscular dose of 1) 1.5 ml of saline (negative control), 2) 0.02 mg GnRH (Fertagyl; positive control), 3) 2 ml of llama seminal plasma (1:1 dilution with PBS), or 4) 1.5 ml of rabbit seminal plasma (undiluted). The same pool of llama/rabbit seminal plasma collected during Experiment 1 was used in Experiment 2. The day of treatment was designated Day 0. As in Experiment 1, no inflammatory reaction was observed at the injection site, but a transient limp was clinically apparent in all animals given rabbit seminal plasma. The limp disappeared in all rabbits within 20-30 min after treatment. Rabbits were examined daily by transcutaneous UBM after treatment to detect ovulation and on Day 8, to detect presence of corpora lutea (CL). Ovulation was not defined by the sudden disappearance of large follicles (≥ 1.8 mm) that were detected during the previous examination. Ovulation was confirmed by subsequent detection of CL. Individual CL were counted and measured as described in Experiment 1.

For measurement of plasma progesterone concentration, blood samples were collected from the marginal ear vein or the lateral saphenous vein every-other-day from Day 0 to Day 8. A

topical lidocaine-prilocaine 5% cream (EMLA, AstraZeneca Canada Inc., Mississauga, Ontario, Canada) was applied over the vein at least 15 min before blood sampling. Blood samples were centrifuged at 1700 x g for 30 min and the plasma was stored at -20°C. Plasma progesterone concentrations were determined using a commercial, double-antibody radioimmunoassay kit (Coat-a-Count progesterone; Siemens Medical Solutions Diagnostics, Los Angeles, California, USA). All samples and standards (100 ul) were assayed in duplicate in a single assay. The intra-assay coefficients of variations were 9.7%, 6.5% and 6.3%, respectively, for reference plasma progesterone concentrations of 1.99, 3.47, and 17.06 ng/ml.

For measurement of plasma LH concentration, blood samples were taken at 0, 1, 2, 3 and 6 hours after treatment. Topical lidocaine-prilocaine 5% cream was applied over the vein before blood sampling and samples were centrifuged and stored as described above. Plasma LH concentrations were determined using an specific homologous double antibody radioimmunoassay for Rabbit LH (A.F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA, USA) (Brecchia *et al.*, 2006). Concentrations of LH are expressed in terms of NIDDK-RbLH (AFP-7818C). The standard curve ranged from 0.5 ng/ml (90% ligand labelled LH) to 16.0 ng/ml (20% ligand labelled LH). The primary antibody (AFP3120489) was raised in guinea pigs, and the anti-guinea pig secondary antibody was raised in sheep (kindly provided by Susan Cook and Dr B. Laarveld, Animal and Poultry Science, University of Saskatchewan, Saskatoon, SK Canada). All samples and LH standards (50 ul) were assayed in duplicate in a single assay. The intra-assay coefficient of variation was 3.9% for the high reference plasma LH concentration (6.33 ng/ml). The intra-assay coefficient of variation was 10.9% for the medium reference plasma LH concentration (0.83 ng/ml). Values that

exceeded more than three standard deviations from the mean for the respective group were considered outliers and removed from further analysis.

4.3.3. Statistical analysis

Analysis of variance was used to compare non-serial data (i.e., number of follicles at the time of treatment, number and size of corpora lutea on Day 8) among groups. Analysis of variance for repeated measures (Proc-mixed in SAS; Enterprise Guide 4.2, Statistical Analysis System Institute Inc., Cary, North Carolina, USA) was used to compare serial data (i.e., plasma progesterone and LH concentrations) to determine the effects of treatment, day, and treatment-by-day interaction. Tukey's multiple comparison was used as a post-hoc test when a main effect of treatment or a treatment-by-day interaction was detected. Ovulation rates were compared among groups by Fisher's exact test. $P < 0.05$ was considered statistically significant. For the purposes of analysis and illustration, hormonal data were centralized to Day 0 (day of treatment).

4.4. Results

4.4.1. Experiment 1

The mean number of follicles ≥ 1.5 mm at the time of treatment did not differ among groups ($P = 0.25$), nor did the proportion of rabbits that ovulated ($P = 1.0$; Table 4.1). Conventional transabdominal ultrasonography was inadequate for evaluating the ovarian response to treatment because corpora lutea were not clearly differentiated from the surrounding tissue. Corpora lutea were clearly distinguishable in the ovaries *ex situ* by UBM. In rabbits that

ovulated, no differences were detected among groups in the number ($P = 0.51$) and size ($P = 0.82$) of CL (Table 4.1).

Table 4.1. Number and diameter (mean \pm SEM) of follicles and corpora lutea (CL) in rabbits treated with saline, GnRH, or seminal plasma of llamas or rabbits (Day 0 = day of treatment). (n= rabbits per group; Experiment 1). Ovarian structures were assessed *in vivo* prior to treatment by transabdominal ultrasonography (12 MHz) and *ex vivo* after treatment, by ultrasound biomicroscopy (25 MHz).

Endpoint*	Saline	GnRH	Llama SP	Rabbit SP
Number of follicles ≥ 1.5 mm at Day 0	15.0 \pm 1.96	13.5 \pm 0.96	17.3 \pm 1.31	14.5 \pm 1.25
Proportion of rabbits that ovulated	5/6	4/4	5/6	6/6
Number of CL on Day 8	10.6 \pm 0.98 (n = 5)	13.0 \pm 1.78 (n = 4)	11.4 \pm 1.54 (n = 5)	10.5 \pm 0.62 (n = 6)
CL Diameter (mm) at Day 8	2.5 \pm 0.21 (n = 5)	2.7 \pm 0.13 (n = 4)	2.6 \pm 0.14 (n = 5)	2.5 \pm 0.17 (n = 6)

*No significant differences among groups for any endpoint

4.4.2. Experiment 2

The mean number of follicles ≥ 1.8 mm at the time of treatment did not differ among groups ($P = 0.98$). As shown in Table 4.2, ovulation occurred only in the group treated by intramuscular administration of GnRH.

The interval from treatment to ovulation was not determined in this study. Ovulation was confirmed by the observation of corpora lutea, which were first detected on Day 1.8 \pm 0.20.

Table 4.2. Number and diameter (mean±SEM) of follicles and corpora lutea (CL) in rabbits treated with saline, GnRH, or seminal plasma of llamas or rabbits (Day 0 = day of treatment). Ovarian structures were assessed *in vivo* by transcutaneous ultrasound biomicroscopy in rabbits that underwent surgical translocation of the ovaries (n= rabbits per group; Experiment 2).

Endpoint	Saline (n = 5)	GnRH (n = 5)	Llama SP (n = 6)	Rabbit SP (n = 7)
Number of follicles ≥ 1.5 mm at Day 0*	12.2 ± 2.06	10.7 ± 0.84	13.0 ± 2.76	11.2 ± 1.32
Number of follicles ≥ 1.8 mm at Day 0*	9.0 ± 2.00	8.2 ± 0.70	9.3 ± 2.68	8.7 ± 1.15
Proportion of rabbits that ovulated	0/5 ^a	5/5 ^b	0/6 ^a	0/7 ^a
Number of CL on Day 8	----	7.0 ± 0.50	----	----
CL Diameter (mm) on Day 8	----	2.6 ± 0.05	----	----

* No significant differences among groups

^{a,b} Proportions differed among groups (P <0.05)

During the 6-hour period following treatment, plasma LH concentration increased in the GnRH group (positive control), but not in any other group, where LH remained at basal levels (treatment-by-time interaction, P<0.01). Plasma LH concentrations in the GnRH group were maximal at 1 h after treatment, began to decrease at 3 h, and were basal at 6 h after treatment (Fig. 4.1).

Plasma progesterone concentrations increased only in the GnRH group and remained basal in the other groups (treatment-by-day interaction, P<0.001). In GnRH group, progesterone concentrations were elevated by Day 2 and increased linearly to the last day of sampling (Day 8; Fig. 4.2).

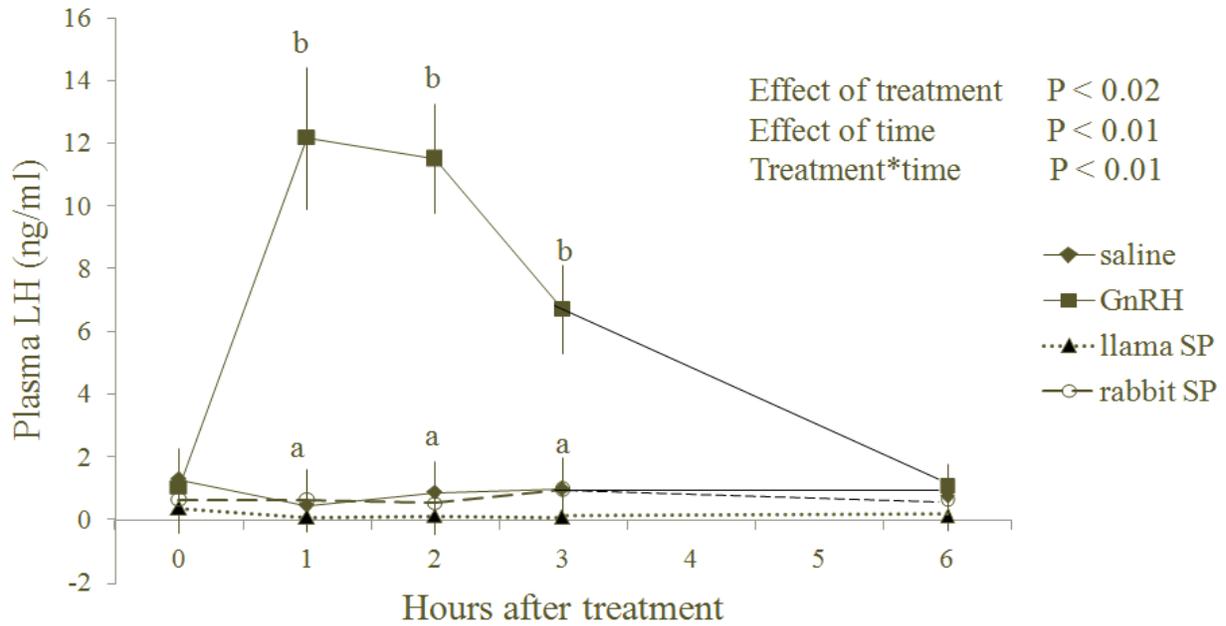


Figure 4.1. Plasma LH concentrations (mean \pm SEM) in female rabbits after intramuscular treatment with saline, GnRH, or seminal plasma of llamas or rabbits. ^{ab} On a given hour, values with no common superscript are different (P < 0.05).

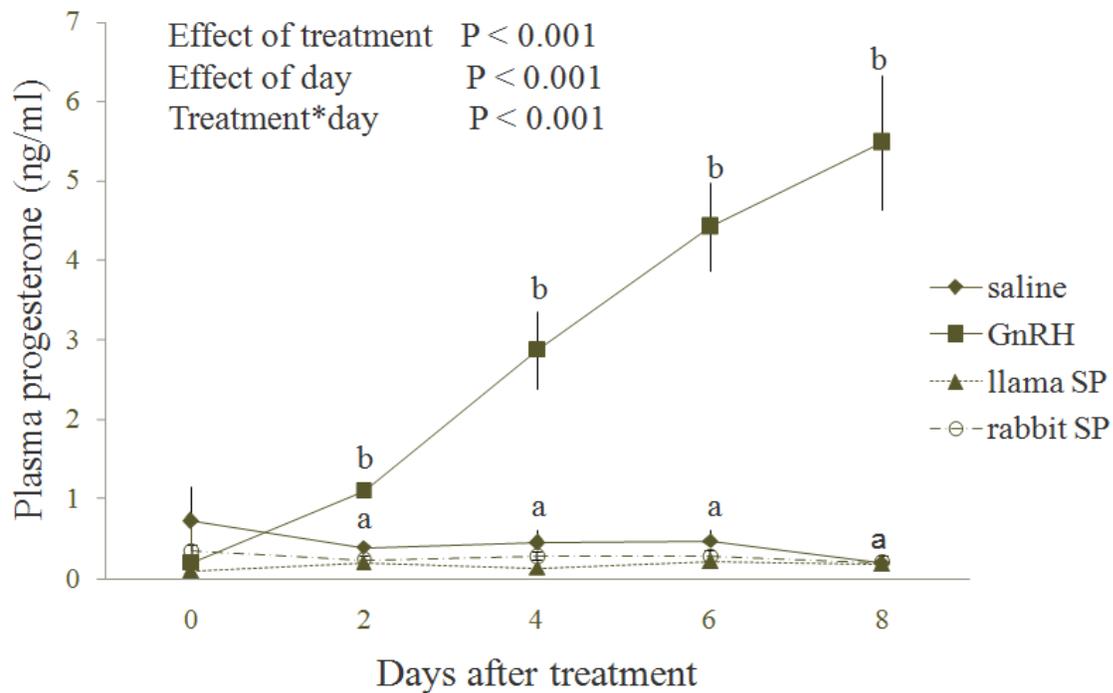


Figure 4.2. Plasma progesterone concentrations (mean \pm SEM) in female rabbits after intramuscular treatment with saline, GnRH, or seminal plasma of llamas or rabbits. ^{ab} On a given day, values with no common superscript are different (P < 0.05).

4.5. Discussion

The existence of an ovulation-inducing factor (OIF) in seminal plasma of llamas and the biological effects of OIF (i.e. ovulation) have been well documented using the female llama and alpaca as animal models (Adams *et al.*, 2005; Ratto *et al.* 2005; 2006b). Presence of OIF has recently been found in seminal plasma of rabbits (Silva *et al.*, 2010), but its effects on female rabbits have not been reported. The present study was conducted to determine the effect of OIF in seminal plasma of llama and rabbit on release of LH and ovulation in female rabbits. The administration of seminal plasma as a single intramuscular dose was chosen based on results reported in alpacas (Ratto *et al.* 2005). Our results did not provide evidence to support the hypothesis that seminal plasma elicits a surge of LH release and stimulates ovulation in rabbits. These findings were not consistent with those documented in llamas and alpacas (Adams *et al.*, 2005; Ratto *et al.* 2005; 2006b).

In order to test our hypothesis, we conducted two experiments in this study. The results obtained from both experiments using similar doses of seminal plasma were contradictory, but may be attributed to differences in housing of the rabbits during the study. The data in Experiment 1, suggested that ovulation-inducing effects of seminal plasma were confounded by the group housing arrangement because ovulation also was detected in the saline group (negative control). This unexpected result may be attributed to behavioral stimuli since the occurrence of ovulation was also observed in previous studies when female rabbits were repeatedly mounted by other females while caged in pairs (Staples, 1967). In a later study, however, pseudopregnancy occurred in rabbits group-housed, even when only occasional matings were

observed (Rommers *et al.*, 2006). In ewes (i.e., spontaneous ovulators) and musk shrews (i.e., induced ovulators) observations suggested that olfactory cues (pheromones) participate in hypothalamic control of pituitary secretions and ovulation (Knight *et al.*, 1980; Rissman and Li, 2000). Whether pheromones play a role in induction of ovulation in rabbits remains unclear.

Previous evidence has supported the notion that ovarian follicular size at the time of treatment is an important determinant of the ovarian response in induced ovulators, such as llamas (Bravo *et al.*, 1991), and must be taken into account in studies involving ovulatory response (Bravo *et al.*, 1992; Adams *et al.*, 2005; Ratto *et al.* 2005, 2006a, 2006b, 2011; Tanco *et al.*, 2011; Bogle *et al.*, 2011). In this sense, ultrasonography was used in the present study to detect the presence of large follicles in similar numbers in all the rabbits at the moment of treatment. Follicle diameter was detected *in vivo* by transabdominal ultrasonography (12 MHz) in Experiment 1 ($\geq 1.5\text{mm}$) but without the clarity and the precision achieved by transcutaneous UBM (25 MHz) in Experiment 2 ($\geq 1.8\text{mm}$). Transcutaneous UBM was used in rabbits that underwent translocation of the ovaries to a subcutaneous position, as described previously (Chapter 3). Ovarian response (i.e., ovulation) was evaluated by UBM in both experiments due to the ineffectiveness of transabdominal ultrasonography for that purpose. In Experiment 1, numbers and size of CL were examined in excised ovaries by UBM *ex situ*, limiting the results to one time examination of the ovaries. In Experiment 2, ovarian response was evaluated in live rabbits by transcutaneous UBM, permitting to perform follow-up examinations (data not shown).

One explanation of the failure of seminal plasma to induce ovulation in rabbits may be that the required dose was not reached in the present study, considering that OIF effects in other species have been reported to be dose dependent (Tanco *et al.*, 2011). In addition, the ovulatory

effect could probably be affected by the route of administration chosen, considering that only the intramuscular route was evaluated in this study. It could be speculated that seminal plasma at the dose or route administered was insufficient to produce a preovulatory peak of LH, and therefore failed to induce ovulation in rabbits. Ovulation occurred only in the GnRH group, where a preovulatory peak of LH was elicited. In this regard, ovulation failure in our study appears to be due to an insufficient stimulation to provoke the LH surge rather than to a lack of mature follicles in the ovaries, as stated by others (Hulot *et al.*, 1988). Whether OIF in seminal plasma of rabbits has an effect on ovulation similar to that documented in seminal plasma of llamas and alpacas remain unclear. In addition, there is a possibility that OIF in seminal plasma of rabbits represents a smaller proportion of the total protein than OIF in the seminal plasma of llamas and alpacas. Likewise, another possibility is that OIF in the seminal plasma of other species does not have an effect on ovulation in rabbits. In summary, data from the present study do not support the hypothesis that llama or rabbit seminal plasma induces a preovulatory surge of LH or ovulation in rabbits. It will be interesting in further studies to determine whether there is a minimum effective dose of seminal plasma and/or purified OIF to stimulate ovulation in rabbits, or if this factor acts in rabbits differently than in llamas and alpacas.

4.6. Acknowledgements

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5.0. GENERAL DISCUSSION

Ovulation inducing-factor (OIF) is a protein present in the seminal plasma which is thought to be conserved among species (Ratto *et al.*, 2005; Bogle *et al.*, 2011). The effects of OIF have been well documented using the female llama and alpaca as *in vivo* animal models - induced ovulating species in which ovarian dynamics and ovulation have been well characterized, and in which detection of ovulation can be performed by ultrasonography (Adams *et al.*, 1989, 1990, 1991; Vaughan *et al.*, 2003; Ratto *et al.*, 2003, 2005, 2006a). In particular, OIF stimulates a preovulatory LH surge and elicits ovulation (Chen *et al.*, 1985; Zhao *et al.*, 2001; Adams *et al.*, 2005; Ratto *et al.*, 2006b, 2011). South American Camelids have been used frequently as animal models in most of the studies of OIF since they are induced ovulators, but several aspects of OIF, such as the mechanism of action and biological pathways are still unknown. We proposed the use of the rabbit as a new animal model for the study of OIF. Rabbits are also induced ovulators, which makes them particularly suitable models for the study of ovulation that occurs normally only after stimulation (Fee and Parkes, 1929; Hill and Parkes, 1931; Brambell and Parkes, 1932; Spies *et al.*, 1997). As animal models, rabbits are small, readily accessible, easy to handle, and easy to maintain (Manning *et al.*, 1994).

Reproductive physiology and ovarian function in rabbits have been studied for many years (Heape *et al.*, 1905; Parkes, 1931; Hunzicker-Dunn *et al.*, 1979; Dharmarajan *et al.*, 1989; Spies *et al.*, 1997; Rashwan *et al.*, 2003; Boumahdi-Merad *et al.*, 2011). These studies were done using invasive methods which in most of cases require the sacrifice of the animal and the observation of response to treatments *ex vivo*. These methods limit the possibility to obtain serial

observations of ovaries in a single rabbit and require a large number of animals to obtain the desired information.

With the constant development of imaging technologies such as ultrasonography, noninvasive *in vivo* evaluation of ovaries has been shown to be a powerful tool for understanding reproductive physiology in different species. Conventional ultrasonography (5-7.5MHz) has also been used successfully in large animals (e.g., cattle) to study ovarian function (Adams and Pierson, 1995). However, the use of this technique for imaging ovarian function in rabbits has not been reported. Recently, a new method for evaluating ovarian physiology has been described in live mice by using ultrasound biomicroscopy (20-25MHz; Jaiswal *et al.*, 2009). This technology enables imaging of the same animal repetitively, thus permitting detection of dynamic processes and decreasing the required number of animals per experiment. With this in mind, we proposed the use of ultrasound biomicroscopy to examine ovarian structures in rabbits as used in mice.

Our general objective was to determine whether rabbit can be a new model for the study of ovulation-inducing factor (OIF) as South American camelids are. To achieve this goal, we conducted two studies in the present thesis. The first study was developed to validate the use of ultrasound biomicroscopy as a tool for assessing ovarian follicles and corpora lutea in rabbits (Chapter 3, Experiment 1) and to determine if surgical translocation of the ovaries to a subcutaneous position would permit serial *in vivo* transcutaneous imaging of the ovaries (Chapter 3, Experiment 2). In the second study (Chapter 4), two experiments were conducted to test the hypothesis that llama and rabbit seminal plasma induces a surge of LH and elicits ovulation in rabbits.

5.1. Surgical translocation and ultrasound biomicroscopy of the ovaries in rabbits

In rabbits, ovarian function has typically been studied *ex vivo* after histological examination of the ovaries (Deanesly, 1930; Armstrong *et al.*, 1978; Kranzfelder *et al.*, 1989; Agrawal and Jose, 2011) or by direct observation of the ovaries by laparotomy (Marshall and Verney, 1939; Kauffman *et al.*, 1998; Agrawal and Jose, 2011) or laparoscopy (Blasco *et al.*, 1994; Argente *et al.*, 2010). Experiment 1 (Chapter 3) was the first report of the use of ultrasound biomicroscopy in rabbits, and showed that this technique provides reliable information of structures of the ovaries (e.g., follicles and corpora lutea) *ex situ*. The identification of ovarian follicles and corpora lutea were easily achieved in the ovaries *ex situ*. Subsequently, the strong correlations between UBM and histology to compare the number and size of follicles and corpora lutea has shown that UBM is a highly feasible technique for assessing ovarian structures in rabbits (Chapter 3). Our results are in agreement with those reported in mice (Pallares and Gonzalez-Bulnes, 2008; Mircea *et al.*, 2009) as well as in humans (Baerwald *et al.*, 2009) where UBM was found to be a valuable tool for examination of ovarian structures.

An initial attempt to use UBM *in vivo* was done in Experiment 1 to evaluate ovarian structures transabdominally. However, we were unable to obtain ovarian images by this transabdominal approach. Explanations for this failure include the low penetration of the high-frequency sound waves through tissue and the high impedance of the rabbit's body wall, as reported previously in rats (Jaiswal *et al.*, 2009). For this reason we decided to seek another approach to assess ovarian structures *in vivo* using UBM in rabbits (Chapter 3, Experiment 2).

Until now, histological examination has been the only way to evaluate accurately ovarian structures in small animals (e.g., rabbits). The use of UBM has been found to be an effective tool to identify ovarian structures in rabbit ovaries *ex situ* (Chapter 3, Experiment 1). To be used in live rabbits, a novel approach needed to be developed to allow the visualization of the ovaries by UBM, because the ovaries are located deep within the body. Therefore, the translocation of ovaries to a more superficial position (the abdominal wall) was performed to facilitate the penetration of high-frequency waves and enable the imaging of rabbit ovaries by transcutaneous UBM (Chapter 3, Experiment 2). The surgical translocation performed without altering blood supply was feasible and without major complications. The time course of follicular growth observed in live rabbits (Chapter 3, table 3.2) may indicate that there was no apparent effect of the surgical procedure on ovarian function (i.e., necrosis, atrophy, lack of ovarian structures). In our study (Chapter 3), after the surgical procedure and after complete postsurgical recovery, there was evidence that the vasculature remained relatively intact, because the ovaries did not shrink in size, and ovarian structures remained visible throughout the observational period. In previous studies, investigators working in other species reported the preservation of ovarian function after relocation of the ovary to the vagina in sheep (Dierschke and Williams, 1970); or after the transposition of the ovary from the pelvis into the vaginal fornix in monkeys (Iffy *et al.*, 1978). Studies in which the procedures were performed taking care not to interrupt the ovarian blood supply as in our study.

In general, UBM in rabbits is safe, and enables the acquisition of good quality images of the ovaries within two months after surgical translocation of ovaries. Although surgical translocation was required to image the ovaries in rabbits, an advantage of the use of a rabbit model compared to mice (Jaiswal *et al.*, 2009), is that transcutaneous UBM in rabbits does not

require anesthesia or sedation during the ultrasound procedure. However, UBM is very sensitive to mechanical interference from surface hair and air, thus the area to be scanned must be shaved thoroughly and coupling gel used generously. Transcutaneous UBM allowed the detection of changes in follicle size and number within rabbits over time, and permitted detection of ovulation - an important factor in determining ovarian response to any treatment. Importantly also, because follicles of determined sizes have to be present at time of mating or treatment for ovulation to occur in other species (e.g., camelids; Adams *et al.*, 1990; Bravo *et al.*, 1991a).

The use of *in vivo* imaging in small animal is a very promising tool in different areas of research such as developmental biology, ocular development and ovarian dynamics (Turnbull and Foster, 2002; Brown *et al.*, 2005; Jaiswal *et al.*, 2009; Pallares *et al.*, 2009). There is a great potential for future studies involving UBM imaging of rabbit ovaries *in vivo*, considering that histology is more complicated, time consuming and can provide only a single observation in time. Although we have developed an efficient method for examination of ovaries in rabbits by using transcutaneous UBM, still need to be determined whether ovarian responses following different ovulatory treatments can be distinguished using UBM. This will help to ultimately determine UBM usefulness in investigating the success of particular treatments in further studies associated with ovulation induction in rabbits. Future improvements of UBM technology will allow for better resolution and penetration of UBM waves and, hopefully, obviate the necessity of surgical ovarian translocation in rabbits.

5.2. Effect of seminal plasma on ovulation in rabbits

In this study, female rabbits were used as a model to study the effects of llama and rabbit seminal plasma on LH release and ovulation (Chapter 4). In induced ovulators (i.e., rabbit, camelids), a preovulatory LH surge normally occurs after mating. This surge of the LH is released from the pituitary (Hilliard *et al.*, 1974; Jones *et al.*, 1976; Meunier *et al.*, 1983; Spies *et al.*, 1997 (Xu *et al.*, 1985; Bravo *et al.*, 1992; Aba *et al.*, 1995). South American camelids have been used intensively as an animal model for the study of ovulation-inducing factor (OIF) in the seminal plasma for several years (Adams *et al.*, 2005; Bogle, 2009; Ratto *et al.*, 2005, 2010, 2011; Tanco *et al.*, 2011; Bogle *et al.*, 2011). Although the camelid model has been the basis for the discovery of several aspects of OIF, there is still much to be learned about the OIF mechanism of action and biological pathways. Data from the present study shows that a small animal model, like the rabbit, may provide some advantages for the study of ovarian function and mechanism of action and pathways of factors that control or alter ovarian function. As has been shown to be the case when mice and rats were the animal models for the study of the effects of chemical substances (e.g., ethyl-methane sulfonate), pharmaceuticals and hormonal agents (e.g., diethylstilbestrol) (Deodhar *et al.*, 1968; Elger and Hasan, 1985; Nef, 2001; Henley and Korach, 2010).

To develop our rabbit model, we studied the effects of llama and rabbit seminal plasma on ovulation in female rabbits grouped together (Chapter 4, Experiment 1) and in females rabbits caged individually (Chapter 4, Experiment 2). Ovarian follicular size was evaluated prior to treatment in all rabbits by using conventional ultrasonography (125 MHz) or ultrasound biomicroscopy (25 MHz), respectively, to determine the presence of largest follicles in the

ovaries of all rabbits prior to treatment. Importantly, as in other species (i.e., camelids) where follicles of determined sizes (≥ 6 mm) have to be present at time of treatment (e.g. mating) in order for ovulation to occur (e.g., camelids; Adams *et al.*, 1990; Bravo *et al.*, 1991a).

Presence of large follicles ≥ 1.5 mm was detected by conventional ultrasonography (Chapter 4, Experiment 1), but not as clearly as the presence of large follicles ≥ 1.5 mm and ≥ 1.8 mm detected by ultrasound biomicroscopy (Chapter 4, Experiment 2). Based on existing literature (Marongiu and Gulinati, 2008) and observations from our first study (Chapter 3), treatment was given when a similar number of large follicles were present in the rabbits at the time of treatment. In our second study (Chapter 4), rabbits were treated intramuscularly with saline, GnRH, or llama or rabbit seminal plasma. We hypothesized that llama and rabbit seminal plasma would elicit a surge of LH release and would be responsible for inducing ovulation in rabbits (Chapter 4). The hypothesis was based on the effects of seminal plasma in other induced ovulators, such as camelids (Chen *et al.*, 1985; Zhao *et al.*, 2001; Adams *et al.*, 2005; Ratto *et al.*, 2006b, 2011) and koalas (Johnston *et al.*, 2004).

In Experiment 1 (Chapter 4), rabbits were maintained together in a room for the duration of the experiment (about 60 days), and we found that animals in all treatment groups ovulated. No differences in proportion of animals that ovulated, or the number and size of corpora lutea, were found among groups (Chapter 4, table 4.1). Our results showed that ovulation occurred regardless of treatment. The unexpected results, in particular the occurrence of ovulation in the negative control group (saline), suggested that the experimental outcome may have been confounded by direct rabbit-to-rabbit contact in group housing. Although group-housing has been shown to be an appropriate method of maintaining rabbits for use in research (Fuentes and Newgren, 2008), factors related to the housing system may play a role in ovulation in rabbits. In

an earlier study (Staples, 1967), ovulation was reported when females were repeatedly mounted by other females while caged in pairs. However, the causes of ovulation in group-housed rabbits still are not clear (Rommers *et al.*, 2006). Even, pheromones can be involved. In Experiment 1 (Chapter 4), mounting and mating behaviour among females were occasionally observed, but because the study was not designed to test this behaviour, no further notation of the frequency or the identification of the individuals involved were recorded. The animals were sacrificed 8 days after treatment to evaluate ovarian response by UBM *ex situ*. Therefore further interpretation of our results was limited to a one time examination of the ovaries at that time.

In Experiment 2 (Chapter 4), animals were caged individually for the duration of the experiment (about 90 days), and after treatment, ovulation was observed only in the positive control group (GnRH). No ovulation was observed in rabbits treated with rabbit or llama seminal plasma, or in the negative control group (saline) (Chapter 4, table 4.2). This result may be interpreted to mean that seminal plasma does not induce ovulation in rabbits, or that the dose of seminal plasma and/or route of administration were insufficient to induce ovulation. The effects of OIF in llamas have been reported to be dose-dependent (Tanco *et al.*, 2011). There is also evidence that route of administration contributes to the variation in the response. In South American camelids, the ovulation rate was significantly higher after intramuscular administration of camelid seminal plasma than after intrauterine administration (Ratto *et al.*, 2005). In gilts, deposition of boar seminal plasma near the utero-tubal junction has been observed to advance the time of ovulation, but no effect was found when it was deposited in the middle of the uterine horn between two ligatures (Wabersky *et al.*, 1999). Some others have reported that ovulation in rabbits may be induced by absorption of GnRH analogues through the vaginal mucosa when added to the insemination dose, and with similar (Viudes de Castro *et al.*, 2007; Ondruška *et al.*,

2008) or better results (Quintela *et al.*, 2008) as those obtained when GnRH is given intramuscularly.

In summary, we have developed a model that will allow us to examine OIF and other factors influencing ovulation in the rabbit *in vivo* and in a serial manner by using the transcutaneous UBM. Seminal plasma administered intramuscularly to females rabbits caged individually did not induce LH release, as in the negative control group (saline). Ovulation occurred only in the positive control group (GnRH), where a preovulatory peak of LH was observed within 1 h after GnRH administration. In our study, only one dose and a route of administration of seminal plasma were evaluated. The discrepancy in ovulation rates between our two experiments (Chapter 4) warrants re-examination of the effects of seminal plasma. The use of different doses and routes of administration, to further test the hypothesis that OIF stimulates LH release and induces ovulation in mature rabbits is required.

6.0. GENERAL CONCLUSIONS

In general, results of the studies presented herein do not support our broad hypothesis that OIF in seminal plasma induces ovulation in the rabbit. However, the data obtained from a single dose and route of administration of seminal plasma may be insufficient to determine the effects of OIF in rabbits. Based on our experimental results thus far, we conclude that:

- Ultrasound biomicroscopy (UBM) *ex situ* is as a valuable tool to estimate the number and size of follicles ≥ 0.6 mm and corpora lutea on the ovaries of rabbits.
- *In vivo* UBM evaluation of rabbit ovaries is made possible by surgical translocation of the ovaries to a subcutaneous position, and is useful for evaluating ovarian follicular size prior to treatment and the ovarian response to treatment.
- Group housing of female rabbits is associated with a high incidence of ovulation and confounded the detection of an ovulation-inducing effect of seminal plasma.
- No ovulation-inducing effect of seminal plasma (rabbit or llama) was observed in female rabbits caged individually.
- LH secretion is essential for the occurrence of ovulation in rabbits.
- The rabbit appears to be a useful animal model for the study of OIF but further studies should be carried out test the hypothesis that OIF stimulates LH release and induces ovulation in mature rabbits.

7.0. FUTURE STUDIES

Further research is needed to investigate the effects of seminal plasma or purified OIF in rabbits and to determine whether these effects are similar to those observed in other species. The following issues need to be addressed:

- The influence of different doses of OIF on circulating LH concentrations and ovarian response in rabbits.
- The influence of different routes of administration of OIF on the LH surge and ovarian response in rabbits.
- The effect of administration of seminal plasma of other species on ovulation in rabbits and vice versa.

In addition, the use of UBM *in vivo* in rabbits provides enormous potential to carry out subsequent studies on the reproductive physiology, such as:

- The characterization of ovarian follicular dynamics in rabbits
- The characterization of luteal dynamics in rabbits
- Response to superstimulatory and ovulatory treatments in rabbits

8.0. BIBLIOGRAPHY

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