Investigation of the Presence of GnRH and GnRH-R System in Bovine Oocytes, Sperm and Early Embryos and their Functional Role in Reproduction

by

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ABSTRACT

The objectives of this study were to investigate: 1) the effect of GnRH agonist on oocyte maturation, sperm function and fertilization, 2) the presence of GnRH-R in bovine oocytes, sperm and early embryos.

To examine the effect of GnRH agonist on sperm function, sperm were incubated in modified Tyrode's medium with 0, 0.2, 0.4, 0.8 and 1.2 μgmL^{-1} of buserelin and with1 μgmL^{-1} P_4 for 3 h. Acrosome status in each group was assessed at 0 h and 3 h. For zona-binding assay, *in vitro* matured oocytes were co-incubated with sperm in different concentrations of buserelin and P_4 for 4 h and the zona-bound sperm in each treatment group was determined. Acrosome reacted percentage were higher in sperm treated with 0.4, 0.8 μgmL^{-1} buserelin than the control group ($p<0.001$). The number of zona-bound sperm were higher in 0.8 μgmL^{-1} buserelin compared to negative control ($p<0.01$). Effect of buserelin was blocked by antide. To investigate the effect of GnRHa on maturation of bovine oocytes 0.8 μgmL^{-1} buserelin was added to the *in vitro* maturation media and maturation rate was obtained after 24 h against control groups with FSH and without FSH. To assess the effect on fertilization rate one group of oocytes undergoing *in vitro* fertilization was treated with 0.8 μgmL^{-1} buserelin, while *in vitro* fertilization without buserelin served as control. Maturation rate was not different among the groups and were 55.14 ± 0.39, 50.69 ± 0.42 and 43.5 ± 0.46 % for oocytes treated with FSH, buserelin and negative control, respectively. No difference was observed on fertilization rates, with fertilization rates being 55.25 ± 0.89 and 51.25 ± 0. 51% for buserelin treated and the control group, respectively. Presence of GnRH-R protein and mRNA on bovine sperm, oocytes, cumulus cells and early embryos were studied using immunostaining technique

and RT-PCR. No receptors for GnRH were detected in bovine sperm, oocytes or early embryos studied and expression of GnRH-R mRNA was also not evident. GnRH-R mRNA was expressed by cumulus cells, immature and mature COC. GnRH receptor mRNA expression by mature COC was higher than immature COC (2.01±0.12 and 0.89±0.2).

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ABBREVIATIONS

AI	- Artificial insemination
ANOVA	- Analysis of variance
AR	- Acrosome reaction
bFGF	- Basic fibroblast growth factor
BSA	- Bovine serum albumin
Camp	- Cyclic adenosine monophosphate
COC	- Cumulus oocyte complexes
cDNA	- Complementary deoxyribose nucleic acid
DAG	- Diacylglycerol
GVBD	- Germinal vesicle breakdown
FITC	- Fluorescein isothiocyanate
FSH	- Follicle stimulating hormone
GnRH	- Gonadotropin releasing hormone
GnRH-R	- Gonadotropin releasing hormone receptor
GnRHa	- Gonadotropin releasing hormone agonist
GPCR	- G protein coupled receptors
IP_3	- Inositol 1, 4, 5, trisphosphate
IVF	- *In vitro* fertilization
IVM	- *In vitro* maturation
LH	- Luteinizing hormone
MAPK	- Mitogen activated protein kinase
mRNA	- Messenger ribose nucleic acid
P_4	- Progesterone
PAI	- Plasminogen activator inhibitor
$PGF_{2\alpha}$	- Prostaglandin $F_{2\alpha}$
PKA	- Protein kinase A
PKC	- Protein kinase
PLA2	- Phospholipase A2
PLD	- Phospholipase D
PSA	- *Pisum sativum* agglutinin
RT-PCR	- Reverse transcription polymerase chain reaction
SCS	- Superovulated cow serum
TALP	- Tyrode's albumin lactate pyruvate
TGF-β_1	- Transforming growth factor-β_1
TIMP-1	- Tissue inhibitor for metalloproteinase-1
Upa	- Urokinase-type plasminogen activator
ZB	- Zona binding

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CHAPTER 1

GENERAL INTRODUCTION AND BACKGROUND

1.1 GENERAL INTRODUCTION

Gonadotropin releasing hormone (GnRH) is a pivotal hypothalamic decapeptide regulating reproduction. Around 23 forms of GnRH have been identified so far in which the NH_2- terminal and COOH- terminal sequences, which are important for receptor binding and activation are conserved. In mammals there are two forms; GnRH I, having an amino acid sequence of pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, and GnRH II, differing from GnRH I by having His^5, Trp^7 and Try^8 residues. In mammals GnRH II has a broad tissue distribution, including central and peripheral nervous systems, reproductive tissues, kidney and tumors (Neill et al. 2001; Millar 2005).

. GnRH I is released from the hypothalamus intermittently in a pulsatile manner and travels to the putative target sites in gonadotropes in the anterior pituitary, via the hypothalamo-hypophyseal portal circulation. Upon binding to its receptor, a member of G protein coupled receptor (GPCR), designated as GnRH-R I, (in the plasma membrane of gonadotropes) a range of intracellular signaling pathways are activated. This lead to the synthesis and secretion of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) in a highly coordinated pulsatile manner. In turn, gonadotropins stimulate sex hormone synthesis and gametogenesis in the gonads to ensure reproductive competence (Fink G 1998).

GnRH II has been postulated to have diverse functions through autocrine and paracrine fashion. In the brain, it appears to be a neuromodulator and stimulates sexual behavior (Millar 2005).

GnRH receptors too have undergone coordinated gene duplications such that cognate receptor subtypes for respective ligands exist in most vertebrates. At least two forms of the receptor have been identified in mammals, GnRH-R I & GnRH-R II (Hapgood et al. 2005). GnRH-R I is distinctive among other GPCRs (including GnRH-R II) for not having a –COOH terminal tail. Interestingly, in man and some other mammals (bovine, sheep and chimp) GnRH-R II appear to be silenced. However GnRH- I and GnRH- II still have distinctive roles in differential signaling resulting in different down stream effects (Millar 2005).

Although the hypothalamus and pituitary are the main source and target site of GnRH, many physiologically important autocrine/paracrine sites of the GnRH-GnRH-R system acting locally have been discovered in different species of animals. With regard to the reproductive system, it is becoming evident that tissues and cells such as the ovary (including cumulus oocyte complexes), placenta oviduct, testes (including germinal cells) and early embryonic stages express functional forms of GnRH and its receptor (Ramakrishnappa 2005). In bovine, GnRH receptor mRNA has been demonstrated in ovarian follicles, corpora lutea (Ramakrishnappa 2001) and also cumulus oocyte complexes (Funston & Seidel 1995). In the human, a dynamic expression pattern for GnRH mRNA occurs in all endometrial cell types and it has been observed that staining patterns are more intense during the luteal phase as compared to follicular phase of menstrual cycle (Raga et al. 1998). Very recently in our laboratory, through

immunohistochemistry and RT-PCR techniques, the presence of GnRH-R protein and mRNA in bovine endometrial and oviductal epithelial cells was elucidated (Singh et al. 2008). Furthermore, *in vitro* fertilization studies by Funston & Seidel (1995) supplementing GnRH agonist have been shown to improve cleavage rate in bovines. It is also well established in bovine reproductive management practices that administration of GnRH or GnRH agonist at the time of artificial insemination or 11-14 days after AI increases pregnancy rates. This timing is significant as it coincides approximately with maternal recognition of pregnancy (Peters 2005).

Due to current bovine reproductive management practices, it is obvious that both bovine sperm and oocytes are exposed to GnRH during maturation, through the course of migration to the site of fertilization during their interaction and early embryonic development. Furthermore, the information accumulating on the expression of GnRH-GnRH-R system across reproductive tissues and cells, suggest that there could be a functional form of GnRH-GnRH-R system present in bovine sperm, oocytes and early embryonic stages.

Therefore, an in depth study to investigate the presence of GnRH-GnRH-R system and its direct effects in bovine gametes and early embryonic stages is required. A future understanding on the meaningful applications of GnRH analogues in bovine reproductive management in terms of improving *in vitro* embryo production is required.

1.2 BACKGROUND

This chapter provides a brief review of GnRH and GnRH receptors, the extrahypothalamic presence especially in reproductive tissues and cells, and related roles in mammalian reproduction. A very brief review on GnRH analogues and their applications in current reproductive management practices in bovine is also included. In addition, sperm functions in fertilization, oocyte maturation, fertilization and early embryonic development are reviewed. The significance and objectives of the study are presented at the end of this chapter.

1.3 FORMS OF GnRH

GnRH was first isolated from the hypothalamus in mammals and it was considered to be a unique structure with a primary role of regulating biosynthesis and secretion of pituitary LH and FSH. Later on, it became evident that different forms of GnRH exist in vertebrates (King and Millar 1980), with 23 different structural forms of the decapeptide being identified in protochordates and vertebrates. In all these forms NH_2- and COOH- terminal sequences which are important for receptor binding & activation are conserved.

In most vertebrate species there are at least two or usually three forms of GnRH which differ in their amino acid sequence, localization and embryonic origins. Amino acids at positions 1, 2, 4, 9 & 10 are conserved while position 8, has the most variable amino acid. Amino acids at positions 6, 5, and 7 are also variable (Powell et al. 1994; Millar 2005).

There are two forms of GnRH in mammals. The hypothalamic/hypophisiotropic form which was first isolated and sequenced in early seventies by Dr. A. Schally, Dr. R. Guillemin, Dr. R. Yallow and co-workers. This has been designated as GnRH I or type I. The second type, mid brain GnRH/ GnRH II was first isolated from chicken brain (Miyamoto et al. 1987) and thus it's referred to as chicken GnRH (Millar 2005). GnRH II is structurally conserved from bony fish to humans and may be the earliest evolved form (Millar and King 1987; Millar 2005). GnRH II differ from GnRH I by the amino acid residues at position 5, 7, & 8. The wide distribution of GnRH II in the central and peripheral nervous systems suggests a possible neurotransmitter/neuromodulatory role (Millar 2005).

A third form of GnRH, GnRH III has been found in many vertebrate species and is also known as Salmon GnRH/telencephalic GnRH. It is clear that each of these three forms has its unique location within the brain suggesting a different developmental origin and/or function (White et al. 1994; Millar et al. 2001).

1.4 FORMS OF GnRH-R

Amino acid sequence of the GnRH-R was first deduced from clones of a mouse pituitary αT3 gonadotrope cell line (Tsutsumi et al. 1992). This provided the basis for the cloning of GnRH-R of the pituitary of many other mammals such as rat (Kaiser et al. 1992. Perrin et al. 1993), sheep (Illing et al. 1993; Brooks et al. 1993) cow (Kakar et al. 1993) and pig (Weesner and Matteri 1994). Receptors from these species share over 80% amino acid identity.

GnRH receptors have the characteristic features of G protein coupled receptors (GPCR) which couple with Gqα and function in the inositol phosphate signaling pathway. The NH_2- terminal domain is followed by seven putative α-helical transmembrane domains connected by three extracellular loop domains & three intracellular loop domains. The extra cellular domains and superficial regions of the transmembrane domains are normally involved in binding of GnRH. The transmembrane domains are believed to under go conformational changes with signal propagation, while the intracellular domains are involved in interacting with G-proteins and other proteins for intracellular signal transduction (Millar 2005). Mammalian GNRH-R I are unique in structure for not having the –COOH terminal tail present in all other GPCRs including non mammalian GnRH-R. This suggests a recently evolved feature which may have an important role in the mammalian GnRH receptor function (Millar 2005).

The presence of three forms of GnRH in vertebrates suggested that a separate and cognate receptor types may be present (Troskie et al. 1998). The cognate receptor for GnRH II was cloned from the marmoset monkey in a recent study and was found to be distributed in the brain areas associated with reproductive behaviors in primates. Subsequent studies demonstrated that GnRH II stimulated sexual behavior in female marmosets (Millar 2005). GnRH-R II transcripts have been shown to be expressed in the majority of gonadotropes and in non-neural reproductive tissues such as the prostate. Unlike the type I receptor which internalize slowly due to the lack of the C-terminal tail, the type II receptor is rapidly internalized (Mc Ardle et al. 2002) and has a distinctly different signaling pathway which preferentially stimulates FSH secretion. This has led to

the suggestion that GnRH-GnRH-R II system may have a specific role in the regulation of gonadotrope function as well (Ramakrishnappa et al. 2005).

However a full length GnRH receptor type II is hypothesized to be absent in human, chimpanzee, cow, horse, sheep, rat and mouse although it is present in marmosets, green monkey, rhesus monkey and pig (Morgan et al. 2003; Millar 2005). It has now been rationalized that the silencing of the type II receptor can be accommodated by GnRH II signaling through the type I receptor, a phenomenon called as ligand-induced-selective signally (Millar 2005). Contrary to this hypothesis van Biljon and co-workers (2002) have stated, that it is possible that the type II GnRH receptor gene in humans (on chromosome 1) may be functional.

1.5 GnRH AND GnRH-R INTERACTION IN THE PITUITARY

GnRH activation of its receptor results in stimulation of diverse signaling pathways in the anterior pituitary. Upon binding to its receptor, GnRH activates Gq/ G11 proteins coupled to the receptor. This leads to increased phospholipase C activation causing a break down of phosphoinositide producing inositol 1, 4, 5, trisphosphate (IP_3) and diacylglycerol (DAG)(Kraus et al. 2001; Ruf et al. 2003). DAG in tern activates the intracellular protein kinase C (PKC) pathway and IP_3 stimulates the release of intracellular calcium. PKC activation leads to increase in the mitogen activated protein kinase (MAPK) in gonadotropes. The active MAPKs move to the nucleus to activate a variety of transcription factors which modulate gene expression. These pathways differentially regulate the synthesis & secretion of gonadotropin subunits α, LHβ and

FSHβ selectively modulating gonadotropin synthesis and/release by the gonadotropes (Harrison et al. 2004).

The frequency and amplitude of GnRH pulse release is a critical and rate limiting factor for the regulation and maintenance of gonadotropin secretion from gonadotropes. The GnRH pulse frequency & amplitude depend on the feedback control of the sex steroids and other Gonadal peptides and growth factors, produced through the course of reproductive cycle. GnRH receptor synthesis and LHβ synthesis is favored at high GnRH pulse frequency and FSHβ synthesis is favored at low GnRH pulse frequency (Kaiser et al. 1997) It has also been suggested that modulation of the frequency of intracellular calcium pulses may also play a role in the differential regulation of LHβ & FSHβ mRNA expression (Haisenleder et al. 1997).

1.6 EXTRAHYPOTHALAMIC GnRH-GnRH-R SYSTEM AND POSSIBLE PHYSIOLOGICAL ROLES

Besides the well established role for GnRH I and GnRH-R I in gonadotropin regulation in the pituitary, the detection of both forms of hormone and receptor in various mammalian nonpituitary tissues and cells suggests numerous and diverse autocrine, paracrine and endocrine extra pituitary roles for GnRH &GnRH-R. These include neuronal migration during development (Romanelli et al. 2004), neuromodulation in the brain affecting sexual behavior (Millar 2003), possible modulation of visual processing in the eye (Wirsig-Weichmann et al. 2002), digestive tract function (WeiQuan rt al. 2001), inhibition of gastric acid secretion (Chen et al. 2005), adhesion chemotaxis and homing in T cells (Chen et al. 2002), hCG release in the placenta (Raga et al. 1998),

steroidogenesis in ovarian cells (Kang et al. 2001, Pellicer et al. 1990;Ramakrishnappa et al. 2003), sperm function and sperm oocyte interactions (Morales et al. 1998), growth inhibition in reproductive tumors (Harrison et al. 2004; Finch et al. 2004; Franklin et al . 2003; Grundker et al. 2002, 2003) and proliferation in melanoma cells (Limonta et al. 2003).

In the last decade or so, an upsurge of information has occurred on extrapituitary presence of GnRH, GnRH-R system in different tissues and in different species of animals. However, the specific roles of each form of the hormone and receptor in these various tissues and cells are only partially delineated. The presence of multiple forms of GnRH and its receptor in mammals, as well as the emerging multiple roles that have presented new therapeutic targets and intensified the search for novel GnRH analogues (Hapgood et al. 2005).

1.6.1 In reproductive tissues and cells

Ovary

The earliest revelation of high affinity binding sites for GnRH in rodents was via radioligand binding assay, in 1979 (Clayton et al. 1979). Since then, there has been a flare of information on the presence of GnRH-GnRH-R in ovarian tissues across various mammals. Through radioligand binding assays, ligand specific binding sites have been demonstrated on rat granulosa and luteal cells (Harwood et al. 1980; Reeves et al. 1980; Jones et al. 1980; Pieper et al. 1981). Later on, these findings were confirmed by subsequent researchers (Latouche et al. 1989; Whitelaw et al. 1995). Expression of GnRH mRNA has been demonstrated in rat gonads (Oikawa et al. 1990; Bahk et al.

1995) and employing in situ hybridization it had been demonstrated that GnRH mRNA localizes in granulosa cells of primary, secondary, and tertiary follicles in the ovary (Clayton et al. 1992; Whitelaw et al. 1995). GnRH-R mRNA has also been demonstrated in human granulosa luteal cells by using reverse transcription polymerase chain reaction (RT-PCR) technique (Minaretzis et al. 1995; Olofsson et al. 1995; Kang et al. 2000) Furthermore, in our lab the presence of GnRH-R mRNA transcripts in bovine follicles and corpus luteum by using RT-PCR technique were demonstrated (Ramakrishnappa et al. 2003). In this study, moderately high levels of GnRH-R transcripts were evident in granulosa cells from small follicles, while slightly lower & uniform expression levels had been detected in medium and large follicles. Whereas, barely detectable expression levels had been detected in stage I and IV luteal tissues while clearly detectable transcripts were detected in stage III luteal tissue and lower expression levels were seen with in stage II. GnRH/GnRH like molecules have been detected in human and bovine follicular fluid (Ying et al. 1981; Aten et al. 1987a; Ireland et al. 1985)

In the ovary, GnRH has been shown to trigger both stimulatory and inhibitory effects on its function (Sharpe 1982; Janssens et al. 2000) GnRH exert its direct effects in the ovary either on its own or in combination with other modulators such as PGF2α, angiotensinogen II or LH. Stimulation of signaling pathways such as PLC, PLA2 and PLD, activate protein kinase C (PKC) causing either inhibitory or stimulatory effects on ovarian steroid out put (Hsueh and Jones 1982). Other researchers have also demonstrated that GnRH modulates both basal and gonadotropin-stimulated steroidogenesis in the ovary (Olofsson et al. 1995). The inhibitory action of GnRH or its agonists on gonadal steroidogenesis involves suppression of gonadotropin receptors or

intermediary enzymes involved in steroidogenic pathway. Reports suggesting GnRH agonist induced suppression of FSH and LH receptors (Tilly et al. 1992; Piquette et al. 1991; Guerrero et al. 1993), gonadotropin induced cAMP levels (Richards 1994; Knecht et al. 1985) or steroidogenic enzyme activity such as peripheral-type benzodiazepine receptor, steroidogenic acute regulatory protein, P450scc enzyme, and 3βHSD (Sridaran et al. 1999a; Sridaran et al. 1999b) and no effect (Casper et al. 1984) of GnRH on progesterone production in human granulosa-lutein cells (hGLCs) have been documented.

In bovine species, similar observations had revealed inhibitory effects of GnRH agonist, buserelin on progesterone secretion from *in vitro* cultured luteal cells (Milvae et al. 1984). *In vivo* studies by D'Occhio et al. (2000) had suggested that the suppressed ovarian function in heifers treated for a long-term with GnRH agonist may have been due, in part, to a direct action of deslorelin (GnRH implant) on the ovaries. On the other hand, there are several reports, both from *in vitro* or *in vivo* studies, in rodent, primate and *in vitro* human granulosa cell culture models providing sufficient evidence to contradict the above mentioned inhibitory effects of GnRH agonist at the gonadal level. Liu et al. (1991) reported dose-related stimulatory effects of GnRH agonist on aromatase activity and progesterone production in monkey granulosa cell cultures and GnRH antagonist suppression of this stimulatory effect. Similar studies demonstrating GnRH agonist induced steroidogenesis in cultured human granulosa cells have been reported (Ranta et al. 1982; Parinaud et al, 1992; Bussenot et al. 1993). Parinaud and co-workers (1998) suggested that GnRH agonist could modulate steroidogenesis by a direct ovarian action. The agonist, buserelin, increased basal and decreased LH induced progesterone

secretion *in vitro*. Guerrero et al. (1993) found an increase in progesterone and decrease in estradiol production, which seemed to be related to a decrease of LH receptor numbers and aromatase activity in GnRH agonist treated cells. Recently in our lab, it was revealed that GnRHa, buserelin resulted in a dose dependent stimulatory effect on steroid hormone output from both granulosa cells and luteal tissues *in vitro.* Further investigations had confirmed that there is an increase in the mRNA expression for both steroid acute regulatory protein (Star) and cytochrome P450 side chain cleavage ($P450_{scc)}$ enzymes, which are rate limiting steps in the regulation of luteal steroidogenesis (Ramkrishnappa 2004). In agreement, an *in vivo* study in pre-pubertal heifers reports an increased plasma concentration of 17β-estrodiol with continuous GnRH agonist treatment for 28 days (Bergfeld et al. 1996a).

Oocytes

In 1988 N. Dekel and co-workers demonstrated the presence of GnRH-R in rat oocytes through photoactivation of rat oocytes with I^{125}- labeled GnRH analog followed by autoradiographic analysis and subsequent displacement by excess unlabeled hormone. This was confirmed via the binding of GnRH-receptor antibody to the oocyte, demonstrated by immunocytochemistry. In the same study specific binding of the GnRH analog was also demonstrated in cumulus cells. In bovine, Funston and Seidel (1995) demonstrated the presence of GnRH receptor mRNA in matured cumulus oocyte complexes by RT-PCR and verified these findings through Southern hybridization with cDNA for the ovine GnRH receptor. Furthermore, supplementation of GnRH at a concentration of 0.8 μgmL^{-1} to the fertilization medium was shown to increase cleavage

rate of bovine oocytes fertilized *in vitro*. Treatment with GnRH agonists also resulted in increased cleavage rates, while treatment with GnRH antagonists were able to abolish the enhancing effect of GnRH on cleavage rate.

Testes

In early 80s, evidence of the presence of GnRH-GnRH-R system in the testicular tissue began to emerge through radioligand biding assays. Ligand specific binding sites were demonstrated in rat interstitial tissue, including leydig cells (Bourne et al. 1980 and 1982; Lefebvre et al. 1980; Sharpe and Fraser 1983). Subsequently presence of GnRH mRNA in the testicular tissue, in both fetal and mature rats, as well as in the adult human, seminiferous tubular cells has been observed (Botte et al. 1998). GnRH-R mRNA have been observed in interstitial cells, in human and rats (Bahk et al. 1995; Clayton et al. 1980). GnRH like molecules have been detected in human seminal plasma (Izumi et al. 1985) and in testicular interstitial fluid of hCG-treated rats (Sharpe and Fraser 1980)

Interestingly, *in vivo* studies administering GnRH to sub fertile stallions has found to result in almost immediate increase in fertility (Pickett et al. 1991) and stallions receiving GnRH in a pulsatile manner had been reported to have increased libido, increase progressively motile sperm and increased sperm velocity& longevity resulting in increased pregnancy rates. Similarly, dramatic increases in sperm motility and fertility have been reported in beef bulls after administration of GnRH in a pulsatile fashion (Funston and Seidel 1995).

GnRH agonist in a supraphysiological, long acting dose appears to exert an inhibitory effect on each step of spermatogenesis. This is thought to be mediated by

suppressing FSH, LH and intratesticular testosterone levels. On the other hand, GnRH agonist/GnRH antagonist treatment enhanced the regeneration of spermatogenesis from damaged testes in irradiated rat (Meistrich and Kangasniemi 1997; Shuttlesworth et al 2000), cryptorchid rat (Koichi et al. 2002), cytotoxic therapy rat (Meistrich et al. 1999), and juvenile spermatogonial depletion (jsd) mutant mice (Matsumiya et al. 1999). It is hypothesized that GnRH agonist treatment stimulated spermatogonial proliferation, and regeneration of spermatogenesis occurred. The exact mechanism for this is still uncertain, however, reduced intratesticular testosterone levels by GnRH agonist may play a role since intratesticular testosterone level was elevated after irradiation or chemical insult (Meistrich and Kangasniemi 1997). Testosterone is necessary for spermatogenesis but suppressive to spermatogonial proliferation (Koichi et al. 2002).

Sperm

In 2000, Bull and co-workers reported the expression of three GnRH-R transcripts of 4.7, 3.5, and 2.2 kb size in testicular germ cells of mouse and two distinct GnRH-R transcripts of 4.4 & 3.5kb size in rat testicular germ cells by means of Northern hybridization technique and Northern blot analysis. Another study by Lee et al. (2000) demonstrated localization of GnRH-R in acrosomal region of the human sperm via immunohistochemical staining. Supporting this evidence another more recent study displays the presence of GnRH-R II, axon 1 containing transcripts in human mature sperm and post meiotic testicular germ cells, using in situ hybridization technique (Biljon et al. 2002), and thereby leading to doubts whether GnRH-R II is functional in humans.

Studies using hemizona assay directed towards the role played by GnRH in sperm function and sperm oocyte interaction reveals that GnRH increases human zona-pellucida binding. In this study, it was revealed that exposure of capacitated sperm to a 20nM GnRH for 5 minutes, increased the number of sperm bound to the zona pellucida by an average of 365% +/- 15%. Prior treatment with GnRH antagonist had inhibited sperm binding to the zona-pellucida (Morales et al. 1998, 1999). The same study reported that GnRH did not change the pattern of sperm movement, frequency of sperm-zona collision or the percentage of acrosome reacted human sperm. As well, there's no effect of GnRH on sperm capacitation (Naz and Sellamuthu 2006). It is hypothesized that increased sperm binding to oocytes is due to exposure and/or change in affinity of zona receptors on sperm membranes (Naz and Sellamuthu 2006). Findings of various studies suggest that sperm may positively interact with GnRH during development as well through the course of its journey from the male reproductive tract to the site of fertilization in the female reproductive tract (Hedger et al; 1985; Hsueh and Schaeffer 1985; Verhoeven and Cailleau 1985). In the oviduct, the sperm may interact with GnRH secreted locally or transported by the products of ovulation by the ovary (Ying et al. 1981; Aten et al. 1987a and b; Ireland et al. 1988; Oikawa et al. 1990).

Embryos

Seshagiri et al (1994) first demonstrated the expression of GnRH in preimplantation rhesus monkey embryos. The presence of GnRH and GnRH-R mRNA and protein expression was reported in human preimplantation embryos (from 8 cell stage to expanded blastocysts) employing RT-PCR and immunohistochemical studies (Casan et

al. 1999). In mouse GnRH was shown to be expressed in the developing mouse embryos from morula to hatching blastocyst stages at the mRNA (via RT-PCR) and protein level (via Immunohistochemistry). GnRH-R mRNA was also shown to be present in developing embryos in mice. Mouse preimplantation embryonic development is demonstrated to be significantly enhanced with GnRHa treatment while GnRH antagonist treatment was shown to have detrimental effects on preimplantation embryonic development, which could be reversed upon agonist treatment, implying a specific action by GnRH rather than a non specific or toxic effect (Raga et al. 1999). The percentage of embryos expressing GNRH-R mRNA is reported not to increase with the developmental stages from morula to hatching blastocyst stage, whereas the percentage of embryos positive for the expression of GnRH mRNA is reported to rise significantly during development from morula (65%) to blastocyst (87%) and hatching blastocyst (90%) stages (Raga et al. 1999). A recent study by Nam and coworkers reported the expression of GnRH and GnRH-R in porcine IVF derived embryos. In this study they also reported embryotrophic effects of GnRH agonists on porcine embryos. A significant increase in the rate of blastocyst formation had been observed while co-supplementation with GnRH antagonist was reported to reverse the embryotrophic effects (Nam et al. 2005)

Recent studies indicate that in early pregnancy both GnRH-1 and GnRH-II stimulate the mRNA and protein levels of urokinase-type plasminogen activator (uPA) in human extravillous and cytotrophoblasts and decidual stromal cells *in vitr*o. These findings are suggestive of the regulatory role this hormone plays in proteolytic degradation of the extracellular matrix of the endometrial stroma which is prerequisite for the decidualization and trophoblast invasion (Tabibzadeh and Babakania 1995; Paria et

al. 2002). GnRH-I and GnRH-II have also been shown to suppress the trophoblastic expression of plasminogen activator inhibitor (PAI-I) in a dose and time dependent manner (Cheng and Leung 2005).

Uterus and oviducts

Revelation of the presence of an immunoreactive GnRH in porcine endometrial tissue was in 1993 by Li et al. Expression of GnRH mRNA has also been observed in rat endometrial stromal cells by Ikeda et al. (1996). Imai and co-workers (1994) demonstrated presence of GnRH-R mRNA in normal human endometrium and endometrial carcinoma through RT-PCR and binding assays. In humans, a dynamic expression pattern for GnRH mRNA has been observed in both the endometrium and isolated endometrial cells. These levels are reported to be significantly higher in the luteal phase of the menstrual cycle (Dong et al. 1998 and Raga et al. 1998). GnRH-I immunoreactivity has been noticed in all endometrial cell types. It has been observed that staining patterns are more intense during the luteal phase as compared to follicular phase of menstrual cycle (Raga et al. 1998). GnRH-II immunoreactivity too is reported to be dynamically expressed in stromal and epithelial cells such that stronger signals are detected in the early and mid secretory phases than in the proliferative and the late secretory phases (Cheon et al. 2001) The presence of GnRH mRNA and protein expression in human fallopian tube had also been elucidated by using RT-PCR and immunohistochemical techniques, during the luteal phase of the menstrual cycle (Casan et al. 2000). Recently in our laboratory, through immunohistochemistry and RT-PCR

techniques, the presence of GnRH-R protein and mRNA in bovine endometrial and oviductal epithelial cells was elucidated (Singh et al. 2008).

Placenta

It has been shown that both GnRH-I and GnRH-II mRNA are expressed in first trimester by the human placenta whereas GnRH-I is expressed by full term human placenta. Immunohistochemistry revealed that both forms localize in mononucleate villi and in distinct subpopulations of the extravillous cytotrophoblast. GnRH-I was also observed in outer multinucleated syncyttiotrophoblast layer and allowed differentiation and fusion *in vitro* in cytotrophoblast cultures (Cheng and Leung 2005). In 1995 Lin and co-workers reported the GnRH-R transcripts in human placenta through in situ hybridization assays. GnRH-R expression was detected in cytotrophoblasts and syncytiotrophoblasts. The signals were abundant at 6 weeks gestation, peaked at 9 weeks, begin to decline in the third trimester and undetectable by term according to this assay (Lin et al. 1995). This observation was in agreement with previous binding studies by Bramley et al. (1994). In contradiction, Wolfhart et al (19980 demonstrated the presence of GnRH-R mRNA in human trophoblasts throughout gestation, using in situ RT-PCR.

Breast

Researchers have shown the presence of GnRH-R immunoreactivity and mRNA having a sequence identical to pituitary receptor in both normal and carcinogenic human breast tissues (Kakkar et al. 1994; Kottler et al. 1997; Moriya et al. 2001). Specific ligand binding sites have also been shown to be present in MCF-7 mammary cancer cell lines

expressing two different binding sites, one specific for GnRH-I having high affinity and the other one for GnRH antagonists (Eidne et al. 1985; Segal et al. 1992). However, mixed opinions seem to exist regarding the presence of GnRH-GnRH-R system and its autocrine or paracrine role in the mammary system, as GnRH mRNA were detected in the mammary gland of pregnant and lactating rats, but not in that of immature rats (Palmon et al. 1994; Ikeda et al. 1995). Due to its expression in pregnancy and lactation, it was hypothesized that GnRH expression might be regulated by prolactin (Palmon et al. 1994). The presence of biologically active GnRH peptides in supraphysiological amounts in milk suggests that the possible target of mammary GnRH may be the offspring (Gore 2002).

1.7 GnRH ANALOGUES

1.7.1 GnRH agonists

The short half-life of GnRH in the general circulation, revealed its multifunctional properties and knowledge on peptide sequence, has led to the discovery of synthetic GnRH agonist (GnRHa). Thereby, analogues with greater stability against enzymatic degradation, increased receptor affinity and higher biological potency have been synthesized. In the beginning, GnRHa had been used for the treatment of hypogonadism due to insufficient endogenous secretion of GnRH. Later on the multidimensional properties such as anti-reproductive effects of GnRH analogues and their potential applications in reproductive physiology and medicine, was quickly realized.

A fundamental feature of agonists is the substitution of L-isomers with D-isomers Substitution with a D-amino acid at position 6 increases the half-life of GnRHa in circulation. Removal of the amino terminal glycine increases affinity for the GnRH-R (Karten and River 1986). GnRH agonists, currently in use for clinical or experimental purposes in human and bovine medicine are: gonadorelin (native-like GnRH; gonadorelin diacetate tetra hydrate or gonadorelin hydrochloride), buserelin (D-serine at position 6 and ethyl amide at position 10), fertirelin acetate (ethyl amide at position 10), deslorelin (D-tryptophan at position 6 and ethyl amide at position 10), lupron (D- leucin at position 6), zoladex (D- ser at position 6), Supprelin (D histidine at position 6) Synarel (D- val at position 6) and triptorelin (D-tryptophan at position 6) (Miller et al. 2004). In veterinary medicine the most widely used compounds are the natural decapeptide and buserelin (Peters 2005).

1.7.2 GnRH antagonists

Essential features required of antagonists are a high affinity for the GnRH receptor, but without activation. Low histaminergic properties and resistance to enzymatic degradation are also preferred (Karten and Rivier1986).

GnRH antagonists currently in use in clinical practice are cetorelix and ganirelix. These compounds have substitution at position 1 to 3 with same and different amino acid at position 6 and 10 for cetorelix and position 6, 8 and 10 for ganirelix. The development of no peptide GnRH antagonists has seen intense endeavors from the pharmaceutical industry. The first described no peptide GnRH antagonist is a fused tetra cyclic benzodiazepine that blocks ovulation in rats when given at a dose of 0.5 mg/kg. The

antifungal drug ketoconazole (Nizoral, Janssen Pharmaceutical, Beerse, Belgium) binds and inhibits the rat pituitary GnRH receptor (Miller et al. 2004).

1.8 APPLICATIONS OF GnRH AND GnRH ANALOGUES IN REPRODUCTIVE MANAGEMENT IN BOVINE

1.8.1 Prevention of early embryo mortality

Approximately 25% of bovine embryos are lost during first 3 weeks of life. Untimely luteolysis is a major cause for it, as maintenance of progesterone levels by a viable corpus luteum is very important for the establishment of early pregnancy (Peters 1996 2005). It has been shown that heifers treated with GnRHa from day 4 to 6 of estrous cycle can ovulate and develop an accessory CL which may be a contributory factor for increased P_4 levels in plasma (Schmitt et al. 1996; Rajamahendran and Siangama 1992; Rajamahendran et al. 1998). The use of a single injection of GnRH as a "holding" injection on the day of insemination has been shown to improve overall pregnancy rates, and more so in repeat breeders (Peters 2005). The rationale behind it could be that it induces ovulation at an appropriate time relative to insemination and also stimulate luteinisation, increasing the chance for successful fertilization and embryo survival. Post insemination administration of GnRHa in early estrous cycle seems to enhance the likelihood of conception, pregnancy recognition and embryo survival, through increased plasma progesterone concentration (Ullah et al., 1996). A number of studies have been conducted to determine whether GnRH administration at the time of insemination can improve pregnancy rates in bovines. In general, GnRH doses used were 100 µg for native GnRH and 8-10 µg for its synthetic agonist, buserelin. There are instances when these

treatments have also been carried out during the luteal phase of the estrus cycle and the main intent behind these experiments was to enhance luteal function for sustaining the embryos. Majority of these experiments indicate increased P_4 production after GnRH administrations, whereas some of these have also demonstrated increased pregnancy rates. Mee et al. (1990) and Stevenson et al. (1990) observed an overall improvement at first service and the repeat breeders to the tune of 6% and 7% respectively in a total of around 14,000 cows studied with GnRH administration at estrus, whereas, Mcmillan et al. (1986) report that GnRH treatments between day 11 and 13 of estrous cycle resulted in improved pregnancy rates. This stage (11-14 days after insemination) is significant as it coincides approximately with maternal recognition of pregnancy, characterized by the embryonic secretion of the antiluteolitic factor, interferon τ. Thus, according to these studies it can be apparent that administration of GnRH/agonists at the time of artificial insemination or during the luteal phase increases pregnancy rates. Drew and Peters 1994 and Peters 2005, report that the lower the background fertility in the herd, the higher the percentage improvement but, the physiological and environmental variables affecting fertility is still to be assessed properly. Peters et al. (2000) carried out a meta analysis on the published data on the use of GnRH and its analogues administered between day 11 and day 14 after first insemination and concluded that response to GnRH treatment in terms of pregnancy outcome varies with cow type (beef or dairy), parity (cow or heifer), use of estrus synchronization (Synchronized or natural), pregnancy diagnosis (method and timing) and effect of individual study.

Therefore, it is imperative to study the effect of GnRHa on fertility in laboratories where the experimental conditions can be controlled and kept uniform, thus providing a

much better chance to know whether these treatments can really result in increased fertilization and embryo development rates.

1.8.2 Estrus and ovulation synchronization

Estrous cycle can be effectively manipulated for synchronized ovulation through usage of GnRH or its synthetic analogues. "Ovsynch" (Pursley et al. 1995), is one of such recently developed and promising method of estrus synchronization involving GnRH treatment on Day 0, $PGF_{2\alpha}$ on Day 5-7 and second GnRH on Day 7-9 followed by timed insemination and resulting in normal fertility. This method is being widely practiced in bovine reproductive medicine but, due to limited knowledge of the multifunctional roles of GnRH or its analogues in the reproductive tissues, still there is no definite treatment regimen for GnRH or GnRHa in the field of both human and domestic animal reproductive medicine. It has been demonstrated that administration of GnRH during the luteal phase would result in LH release and cause either ovulation or atresia of the dominant follicle (Webb et al. 1992; Peters et al. 1999) depending upon the follicular status. Administration of PG thereafter would result in emergence of a new follicular wave in a synchronized manner. For those cows in late luteal phase, GnRHa administration could delay luteolysis so that the animals are responsive for PG injection along with cows in the earlier phase of estrous cycle (MacMIllan et al. 1985). Experimental studies have shown that low pulse frequency of GnRH supports FSH synthesis and release and it does not increase LH levels but high GnRH pulse frequency results in inhibition of FSH synthesis and release (Vizcarra et al. 1999). Size of the largest ovarian follicle was noticed to be greater in heifers treated with GnRHa (Pursley

et al. 1997; Taponen., 1999 and 2000), and it was associated with increased E_2 concentrations in plasma (Bergfeld et al. 1996; Dufour et al. 1999). A second dose of GnRHa administered 24 h after the prostaglandin injection, seems to cause an LH surge, stopping E_2 secretion in preovulatory follicle (irrespective of the fact whether the follicle has reached maturity or not) (Taponen et al. 1999, 2002, 2003). Peters et al. (1999) have shown that a second injection of GnRH subsequent to PG in "Ovysynch" protocol significantly advances the timing of ovulation and results in a rise in progesterone concentrations.

1.8.3 Induction of ovulation in the post-partum period

Resumption of the ovulatory cycle is important to the re-establishment of pregnancy and it's desirable for ovarian cycles to begin as early as possible after parturition in an economical point of view (Peters 2005). Thus, over the past few decades numerous studies have been conducted using large doses (100-500 µg) of GnRH or equivalent doses of agonists (eg. buserelin10-20 µg) to stimulate ovarian activity in acyclic cows. It has been observed to be more successful in dairy cows (Bulman and Lamming 1978) than in beef animals (Peters 2005). Bracket and Lean (1997) observed a small but significant reduction in days open (2.75 days) and in the number of services per conception with the use of single large dose of GnRH in post partum dairy cows. Later on, the use of ≥100µg of GnRH was regarded un-physiological as preovulatory gonadotropin surge is induced within 30 minutes after the injection irrespective of the follicular status (Foster et al. 1980). Thus, research moved on to the use of prolonged treatment with lower doses to induce more physiological episodic secretion of

endogenous GnRH and gonadotropins to influence follicular development and maturation. Although some success was achieved in inducing ovulation no regimen was found to give consistent results in a high proportion of animals until real time ultrasound studies revealed the occurrence of regular waves of follicular development during post-partum anestrus. Jagger et al. (1987) has shown that the highest response occurred in cows that had the highest etradiol and the lowest FSH concentrations before treatment.

1.8.4 Cystic ovarian condition

Use of GnRH in cystic ovarian disease dates back to late 1970s, but the efficacy of the treatment in resolving cysts and restoring normal fertility is still not well established (Peters 2005). The definition of a cystic follicle is persistence of a follicle like structure (greater than 25 mm diameter) in the absence of corpus luteum for at least 7-10 days. Luteal cysts have thicker walls and have undergone some luteinisation compared to follicular cysts. Real time ultrasonographic procedure has proved to be a useful technique in the diagnosis of ovarian cysts at farm level (Peters 2005). It is assumed that there is an interaction between genetics and environment in the development of cysts and cows are susceptible for the formation of cysts as a result of environmental stressor effects on the neuroendocrine system which may inhibit the GnRH and/or gonadotropin secretions in a way that ovulation is inhibited (Cole et al. 1986). Thus, it is logical to use GnRH in the treatment of cysts.

GnRH treatments in cystic ovarian disease appear to be effective in that it stimulates a new ovulation or luteinisation similar to non-cystic cows (Kesler et al. 1981; Nanda et al. 1989; Hooijer et al. 2001). Cairioli et al. (2002) noticed that response to

GnRH treatment was better and lowered the progesterone contents in cystic fluid. On the other hand, a group of researchers observed that cysts may luteinize, but do not regress with GnRH treatment (Kesler et al. 1981; Jeffcoate and Ayliffe 1995).

1.8.5 Modulate testicular function and reproductive management in male animals

Although GnRH and GnRH analogues are most commonly used to control fertility and reproduction in female animals, they are increasingly used to modulate fertility, behavior and productivity of male animals as well (Adams 2005). The importance of GnRH in the humoral cascade leading to testicular development, maturation and function is indicated by the phenotype of individuals deficient in GnRH or critical components of the intracellular signaling pathways normally triggered by GnRH. A defect in the gene encoding GnRH where affected animals show profound hypogonadism, has been identified in a murine line (Mason et al., 1986). Similarly, aberrant or incomplete migration of GnRH neurons during embryonic development leads to hypogonadism (Tobet et al. 2001). Phenotypic characteristics in this condition include defects in gonadotropin secretion, failure to complete pubertal transition, retarded development of external genetalia, incomplete testicular growth & maturation, cryptorchidism, oligo/azoo- spermia, androgen deficiency, and reduced libido (Mason et al. 1986; Pitteloud et al. 2002). Integrity of the hypothalamic-pituitary connection is essential for testicular maturation and function. A study conducted by Anderson (1977) showed that in bull calves, transaction of infundibular stalk results in arrest of further testicular growth, reduced androgen synthesis and caused blockade of spermatogenesis. Neutralization of endogenous GnRH with specific antibodies has been shown to have

similar effects in domestic animals (Huxsoll et al. 1998; Schanbacher 1982; Bonneau et al. 1994).

It was demonstrated that the level of gonadotropin exposure during the prepubertal period is an important indicator of mature testis size and spermatogenic potential and gonadotropins seem to influence many aspects of spermatogenesis. LH is responsible for stimulating testicular growth and testosterone secretion and thereupon increases spermatogonial proliferation, whereas FSH supports several phases of spermatocyte maturation (Moura and Erickson 1997). These researchers also suggest that the magnitude of basal and GnRH-induced FSH secretion at 2 months of age may be an effective means of identifying bull calves with higher spermatogenic potential and fertility as yearlings. Furthermore, it has also been demonstrated that increased testicular size and sperm production in yearling bulls can be achieved by immunoneutrilization of inhibin, which in turn results in increased serum FSH levels during prepubertal period (Review by Adams 2005). Moura and Erickson (1997) have suggested that gonadotrophin secretion during prepubertal period may be an indicator in predicting reproductive potential at maturity and episodic administration of GnRH for 2 weeks in early neonatal life appears to have a positive effect on testicular growth and function. Work by Chandolia et al. (1997) also supports this concept that yearling bulls receiving supplemental GnRH as calves had increased testicular growth, spermatogenesis and sertoli cell numbers. On the other hand, immunocastration has been shown to be viable alternative to physical castration in the management of bull calves and active immunization of bull calves against GnRH results in arrest of testicular development, reduced spermatogenesis and testosterone secretion (Adams 2005).

1.9 SPERM FUNCTIONS IN FERTILIZATION AND MEANS OF ACHIEVING THEM *IN VITRO*

1.9.1 Capacitation of sperm

Mammalian sperm although potentially fertile is unable to fertilize an oocyte immediately after ejaculation and requires a period of incubation either in the female genital tract or in an *in vitro* capacitation medium to acquire fertilizing capacity (Yanagimachi 1994; Eisenbach 1999). Removal of decapacitating factors such as cholesterol and other components of the seminal plasma that coat the surface of the sperm in the female reproductive tract or in the *in vitro* capacitation medium triggers many sequential biochemical and physiological changes by which sperm gains the ability to acquire hypermotility, bind to zona pellucida, undergo acrosome reaction ,fuse with and fertilize a mature oocyte (Eisenbach 1999; Abou-Haila and Tulsiani 2000; Baldi et al. 2002; Visconti et al. 2002) which is collectively known as capacitation. The cellular changes that are known to occur include increased cholesterol efflux and increased membrane fluidity increased intracellular HCO_3^-, pH, Ca^{2+}, cAMP and protein tyrosine phosphorilation. The lipid and protein redistribution in the plasma membrane and membrane destabilization during capacitation results in either exposure or hiding of specific receptors and these changes enhance the binding ability of the sperm to its receptors on the ovum (Eisenbach 1999). It has been suggested that the ampula is the main capacitation site. An active capacitating agent in oviductal fluid is thought to be heparin like glycosaminoglycans (Parrish et al. 1988). *In vivo*, the time required for capacitation depends on the etradiol/progesterone ratio. Thus, the duration for capacitation is the shortest at the end of ovulation (Yanagimachi, 1994) and doesn’t occur

in the full luteal phase (Fournier and Thibault 1993). *In vivo* capacitation time of bull sperm have been reported to be 8 hours (Fournier and Thibault 1993). Capacitation is a reversible phenomenon, as capacitated sperm can be decapacited by re-introducing them to seminal plasma.

Discovery of the necessity for sperm capacitation in order to acquire the ability to fertilize an oocyte was in 1951 by Chang & Austin. This was a significant achievement towards the development of IVF, but still the molecular basis of this process remains obscure (Giritharan 2004; Vadnais et al. 2007)). The fact that capacitation *in vitro* can be induced spontaneously in a defined medium without the addition of biological fluids implies that this is intrinsically modulated by the sperm itself in a way that they are programmed to undergo capacitation when incubated in an appropriate medium. However, the capacitation process seems to be tightly controlled by both intrinsic and extrinsic regulators and the regulation of capacitation seem to lie more in the de-repression of inhibitory modulators through the removal of decapacitating factors than in the stimulation of this process (Yanagimachi 1994; Visconti and Kopf 1998).

There are several media constituents that are involved in the enhancement and regulation of the capacitation process. Bovine sperm capacitation can be achieved *in vitro* incubating in physiological temperature in the presence of bicarbonate, calcium, albumin, energy substrates (pyruvate, lactate) in a balanced salt solution and sulphated glycoconjugates such as heparin enhances the process (Parrish et al. 1986; Therien et al. 1995; Harrison 1996; Visconti and Kopf 1998). Caffeine demonstrates a synergistic effect on actions of heparin (Niwa & Oghoda 1988).

The first event in capacitation is cholesterol efflux leading to elevation of intracellular calcium and bicarbonate to activate adenylyl cyclase to produce cyclic adenosine monophosphate (cAMP). This, in turn, activates protein kinase A (PKA) to indirectly phosphorilate certain proteins on tyrosine (Miyamoto and chang 1973; Fraser and dandeker 1973; Bavister 1973; Monks et al. 1986; de Jonge et al. 1991; Breitbart 2003; Hess et al. 2005). Serum albumin acts as a receiving agent for sterols of the sperm plasma membrane and albumin can be replaced by more efficient cholesterol binding proteins such as high density lipoproteins present in oviductal or follicular fluid (Fournier and Thibault 1993; Therien and Manjunath 1996). Mechanism of action of heparin/heparin like glycosaminoglycans in sperm capacitation is suggested to be by binding to and thus removing sperm plasma membrane adsorbed capacitation inhibiting seminal plasma proteins (Fournier and Thibault 1993; Therien et al. 1995). Also, heparin increases cAMP synthesis (Parrish et al. 1994), elevates pH and regulates changes in protein tyrosine phosphorilation (Galantino-Holmer et al. 1997).

Activation of PKA during capacitation also results in the activation of phospholipase D (PLD) which in turn stimulates filamentous actin (f actin) polymerization (Cohen et al. 2004) and there's also an in crease in protein tyrosine phosphorilation dependent actin polymerization and in the membrane bound phospholipase C (PLC).

Among numerous events occurring during capacitation, regulation of intracellular Ca^{2+} is suggested to be one of the most important. The calcium influx due to increased permeability to calcium is reported to occur in a biphasic manner with an initial small elevation during capacitation followed by a much larger elevation around the time of the

acrosome reaction (Das Gupta et al. 1993; Adeoya and Fraser 1993;Parrish et al. 1999; Ladium-Avarenga et al. 2004) and initial influx is thought to be utilized to fill the intracellular calcium stores in the acrosome (Parrish et al. 1999; Dragileva et al, 1999). Voltage-gauged calcium channels, calcium-ATPase inactivation and the Na-Ca pump have been postulated to result in this Ca^{2+} influx (Florman etal. 1992; Vadnais et al. 2007). Calcium has been shown to have a role in acquiring hypermotility in mouse sperm (Suarez and Ho 2003). However, *in vitro* capacitation of bovine sperm can be induced by inclusion of divalent cationic ionophores such as calcium ionophore (A23187) and ionomycin. Both dilamyl-phosphatidylcholine(pC-12), thapsigargin (an inhibitor of acrosomal Ca2+-ATPase) and Angiotensin II experimentally(Parrish et al. 1999; Dragileva et al. 1999; Breitbart 2003).

Reactive oxygen species mediated signaling pathways are also proposed to have a role in capacitation as direct addition of hydrogen peroxide at low concentrations is shown to promote capacitation which could be blocked by inclusion of catalase (Aitken et al. 1998; Banfi et al. 2001).

1.9.2 Sperm-zona binding

After penetrating the cumulus oophorus of the ovum, the sperm bind to the zona pellucida (ZP). Zona pellucida of the oocyte is an extra-cellular matrix of mainly three highly conserved glycoproteins named ZP1, ZP2 and ZP3. The ligand, which binds to sperm receptors is suggested to be O-linked carbohydrates of the glycoprotein ZP3 (Wassarman 1999) and it has been identified as the natural agonist which triggers the signal transduction pathways leading to AR (Tulsiani et al. 1998). The changes in the

structure of O-linked oligosccharides in ZP3 are responsible for the species specificity in sperm –zona binding (Wassarman 1999). At least one receptor on the sperm head is believed to be a tyrosine kinase. Sperm binding causes further activation of cAMP/PKA and PKC respectively leading to higher elevation in cytosolic Ca resulting in f actin dispersion which enable the outer acrosomal and plasma membrane to fuse to progress to the completion of the acrosome reaction (Breitbart 2003).

Sperm zona binding assays or oocyte penetration assays have been reported as predictors of fertility (Bosquet et al. 1983; Graham and Foote 1987a, 1987b; Wheeler and Seidal, 1987; Boatman et al. 1988 Fazeli et al. 1993, 1995). Thus, factors that enhance or inhibit sperm zona pellucida binding could alter the probability of conception (Morales 1998).

1.9.3 Sperm acrosome reaction (AR)

The acrosome reaction is fusion and fenestration of the outer acrosomal membrane with the plasma membrane leading to exocytosis of the acrosomal contents. This reaction is a result of capacitation followed by signal transduction pathways initiated by carbohydrate mediated sperm binding to the zona pellucida of an oocyte or suitable ligand (Yanagimachi 1994; Baldi et al. 2002 Breibart 2002; Lukoseviciute 2007) and this result in the release of trypsin- like acrosin and other enzymes such as acid glycohydrolases, proteinases, phosphatases, estarases and aryl sulfatases and also the exposure of new membrane domains, both of which are essential for the successful fertilization process (Allen and Green 1997; Tulsiani et al. 1998). Migration of proteins in the sperm head plasma membrane during capacitation is said to be a preliminary to

apposed membrane fusion and outer acrosomal membrane (Fournier and Thibault 1993). In human sperm ,substrates of tyrosine phosphorilation such as valosin-containing protein(VCP) and a homolog of Snare-interacting protein NSF has been proposed to mediate acrosomal exocytosis as they have been detected to move from principal piece of sperm to the head region during capacitation (Ficarro et al. 2003)

Progesterone, follicular fluid, prostaglandins, glycosaminoglycans and neoglyoprpteins have been shown to induce the AR *in vitro* (Abou Haila and Tulsiani 2000; Lukoseviciute 2007). And also a definite relationship has been reported between fertility and the ability of sperm to acrosome react under the influence of heparin, calcium ionophore A23187 and lysophosphatidylcholine (Ax et al. 1985; Parrish et al. 1985a 1985b, 1985c; Ax and Lenz 1986; Graham and Foote 1987a, 1987b; Whitfield and Parkinson 1992).

1.10 MATURATION OF OOCYTES

Mammalian oocytes are arrested at the diplotene stage of the first meiotic prophase during prenatal life. The oocyte remains in meiotic arrest for years until the meiotic process is resumed by the preovulatory surge of LH (Hyttel et al. 1997) or removal from the follicle (Pincus and Enzmann 1935). Oocyte maturation refers to the processes occurring as it progresses from the diplotene stage of the first meiotic division to the metaphase stage of the second meiotic division with the formation of the first polar body. This is a complex phenomenon during which immature oocytes undergo nuclear maturation and biochemical changes in the ooplasm, known as cytoplasmic maturation, which renders them fertilizable.

During nuclear maturation the nuclear membrane starts to fold, the nuclear pores disappear and then the nuclear membrane fragments, before rapidly disappearing to leave only small sacs with double walls (Kubelka et al. 1988; Szollosi et al. 1972). These events are known as germinal vesicle breakdown (GVBD), which is the first visible sign of meiotic resumption. The nucleolus disappears rapidly after coming in contact with the cytoplasm. In cattle, GVBD occurs within hours after removal from the follicle or the ovulatory LH surge. In cattle, by 6.6 h of culture, 50% of the oocytes had undergone GVBD (De Loos et al. 1994; Sirard et al. 1989). Then, the chromosomes condense further. The kinetochores appear and the microtubules pull the chromosomes and form the metaphasic plate of MI. The separation of the homologous chromosomes and the migration of the chromosomes to their respective poles take place during anaphase I. During telophase I, the chromosomes found at each pole are surrounded by a nuclear membrane and the oocyte undergoes asymmetric cytokinesis and extrudes the first polar body. The second meiotic division without chromosome replication takes place immediately and the oocyte reaches the metaphase II (Kubelka et al. 1988). The oocyte remains arrested at the metaphase II stage until fertilization takes place, but the completion of nuclear maturation alone does not guarantee subsequent embryo development (Sirard et al. 1989; Yang et al. 1998). A period of approximately 24 h is necessary for a bovine oocyte to complete nuclear maturation *in vitro* (Sirard et al. 1989). It appears that nuclear maturation follows the same pattern *in vivo* and *in vitro* (Hyttel et al. 1986b). Nuclear maturation also involves changes in protein synthesis patterns (Hunter and Moor 1987).

The ability of the oocyte to complete meiosis is known as meiotic competence. Meiotic competence is acquired gradually during follicular growth. Oocytes first acquire the capacity to undergo GVBD and chromosome condensation, then further follicular development is required to acquire the ability to progress to the metaphase I (Tsafriri and Channing 1975) and finally they acquire the ability to reach metaphase II (Sorensen and Wassarman 1976).Thus, meiotic competence is closely correlated with oocyte size, which in turn is correlated with follicle size (Armstrong 2001). The size of the antral follicle at which the oocyte acquires meiotic competence is species-specific (Wickramasinghe and Albertini 1993). Bovine oocytes acquire the ability to complete GBVD and meiosis by the time the antral follicle reaches 2-3 mm in diameter (Fair et al. 1995; Lonergan et al. 1994b; Motlik and Fulk 1986). Meiotic competence is also related to oocyte diameter, since bovine oocytes must have a diameter of 110 µm to complete nuclear maturation to the MII stage (Fair et al. 1995; Otoi et al. 1997).Bovine oocytes with an inside-zona diameter smaller than 95 µm are unable to resume meiosis *in vitro*. A high proportion of bovine oocytes are able to resume meiosis to the MI stage once the oocyte diameter is at least 100 µm (Fair et al. 1995; Otoi et al. 1997). However, the oocyte must measure 110 µm or more to reach the MII stage (Fair et al., 1995). The ability to develop to the blastocyst stage *in vitro* increases with oocyte growth (Arlotto et al. 1996; Fair et al. 1995; Harada et al. 1997). Cleavage and blastocyst rates increased in parallel with meiotic competence and significantly higher developmental rates have been obtained when the diameter of fertilized oocytes is greater than 120 µm (Hazeleger et al. 1995). The acquisition of full meiotic competence coincides with the reduction of the nucleolar transcriptional activity in bovine oocytes (Hyttel et al. 1997; Motlik et al. 1984). The

developmental potential is said to be similar in oocytes originating from non-atretic and early atretic follicles (Blondin et al. 1996a; Blondin and Sirard 1995; Fair et al. 1995). Once the oocyte becomes meiotically competent, inhibitory factors are necessary to maintain the oocyte in meiotic arrest.

Cytoplasmic maturation describes ultra structural changes that take place in the oocyte from the GV to the MII stage and the acquisition of developmental competence of the oocyte (Calarco 1995; Ducibella et al. 1994; Duranthon and Renard 2001; Hyttel et al. 1986a; Hyttel et al. 1986b; Shamsuddin et al. 1993). Cytoplasmic maturation is indirectly assessed as the ability of the mature oocyte to undergo normal fertilization, cleavage and blastocyst development. Other indirect morphological parameters taken into account to evaluate cytoplasmic maturation include cumulus cell expansion, expulsion of the polar body and an increased perivitelline space (Kruip et al. 1983).Ultra structural changes that take place during oocyte maturation include changes in location of the germinal vesicle and mitochondria. The germinal vesicle is eccentrically located in bovine oocytes with a diameter smaller than 110 µm, whereas the germinal vesicle of oocytes with a diameter greater or equal to 110 µm is located close to the zona pellucida (Fair 1995). Mitochondria are centrally located in oocytes with a diameter smaller than 100 µm (Fair 1995). Mitochondria are located in the periphery of oocytes larger than 110 µm (Fair 1995; Fair et al. 1997). The movement of mitochondria is dependent on the microtubules (Kim et al. 1996). Mitochondria morphology is different according to oocyte diameter. Mitochondria are round when the oocyte has a diameter smaller than 100 µm; oval when the oocyte diameter is between 100 and 110 µm and hooded when the oocyte diameter is 110 µm (Fair 1995; Zamboni 1974). The Golgi apparatus is

responsible for producing the cortical granules and the zona pellucida. The number of Golgi apparatus present in the oocyte increases as the diameter of the follicle increases (Fair, 1995). The change in location of cortical granules is reported to constitute the most obvious ultra structural sign of cytoplasmic maturation. The cortical granules that originate from the Golgi apparatus are originally located in the center of the oocyte, and as the oocyte progresses to the metaphase I stage, the cortical granules translocate to the periphery of the oocyte and become attached to the plasma membrane (Cran 1989). The pattern and location of cortical granules seem especially crucial for normal fertilization (Hyttel et al. 1986a; Hyttel et al. 1986b; Hyttel and Madsen 1987). Nucleolus inactivation too occurs during the growth of the bovine oocyte from about 110 to 120 µm (Fair et al. 1996).

1.10.1 Oocyte maturation *in vitro*

The first major break through for the *in vitro* maturation of oocytes was the discovery by Pincus and Enzmann in 1935 that the release of the immature oocyte from the inhibitory influence of the follicle triggered the resumption of maturation *in vitro* culture media. Another significant finding was the determination of the optimal temperature for maturation of bovine oocytes as 39° C (Lenz et al. 1983). Hitherto, a lot of research has addressed in fine tuning *in vitro* maturation conditions and improving the harvest of competent embryos. As a result, there’s a significant amount of information on various maturation media & factors influencing oocyte maturation.

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) aw well as transforming growth factor - α (TGF- α), epidermal growth factor (EGF) have been shown to enhance cumulus expansion and oocyte fertilizability *in vitro* (Kobayashi 1994). Whereas, activin A, inhibin A (Angelika et al. 1997) and retinol (Livingston et al., 2004) has been shown to increase blastocyst formation and also addition of cysteamine (Oyamada 2004) in to maturation medium is reported to increase GSH synthesis leading to improved fertilizability, developmental competence & cryoressistence.

Interestingly 17-β estradiol, growth hormone releasing hormone and vasoactive intestinal peptide, are found to have no effect on nuclear maturation or cumulus expansion on bovine COCs but retard cytoplasmic maturation as reflected by delayed cortical granule migration (Beker 2002). While the absence or the presence of only a small number of cumulus cells have a negative effect on embryo production (Blondin and Sirard, 1995; Sirard et al. 1988), as denuded oocytes in culture are capable of reaching the MII stage, but they are incapable of undergoing normal fertilization and development (Zhang et al. 1995).

Although oocytes matured *in vitro* and *in vivo* are shown to have similar fertilization and cleavage rates, they clearly differ in their developmental potential (Blondin et al. 1996a; Sirard and Blondin 1996). Differences in development between *in vivo* and *in vitro* cultured bovine oocytes are expressed 4 to 5 days post-fertilization at the morula-blastocyst stage (Blondin et al. 1996a; Hyttel et al. 1997). Only one third of *in vitro* matured oocytes develop to the morula-blastocyst stage regardless of whether they are fertilized *in vivo* (Gordon and Lu 1990) or in vitro (Brackett and Zuelke 1993; Dominko and First 1997; Gordon 1994).Even stringent selection procedures do not

always yield oocytes capable of developing to the blastocyst stage after standard *in vitro* maturation/*in vitro* fertilization (IVM/IVF) procedures. It is hypothesized that these oocytes that fail to undergo normal fertilization and development is a result of an incomplete cytoplasmic maturation .Germinal vesicle breakdown also has been shown to occur earlier and meiotic maturation proceeds more rapidly during *in vitro* than *in vivo* maturation (Hyttel et al. 1997). Different patterns of protein synthesis (Kastrop et al. 1991a) and the localization of cortical granules (Szollosi 1967) have also been reported for oocytes matured *in vivo* versus those matured *in vitro*. In addition cumulus cell expansion is shown to be more extensive *in vivo* than *in vitro* (Hendriksen et al. 2000).

1.11 FERTILIZATION

Mammalian fertilization is a complex process in which the sperm and oocyte unite restoring the somatic chromosome number and ultimate development of a new individual exhibiting the characteristics of the species (Yanagimachi 1994; Wassarman 1999). *In vivo* this occurs in the ampulla region of the oviduct. As described earlier both sperm and oocyte should undergo a series of maturational changes before they are able to fuse successfully to form a viable zygote (Visconti and Kopf 1998). The binding of the capacitated sperm with the zona pellucida of the mature oocyte initiates the sperm acrosome reaction, which results in the exocytosis of the acrosomal contents. Thereby with the help of the acrosomal enzymes the sperm crosses the zona pellucida and fuse at the region known as the equatorial segment with the vitelline membrane of the oocyte. At first, the oolemma engulfs the sperm head and subsequently the two membranes fuse.

The vitelline membrane contains microvilli on the surface of the oocyte, except the area overlying the mitotic spindle (Longo and Chen 1984) and in the region with microvilli that sperm prefer to fuse, indicating that microvilli of oocytes and equatorial segment of the sperm consists of molecules involved in sperm oocyte fusion (Yanagimachi 1994). Further studies suggested that substances like glycosylphosphotidylinositol (GPI)-anchored proteins, epididymal protein DE/cystein rich secretory protein-1, fertilin-α ,fertilin-β and cyritestin are involved in sperm oocyte fusion (Choert al. 1998; Miler et al. 2000; Nishimura et al. 2001; Alfieri et al. 2003; Kaji and akudo 2004). This interaction between the sperm and oocyte triggers a series of biochemical events in the oocyte known as 'egg activation', which include an initial transient rise in intracellular Ca^{2+} concentration resulting from release of calcium from intracellular stores (Swann and Parrington 1999) followed by several hours of Ca^{2+} oscillation leading to the induction of cortical granule exocytosis to the perivitelline space and resumption of meiosis (Ben-Yosef and Shalgi 1998). The release of cortical granular enzymes in to the perivitelline space alters the structure of zona pellucida glycoproteins and inactivates ligands for sperm receptors, and thereby zona block to polyspermy is accomplished (Ducibella et al. 1993). The events following lead to ultimate completion of meiosis with the extrusion of the second polar body leaving the egg with a haploid number of chromosomes, thus producing the female pronucleus. The chromatin material derived from the sperm head decondenses, and the male pronucleus is formed. The male and female pronuclei migrate towards each other, and as they move in to close proximity the limiting membranes break down and a spindle is formed on which the chromosomes of the zygote become arranged

.Thus setting the stage for the first cell division immediately (Xu et al. 1994; Schultz and Kopf 1995).

Experimentally transient increase in intracellular Ca^{2+} concentration of mammalian eggs can be achieved by treatment with Ca^{2+} ionophores, ethanol or by induction of IP3 in to the ooplasm (jones and Nixson 2000), but not the Ca^{2+}oscillations.

1.12 EARLY EMBRYONIC DEVELOPMENT

In vivo, ampulla of the oviduct provides the optimum microenvironment for the development of the early embryo, while paracrine and/ autocrine systems mediated by growth factors such as basic fibroblast growth factor(bFGF), transforming growth factor-β_1 (TGF-β_1) and tissue inhibitor for metalloproteinase-1(TIMP-1) and glycoproteins(oviductins) secreted by oviductal epithelial cells contribute to the embryotrophic ability of the oviductal microenvironment (Hoshi, 1996; Martus et al. 1997; Pushpakumara et al. 2002; Kane 2003). *In vitro*, similar environmental conditions are mimicked employing co-culture systems including oviductal epithelial cells, granulosa cells and bovine rat liver cell lines or use of synthetic oviductal fluid (Eyestone and First 1989; Gandolfi 1994). *In vivo* studies have revealed that duration of mitotic divisions for 1-4 cell cycles to be around 32, 13, 14 and 24 h, but the durations for their *in vitro* counterparts have been shown to take 32-34, 9-14, 10-14 and 48-54 h. This reveals a comparatively longer fourth cell cycle resulting in a delay in development after 8-16 cell stage for *in vitro* produced embryos (Gristart et al. 1994; Holm et al. 1998). As embryonic cell division

proceeds past the 8 cell stage the developmental potency gradually declines (Johnson and Ziomek 1981). Compaction starts to occur approximately at the 32 cell stage in both *in vivo* and *in vitro* embryos. After the fifth cell cycle a cluster of apolar cells devoid of microvilli located on the inside of the compacted embryo begin to organize, while outer cells under go changes in the cytoskeleton and differentiate in to trophoectoderm, establishing apical junctional complexes between them (Van Soom et al. 1997; Wiley et al. 1990). Formation of a tight seal around the embryo and the vectorial transportation by the trophoectoderm lead to the accumulation of fluid between cells and progressive coalition forming a central cavity, known as blastocoel, displaces the cluster of inner cells to an eccentric position (Bigger et al. 1988). Thus the inner cell mass and trophoectoderm with distinct functional and morphological characteristics is formed.

In vivo, the developing embryo enters the uterine cavity by 72-84 h after fertilization and *in vitro* by about 110-140 and 135-155 h post insemination the embryo reaches the compact morula and blastocyst stage (Holm et al. 1998). The developmental stage in which bovine genome activates is reported to be the fourth cell cycle (Laurincik et al. 2000) but, some embryonic transcripts are shown to be detectable as early as the first cell cycle (Hay-Schmidt et al. 2001).

1.13 SIGNIFICANCE

Studies so far, clearly show the importance of GnRH and its agonists in bovine reproductive management and medicine. A recent flare of information regarding the presence of extra pituitary GnRH and GnRH-R systems across different mammalian

species and availability of potent and long acting synthetic analogues give forth to the potential applications in the field of mammalian reproduction and in modulation or regulation of certain physiological processes of reproduction. There are evidences suggesting that this hormone acts in an autocrine or paracrine manner in reproductive tissues. The presence of GnRH and GnRH-R system has already been documented in reproductive tissues and early developmental stages of human, porcine and rodent embryos. There are some reports about the presence of GnRH and GnRH-R system in bovine ovarian tissues and ovarian structures and the existence of GnRH receptors in bovine oviductal and endometrial epithelial cells was also elucidated very recently. Existence of this system in bovine oocytes, sperm and early embryonic stages is still to be explored comprehensively. In one report GnRH has been shown to have a positive effect on cleavage rates in bovine *in vitro* fertilization. Therefore, it is still an area of utmost interest which needs to be discovered soon. Hence, we explored the presence of GnRH and GnRH-R system in bovine gametes and early embryos and also whether GnRH agonist could have an effect on sperm and oocyte functions in female reproductive tract in terms of acrosome reaction, sperm zona binding, fertilization and early embryonic development, when these conditions are mimicked in laboratory.

Therefore, the expected outcome from this study will strengthen our knowledge about the presence of this system and the effects of GnRH agonists in bovine reproductive physiology and add to the existing knowledge on the role of GnRH in reproductive biology and mammalian reproduction. Knowledge gained through this study would aid in a meaningful usage of GnRH, or its potent synthetic analogues, in both domestic animal and human reproductive management and in vitro fertilization

programs. This will broaden the basis for future studies with respect to pro-and anti-fertility effects, as well as therapeutic roles, for GnRH or its analogues in the field of reproductive medicine. Thus, the findings of this research would benefit the scientific community, food animal production industry as well as human infertility treatment.

1.14 HYPOTHESIS

I hypothesized that:

i) Bovine sperm express GnRH-R therefore GnRH agonists would have a positive effect on sperm function.

ii) Bovine oocytes express GnRH-R therefore GnRH agonists would have a positive effect on oocyte function.

iii) GnRH agonists would improve oocyte sperm interaction.

iv) Early embryonic stages express GnRH-R.

1.15 OBJECTIVES

In order to test these hypotheses, the following objectives in this study were initiated, namely,

1. To investigate the presence of GnRH-R in bovine:

    1.1 Oocytes

    1.2 Cumulus Oocyte Complexes

    1.3 Sperm

    1.4 Early embryonic stages

2. To investigated the effect of GnRH agonist on

   2.1 Oocyte maturation

   2.2 Sperm function: Sperm Acrosome Reaction

   Sperm Zona Binding

   2.3 Oocyte Sperm interaction: Fertilization

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CHAPTER 2

EFFECT OF GnRH AGONIST ON BOVINE SPERM FUNCTION *IN VITRO*

AND

INVESTIGATION OF GnRH RECEPTORS IN BOVINE SPERM

2.1 INTRODUCTION

Gonadotropin releasing hormone is a key hormone regulating mammalian reproduction. It is now becoming increasingly evident that in addition to the hypothalamo-pituitary GnRH axis, there exists extra-hypothalamic GnRH as well as extra-pituitary GnRH receptors in mammals. It is apparent that GnRH can affect organs other than "traditional" target sites and exerts more specific and localized, regulating roles in autocrine/paracrine manner. With regard to the bovine reproductive system, GnRH-R mRNA has been reported in ovarian follicles, granulosa cells, matured cumulus oocyte complexes, corpora lutea, oviductal epithelium and endometrium (Ramakrishnappa et al. 2005: Singh et al. 2008). In addition, GnRH like molecules have been detected in follicular fluid (Aten et al. 1987; Ireland et al. 1988).

Due to the fact that it is secreted in minute quantities and has a very short half life, it is questionable whether hypothalamic GnRH can be a physiological ligand for extra pituitary receptors. The expression of GnRH–GnRH-R system across different reproductive tissues/cells and current bovine reproductive management practices (such

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as, estrus and ovulation synchronization and induction of post partum ovulation) make it obvious that reproductive tissues and cells are exposed to GnRH/GnRHa (Ambrose et al. 2005; Peters 2005). It is well established that administration of GnRH or GnRH agonist at the time of artificial insemination or 11-14 days after artificial insemination increases pregnancy rates (Peters 2005). Furthermore, *in vitro* fertilization studies by Funston & Seidel (1995) supplementing GnRH and GNRH agonist (GnRHa) have shown to improve cleavage rate in bovines. In human type II GnRH receptor transcripts have been detected in mature sperm and post meiotic testicular cells by in situ hybridization (van Biljon et al. 2002) and GnRH receptor protein had been immunolocalized in the acrosomal region of the sperm by immunohistochemistry (Lee et al. 2000). Long before these discoveries, positive effects of GnRH on human sperm function was elucidated by Morales (1998) by showing that incubating with GnRH increases the binding of sperm to the zona pellucida of ovum and, GnRH receptor expression has been reported in mouse and rat testicular germ cells (Bull et al. 2000).

Neither the presence of GnRH receptor nor effects of GnRH on bovine sperm has been revealed so far. Effects of other reproductive hormones such as progesterone on mammalian sperm function have also been elucidated (Melendrez et al. 1994; Roldan et al. 1994; Kirkman-Brown et al. 2002). In bovine, progesterone is known to enhance sperm-zona binding and acrosome reaction, while estrogen is shown to impair the effect of progesterone on zona binding (Lukoseviciute et al. 2007). Therefore, this *in vitro* study was performed to investigate the presence of GnRH receptors in bovine sperm and to study the direct effect of GnRHa on sperm acrosome reaction and sperm zona pellucida binding, two crucial events in the process of fertilization. A more complete

understanding of this system would enable more meaningful application of GnRH analogues in reproductive management as well as contribute to improve *in vitro* embryo production.

2.2 MATERIALS AND METHODS

2.2.1 Sperm preparation

Semen from a Holstein AI sire was obtained from Westgen, Milner, British Columbia, Canada. Frozen semen straws were thawed in 37^0C water bath for 14s followed by swim-up separation in modified Tyrode's medium (Sp/Hepes-TALP) to obtain the motile fraction of the sperm. The sperm were then washed twice by centrifugation at 500g for 5 minutes. Resulted sperm pellet was diluted in modified Tyrode's medium with 10 μgmL^{-1} heparin and no glucose or hypotaurine (IVF-TALP) to the desired concentrations (Parrish et al 1988).

2.2.2 Recovery of oocytes and *in vitro* maturation

Ovaries were collected from a local slaughter house and brought to the laboratory in 0.9% NaCl supplemented with penicillin-G (100 IU mL^{-1}; Sigma-Aldrich Canada Ltd., Oakville, ON) and streptomycin sulphate (0.2 μgmL^{-1}; Sigma-Aldrich, Canada) at 35-37^0C in a thermos flask. Cumulus oocyte complexes from 2-8mm follicles were collected in to an aspiration medium containing Dulbecco's phosphate buffered saline (DPBS ; GIBCO BRL, ON, Canada), 0.3% bovine serum albumin (BSA; Sigma-Aldrich, Canada) and 50 μgmL^{-1}gentamicin (Sigma-Aldrich, Canada). Oocytes with evenly granulated cytoplasm surrounded by more than three layers of cumulus cells were selected for

maturation. Selected cumulus oocyte complexes were incubated at 38.5^{0}C and 5% CO_2 in a humidified atmosphere for 24h in a maturation medium consisted of tissue culture medium 199 (TCM199; Sigma-Aldrich, Canada), 10 μgmL^{-1}follicle stimulating hormone (Folltropin; Vetrepharm Canada Inc, ON, Canada), 5% super ovulated Cow serum (SCS) and 50 μgmL^{-1}gentamicin.

2.2.3 Sperm acrosome reaction assay

Swim-up separated and washed sperm were diluted to 0.5X 10^6 mL^{-1} in IVF-TALP medium and smeared on teflon coated multi-well slides to asses the percentage of acrosome reaction at 0 h (before incubation) and then sperm samples were treated with buserelin (GnRH-a) at the concentrations of 0, 0.2, 0.4, 0.8 and 1.2 μgmL^{-1} and 1 μgmL^{-1} progesterone (positive control) followed by incubation of 50 μL droplets under mineral oil at 37.5^{0}C in a humidified atmosphere of 5% CO_2 in air for 3 hours. After incubation, sperm samples from each treatment was smeared on teflon coated multi well slides (Fisher Scientific, ON, Canada), air dried and methanol fixed. They were then washed thrice in 0.5% BSA in PBS before incubating with 10 μgmL^{-1} fluorescein isothiocyanate labeled *Pisum sativum* agglutinin (FITC-PSA; Sigma-Aldrich, Canada) in PBS for 45 minutes in a dark chamber. Slides were washed thrice in PBS, mounted in 80% glycerol in PBS and were examined under epifluorescence microscope (excitation filter 450-590 and barrier filter 520) at 400X magnification. Sperm counts were taken on random fields until a total of 100 sperm were counted for each smear. In each field, the number of total sperm was counted under bright field illumination immediately followed by counting the number of FITC-labeled sperm in fluorescent illumination to obtain the acrosome intact

sperm. Sperm with uniform bright fluorescence of acrosome were scored as acrosome intact, while sperm showing patchy/band-like fluorescence and no fluorescence were counted as acrosome reacted (Giritharan et al. 2005). The experiment was repeated six times.

In a follow up experiment (which was repeated six times) sperm samples treated with 0.4 and 0.8 μgmL^{-1} buserelin, which were able to induce acrosome reaction significantly were challenged with different concentrations of antide, a GnRH antagonist.

2.2.4 Sperm-zona binding assay

Matured oocytes with expanded cumulus cells were denuded with 0.1% hyaluronidase (Sigma Aldrich, Canada) in DPBS by vortexing for 3 min. Denuded oocytes (5 each) were placed in 50 µL droplets of in vitro fertilization media with 0, 0.2, 0.4, 0.8 and 1.2 $\mu gmL^{-1}$of buserelin and 1 μgmL^{-1} progesterone (positive control) respectively followed by insemination at a final concentration of $0.3X10^6$ mL^{-1} motile sperm and then incubated at 38.5^0C in a humidified atmosphere at 5% CO_2 covered in mineral oil for 4 h. After incubation sperm oocyte complexes were washed 10 times in DPBS containing 0.5% BSA to remove loosely attached sperm and fixed in 2.5% glutaraldehyde (Sigma-Aldrich, Canada) followed by washing 3 times in 0.5% BSA in DPBS. Sperm oocyte complexes were then incubated in 100 µL drops of HOECHST 33342 stain solution (0.1 $mgmL^{-1}$; Sigma-Aldrich, Canada) in a dark humidifying chamber for 10 min and washed in PBS before mounting on glass slides. Number of sperm attached to zona pellucida were counted under a fluorescent microscope using UV-2A filter combination (excitation filter of 330-380 nm and barrier filter of 420 nm) at

100X and 400X magnification (Giritharan et al. 2005). The experiment was repeated six times.

In a follow up experiment, 0.8 μgmL^{-1} buserelin treatment, which was shown to enhance sperm zona-binding was challenged with 1 μgmL^{-1} antide to determine whether GnRH antagonist could diminish the effect of GnRH agonist on sperm-zona pellucida binding. This experiment was repeated five times.

2.2.5 Investigation of the presence of GnRH receptors in sperm

2.2.5.1 Semi-quantitative RT-PCR to detect GnRH receptor mRNA

Total RNA was extracted from both fresh and frozen semen samples after swim-up separation, using the PicoPure RNA Isolation Kit (Arcturus Bioscience, Inc., CA., USA) according to the manufacturer's instructions. Around $2.5X10^6$ sperm were briefly lysed using 10µl of lysis buffer at 42 ºC for 15 minutes. Extraction was centrifuged (3000 x g for 2 min) and supernatant transferred into a new microcentrifuge tube and equal volume of ethanol was added and the mixture pipetted in to the preconditioned purified column, centrifuged (100 x g for 2min and then 16000 x g for 30 seconds) washed twice with wash buffers and the RNA was extracted by centrifuging (1000 x g for 1 min) with elution buffer (11µl). The quality and quantity of the RNA were assessed by measuring optical densities using Nanodrop ND-1000 spectrophotometer. Reverse transcription (RT) was accomplished by using the commercially available first strand cDNA synthesis kit (Cells to cDNA II kit, Ambion Inc., Austin, TX, USA). RNA samples were DNAse-treated and first-strand cDNA was synthesized by incubation of a 20 µL reaction mixture containing 5-10 µL of cell lysate, 2 µL of random decamers, 4 µL deoxyribonucleoside

triphosphate mixture, 2 µL of X10 RT buffer pH 7.4, RNase inhibitor (0.5 UμL^{-1}), M-MLV reverse transcriptase (0.5 UμL^{-1}) and nuclease free water at 42 °C for 1 h. The reverse transcriptase was inactivated by incubation of the reaction mixture at 94 °C for 10 min (Giritharan et al. 2007). Mature bovine cumulus oocyte complexes served as positive controls for this experiment.

Polymerase chain reaction (PCR) was performed by using Jumpstart RED Taq Ready Mix PCR reaction mix (Jumpstart; Sigma-Aldrich Canada Ltd.) and two sets of gene specific primers for bovine GnRH-R (Funston & Seidel 1995, Ramakrishnappa et al. 2003) and GAPD was used as a house keeping gene and an internal control during the experiment(Giritharan et al. 2007). The primer sequences, annealing temperature, number of cycles and fragment size are given in Table 1. Briefly; gene specific primers, 8.5 µL PCR water and 2 µL of cDNA template were added to 12.5 µL of Jumpstart to make a 25 µL reaction mixture. The PCR products were fractionated on a 2% agarose gel with 0.2µg μL^{-1}ethidium bromide and visualized under ultra violet illumination.

2.2.5.2 Immunostaining

Swim-up separated sperm from fresh and frozen semen samples were diluted to a concentration of 1x 10^6 mL^{-1} and smeared on polylysine coated glass slides, air dried and fixed in -20^0C cold 100% methanol and then examined by both immunocytochemistry and immunofluorescence staining techniques.

For immunofluorescence examination, fixed smears were washed 3 times in PBS and blocked non specific binding by incubating in 5% normal goat serum (NGS) with 0.1% TritonX100 in PBS for 60 min at room temperature. Then, washed thrice in wash

buffer (0.005% Triton X100 and 0.5% NGS in PBS) followed by incubation with primary antibody (ready to use GnRH receptor mouse monoclonal antibody, Thermo Fisher Scientific, Fremont, CA, USA) at 4^0C over night. Four negative control smears were incubated with 1:100 mouse IgG (Millipore, Victoria, Australia). Sections of pituitary (Thermo Fisher Scientific, USA) served as positive control. After rinsing the slides 3 times with PBS, they were incubated with FITC labeled goat anti-mouse antibody at 37^0C for 90min in a dark humidified chamber, washed in PBS, mounted on glass slides and examined under fluorescent microscope.

For immunohistochemical examination DakoCytomation EnVision+System-HRP (DAB) kit (Dako North America, Carpinteria, Ca, USA) was employed. Fixed smears were washed three times with PBS and endogenous peroxidase activity was quenched by incubating for 10min in peroxidase block. Smears were then rinsed three times in PBS followed by incubating in 5% normal goat serum (NGS) with 0.1% TritonX100 in PBS for 60 min at room temperature to block non specific binding. After washing the smears in PBS, they were incubated with GnRH receptor mouse monoclonal antibody (Thermo Fisher Scientific, USA) at 4^0C over night. Instead, the negative controls were incubated in 1:100 mouse IgG (Millipore, Australia). Sections of pituitary (Thermo Fisher Scientific, USA) served as positive controls. Smears were washed in PBS (3 X 5min) followed by incubating with polymer-HRP for 30 min at room temperature in a humidified chamber, washed in PBS (3 X 5min) and incubated for 7min in DAB-chromogen and rinsed in distilled water. Then, they were counter stained with hematoxylin, washed in distilled water and tap water, dehydrated passing through graded alcohol (70%, 80% and 90%), cleared in xylene, mounted and examined under a

microscope for the development of a brown colored precipitate. The staining procedure was repeated 4 times for both fresh and thawed frozen sperm.

2.2.6 Statistical analysis

Data analysis was done by one way ANOVA using JMP statistical software (SAS Institute Inc., USA). Mean separation was done by Turkey-Kramer HSD test. For all experiments, results are reported as mean values for each set of data ± standard error of the means. Differences were considered significant for $P \leq 0.05$.

2.3 RESULTS

2.3.1 Effect of buserelin on sperm acrosome reaction

Percent acrosome reacted was significantly higher ($p<0.001$) in the presence of 0.4 and 0.8 μgmL^{-1} buserelin when compared with the control. Exposure to 0.2 and 1.2 μgmL^{-1} buserelin did not significantly ($p > 0.05$) increase the percent acrosome reacted. Increase in the percentage of acrosome reacted sperm after 3 h incubation in different treatment groups were 24.12 ± 1.20, 29.65 ± 0.87, 36.39 ± 2.16, 48.32 ±1.25, 27.05 ± 0.61 and 34.84 ± 1.14 for 0, 0.2, 0.4, 0.8, 1.2 μgmL^{-1} buserelin and 1 μgmL^{-1} progesterone respectively (Fig.3.1). It was also revealed in the follow up experiment, that antide did not have any effect on AR but could block the stimulatory effect of buserelin (at 0.4 and 0.8 μgmL^{-1}) on sperm acrosome reaction (Fig.3.2).

2.3.2 Effect of buserelin on sperm-zona binding

Buserelin at 0.8 μgmL^{-1} concentration significantly increased ($p<0.01$) the number of sperm bound to zona pellucida. Buserelin at other concentrations tested did not show any significant ($p>0.5$) effect (Fig.3.3). Introduction of 1 μgmL^{-1}antide to the medium did not affect zona-binding ability, but did block the stimulatory effect of buserelin on sperm-zona pellucida binding, suggesting a receptor mediated effect by this GnRH agonist (Fig. 3.4).

2.3.3 GnRH-R mRNA and protein expression

RT-PCR results showed no expression of GnRH receptor mRNA by sperm (Fig.3.5). Neither immunohistochemical staining nor immunofluorescence staining revealed the presence of GnRH receptor proteins on sperm plasma membranes

2.4 DISCUSSION

Acquisition and completion of capacitation, recognition and binding to specific ZP receptors and the induction of AR are critical prerequisites for a successful interaction between sperm and oocyte leading to fertilization (Yanagimachi 1994; Oechninger 2003). The number of sperm binding to the ZP depends on sperm capacitation and the ability of sperm receptors to bind to the receptors in the ZP of the oocyte. Thus, molecules that modify the ability of sperm to bind to the ZP and undergo AR are of physiological significance as they could modify the outcome of fertilization (Morales et al. 2000; Oechninger 2003). The results of this study provide evidence that GnRHa has a direct stimulatory effect on bovine sperm function *in vitro*. The first experiment demonstrated

that GnRHa induces acrosome reaction in bovine sperm *in vitro*. Furthermore, it was also revealed that GnRH antagonist could diminish this stimulatory effect, suggesting a specific effect on bovine sperm. In the second set of experiments, GnRHa appeared to enhance sperm-zona binding ability more than 2 fold (90.02±3.21, 259.6±6.50 in control and 0.8 μgmL^{-1} respectively). Furthermore, it could be blocked by antide at a similar concentration which diminished the effect on AR, further strengthening the point that GnRHa must have acted through their specific receptors on sperm.

Never-the-less, it was not possible to isolate GnRH receptor protein and to detect mRNA expression. Funston and Seidel (1995) also could not detect GnRH receptors on bovine sperm through radioreceptor assays. However, this is different from what has been observed in human sperm, the only other mammalian species in which direct effects of GnRH on sperm functions have been reported so far (Morales 1998; Morales et al. 1999). In contrast to bovine sperm, native GnRH had not been able to induce AR in human sperm *in vitro* but it did enhanced sperm-zona binding (Morales 1998). This could be a species variation as mammalian sperm show unique differences as well as share unique similarities (De Jonge 2005). It is also well known that acrosome reaction occurs less frequently in human sperm than in other animals, even under capacitating conditions (Mallett et al. 1985; Morales 1998). Recently, immunohistochemical staining by Lee et al. (2000) demonstrated the presence of GnRH-R in the acrosomal region of human sperm. However, a more recent and detailed study investigating the expression of type II GnRH-R by different human tissues reveals the detection of transcripts in post-meiotic testicular cells and mature sperm by in situ hybridization (van Biljon et al. 2002). RT-PCR screening by the same group not only confirmed the presence GnRH-R mRNA but

also identified ejaculated sperm as the only source of a potential full length intronless type II GnRH-R gene transcript. Based on their observation and considering other evidence (Miller et al. 2001), it was suggested that GnRH-R type II gene on chromosome 1 in humans might not be a pseudogene after all. It has also been elucidated that putative GnRH binding sites in the human placenta may be significantly different from their pituitary counterparts in several important respects including a 100 fold lower affinity for GnRH agonists compared to the pituitary GnRH receptors (Rama and Rao, 2001). While recent studies involving human placental GnRH receptor has pointed to the presence of a low affinity GnRH in the human placenta that is distinct from the typical high affinity receptors in the pituitary (Belisle et al. 2001) some assays have been not able to detect receptor expression through out various stages of gestation, (Lin et al. 1995) while more sensitive assays have demonstrated the presence of GnRH-R mRNA in human trophoblast through out gestation (Wolfahrt et al.1998). Therefore, we feel it would be premature to conclude that bovine sperm do not express GnRH-R based solely on the observation of the present study. Further experiments should be done to elucidate whether bovine sperm express a different form of GnRH-R than the GnRH-R type I, the form expressed in the pituitary. As both RT-PCR primers and primary antibodies used for immunostaining procedures in this study targeted pituitary GnRH receptor. Presence of a different isoform of the GnRH-R would explain the high concentrations of GnRH analogues required to elicit the effects observed. Mature sperm are also translationally and transcriptionally quiescent and most of the receptors, especially the hormone receptors which have been detected in sperm are reported to be nongenomic (Naz and Sellamuthu 2006). It is difficult to eliminate the fact that presence of GnRH-R mRNA in

very minute quantities did not resulted in failure of detection. Therefore, more sensitive detection methods as real time PCR could therefore be employed in future studies. At the same time it would be interesting to extend my observations to see whether GnRH/GnRHa could act synergistically with ZP receptors to induce AR in the zona bound sperm. Studies are underway in our lab to address these issues.

2.5 CONCLUSION

I conclude that GnRHa enhances bovine sperm function *in vitro*. Although observations suggest a direct receptor mediated effect on sperm, further experiments are necessary to elucidate how GnRHa mediates its action, as we did not detect GnRH receptors on bovine plasma membrane through immunostaining procedures or GnRH receptor mRNA expression through RT-PCR.

ACKNOWLEDGEMENT

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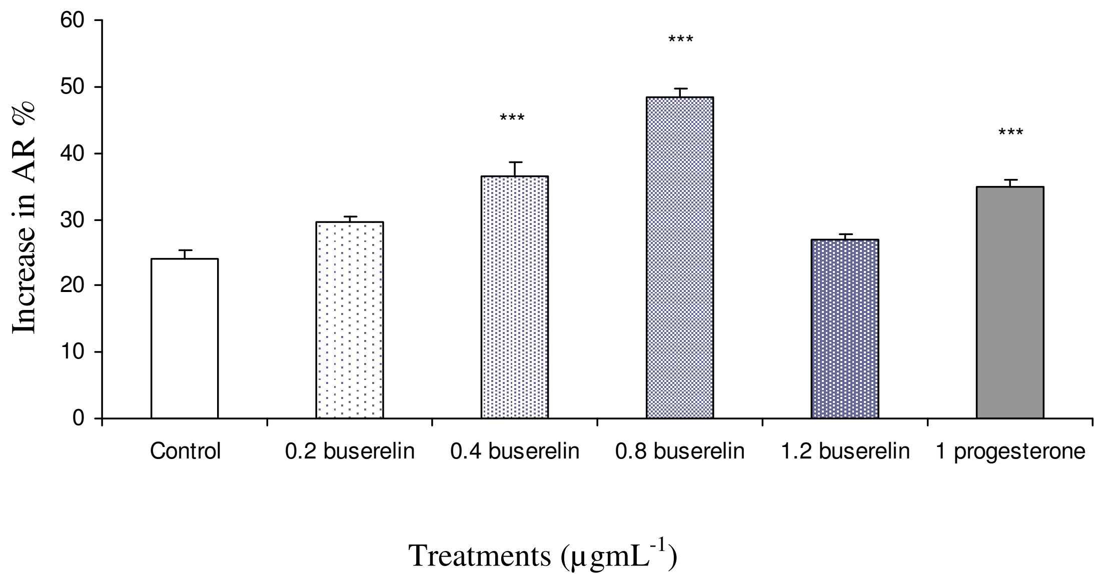


Fig. 2.1. Effect of buserelin (GnRHa) on acrosome reaction of bovine sperm *in vitro*. Data expressed as mean ± SEM. Means are significantly different from the control (***p<0.001)

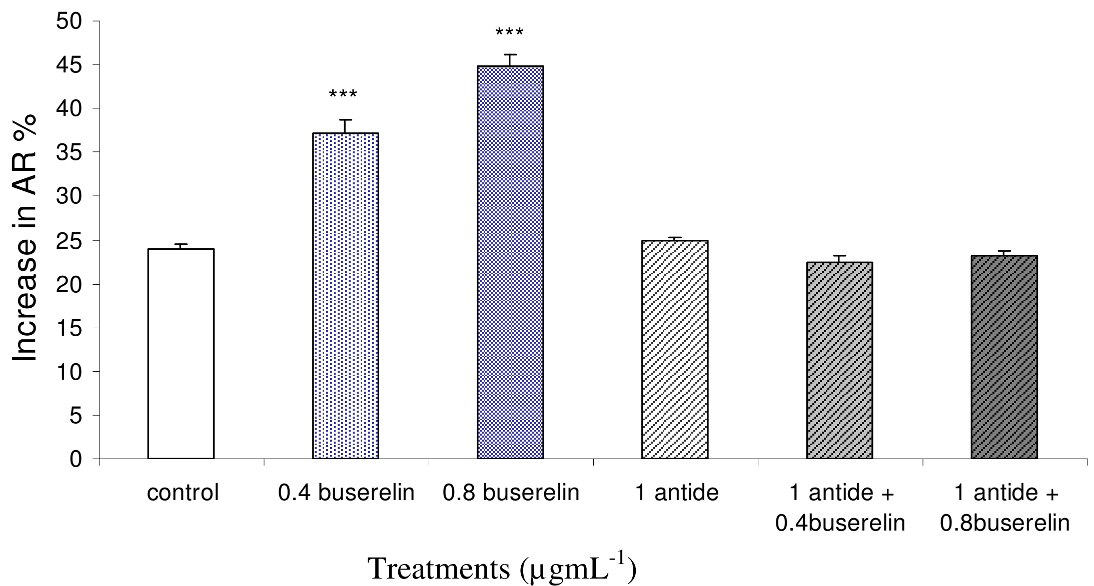


Fig. 2.2. Effect of antide (GnRH antagonist) on the acrosome reaction of bovine sperm *in vitro*. Data expressed as mean ± SEM. Means are significantly different from the control (***p< 0.001)

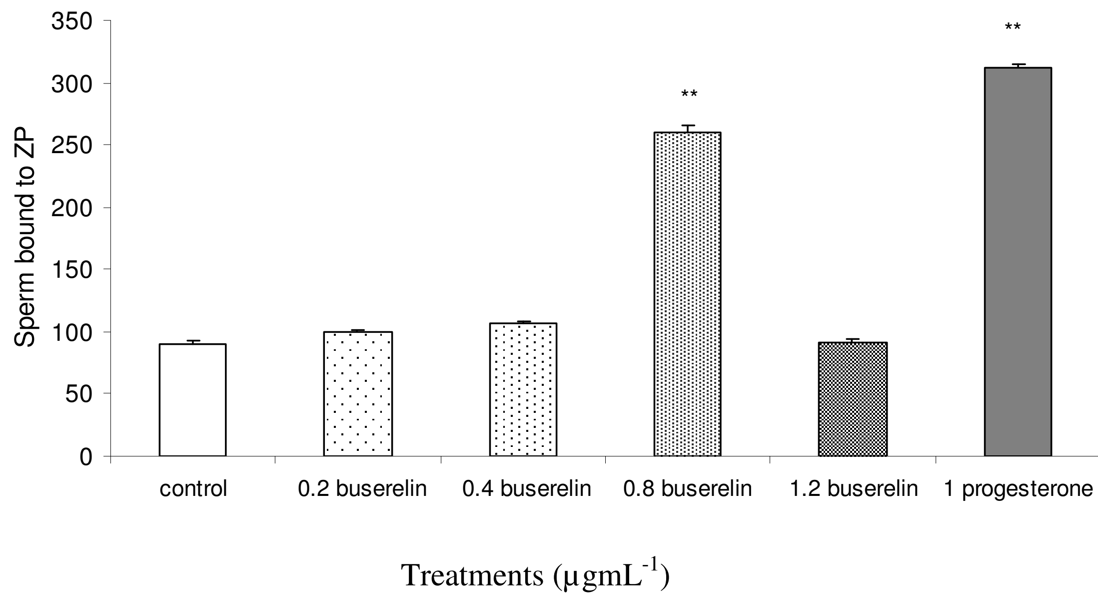


Fig. 2.3. Effect of buserelin (GnRHa) on sperm-zona binding in bovine sperm *in vitro*. Data expressed as mean ± SEM. Means are significantly different from the control (**$p < 0.01$)

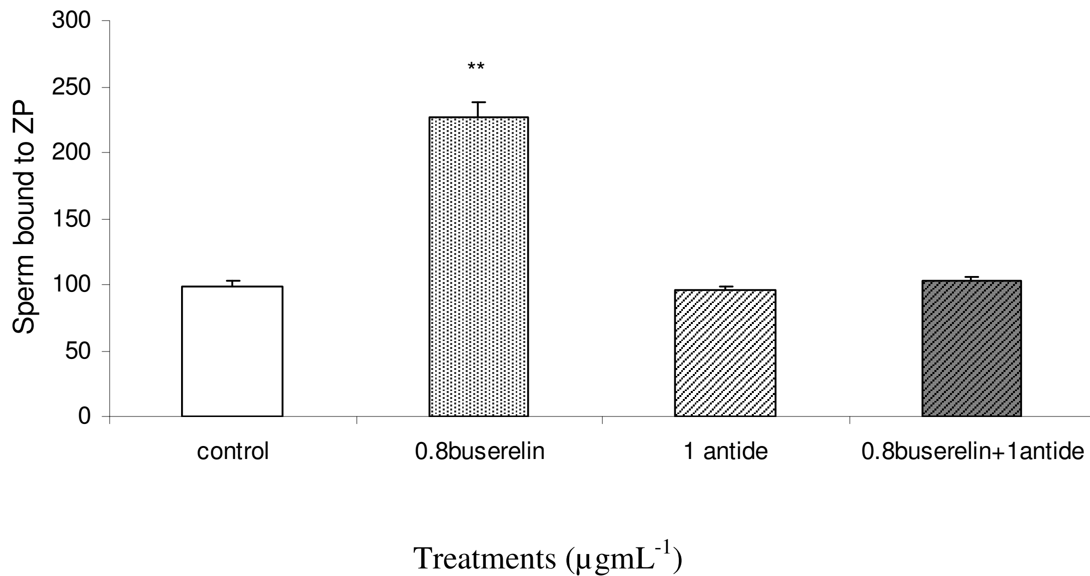


Fig.2. 4. Effect of antide (GnRH antagonist) on sperm-zona binding in bovine sperm *in vitro*. Data expressed as mean ± SEM. Means are significantly different from the control (**$p < 0.01$)

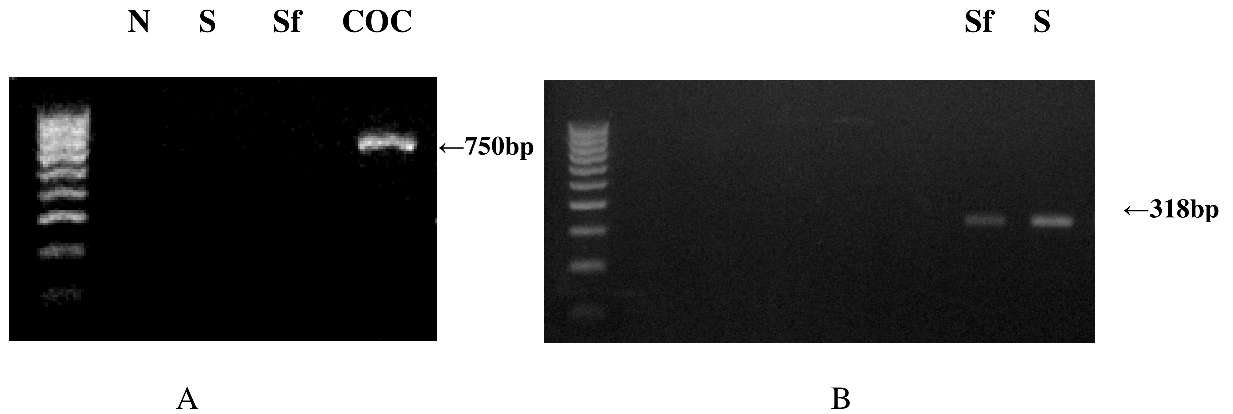


Fig. 2.5. Reverse transcription –polymerase reaction (RT-PCR) amplified products of total RNA isolated from fresh (S), thawed frozen (Sf) bovine semen, cumulus oocyte complexes(COC) and negative control(N) resolved on ethidium bromide stained 2% agarose gel. Photograph (A) shows no expression of GnRH receptor mRNA by both fresh or frozen sperm PCR products and photograph (B) shows expression of GAPD by sperm.

Table 2.1. Details of the gene specific primers used for PCR amplification

Gene symbol	*Primer sequence*	*Anneal T (^oC)*	*PCR cycles*	*Fragment length*
GAPD	5´TGTTCCAGTATGATTCCACCC	58	36	318bp
	3´AGGAGGCATTGCTGACAATC			
1.GnRH-R	5´ GAGTGACAGTTACTTTCTTCC	58	36	920
	3´ GGAAGAAGCGTAACATTACC			
2.GnRH-R	5´ ACTCTGATTGTTATGCCACTG	55	40	750bp
	3´ CCTTTCTTTGACTTTCTATGC			

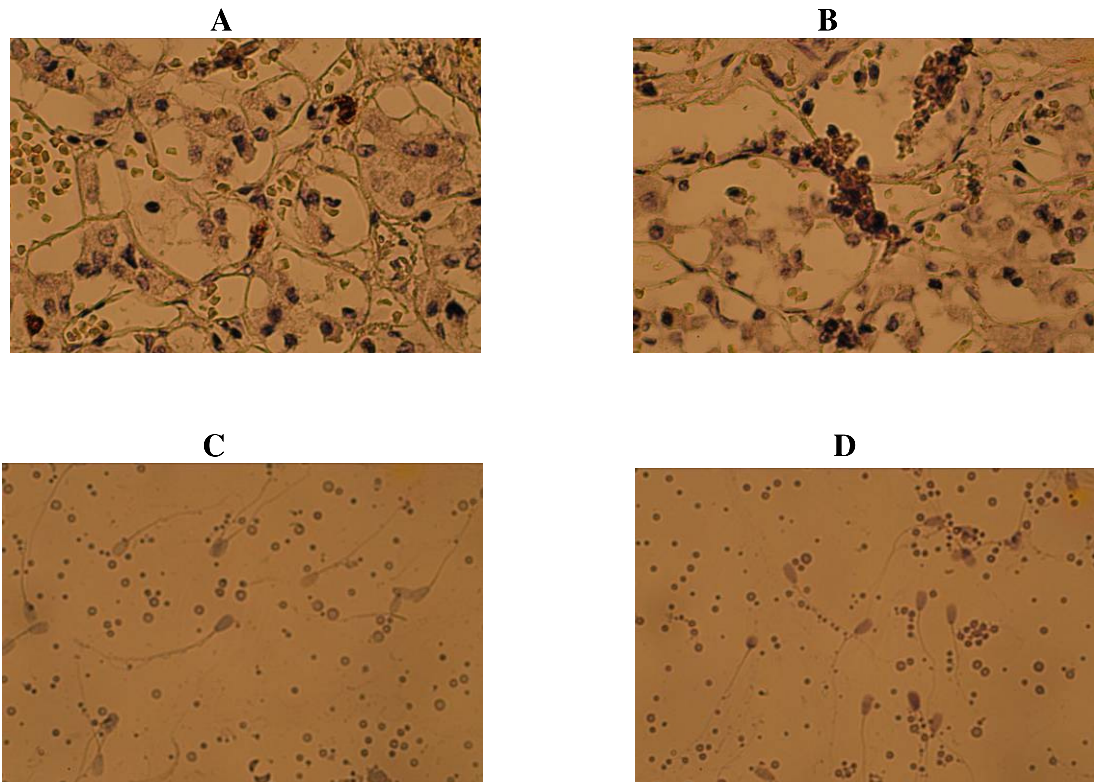
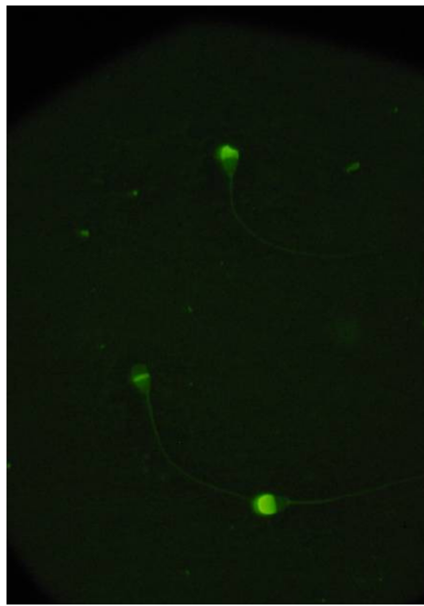
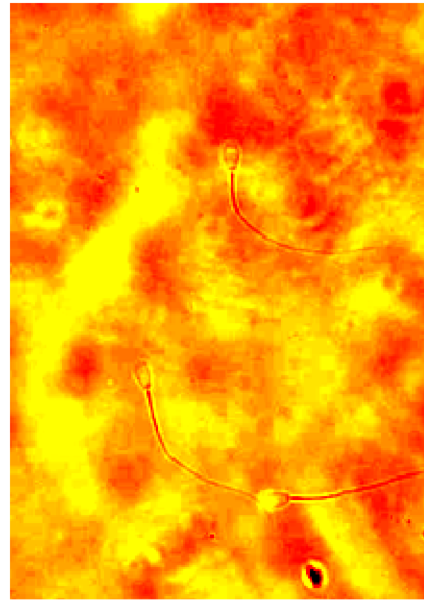


Plate 2.1 Light microscopic images of immunohistochemically stained **A)** sections of pituitary at X100, **B)** pituitary at X400 showing brown colored precipitate (positive control) and sperm showing no development of brown color precipitate upon immunohistochemical staining: **C)** Negative control, **D)** Positive control

Plate 2.2 Pituitary gonadotropes showing green fluorescence upon immunofluorescent staining

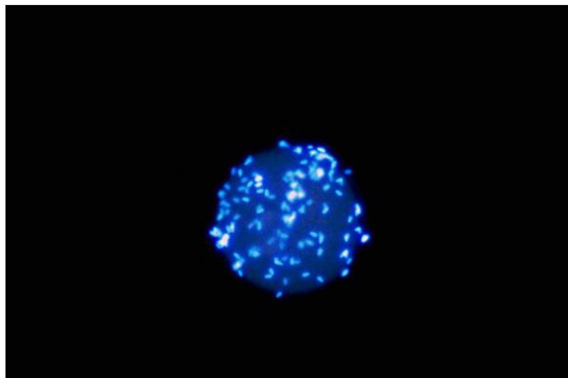


A

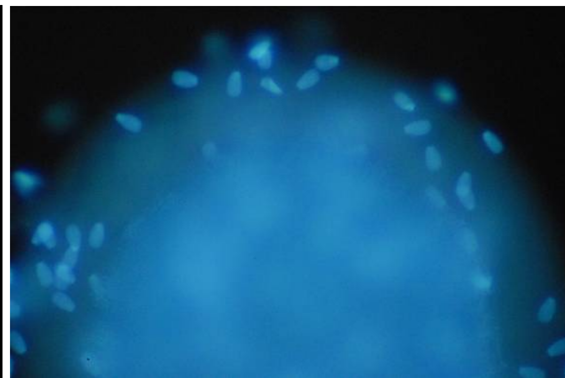


B

Plate 2.3 Fluorescent A) and light microscopic B) images of FITC stained bovine sperm under X400 magnification. Acrosome of sperm shows green fluorescence (excitation filter 450-590 and barrier filter 520). Acrosome intact and acrosome reaction partially completed sperm are seen here.



A



B

Plate 2.4 Sperm bound to zona pellucida of mature oocyte stained with HOECHST 33342. Stained sperm heads **A)** X100 and **B)** X400 show blue fluorescence (excitation filter 330-380m and barrier filter 420nm)

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CHAPTER 3

EFFECT OF GnRH AGONIST ON *IN VITRO* MATURATION OF OOCYTES AND FERTILIZATION AND INVESTIGATION OF GnRH RECEPTORS IN OOCYTES AND EARLY EMBRYOS

3.1 INTRODUCTION

The role of GnRH on regulation of reproduction by stimulating the synthesis and release of LH and FSH by the pituitary is well documented and has been known for many years (Millar 2005). GnRH and its analogues are used extensively used both in therapeutic and agricultural reproductive management practices, especially in cattle (Peters 2005). In recent times, there has been a surge of emerging information regarding its diverse local roles in extra pituitary sites in mammals (Ramakrishnappa et al. 2005). This has attracted more detailed studies in search of its various physiological roles as a better understanding of GnRH-GnRH-R system would lead to optimal application of GnRH and GnRH analogues for reproductive management (Millar 2005).

It has been revealed that GnRH receptors are present in mature cumulus oocyte complexes in cows and that incubation with GnRH increased the cleavage rate in oocytes fertilized *in vitro* (Funston and Seidel 1995). Hillensjo and LeMarie (1980) found that

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GnRH agonists stimulate meiotic maturation in follicle enclosed rat oocytes, and soon after Dekel and coworkers (1988) demonstrated the presence of GnRH receptors rat oocytes. Expression of GnRH receptor mRNA has been demonstrated in murine morula to hatching blastocysts while GnRH agonists enhanced the hatching blastocyst development rate (Raga et al. 1998). GnRH receptors have been shown to present in human preimplantation stage embryos as well (Casan et al. 1999). Recently, embryotrophic effects of GnRH agonists on porcine blastocyst development and expression of GnRH and its receptor by porcine blastocysts have also been studied by Nam and co-workers (2005). In reproductive management it is found that GnRH presents a positive effect on pregnancy rates in farm animals including sows, ewes, mares and cows in clinical trials. In cows, it is suggested that GnRH is effective in increasing pregnancy rates when given either at the time of insemination or 11 to 14 days after (Peters 2005; Ambrose et al. 2005).

This experiment was performed to find out the direct effects of GnRH agonists on maturation of bovine oocytes and *in vitro* fertilization of bovine oocytes. I also investigated the expression of GnRH receptors in bovine oocytes and early embryos.

3.2 MATERIALS AND METHODS

3.2.1 Recovery of oocytes and *in vitro* maturation

Bovine ovaries were collected (immediately after slaughter) from a local slaughter house and transported to the laboratory within 2 h in normal saline (0.9 NaCl) supplemented with penicillin-G (100IU mL^{-1}; Sigma–Aldrich Canada) and streptomycin sulphate (0.2 μgmL^{-1}; Sigma-Aldrich Canada) at 35^0 C in a thermos flask.

Cumulus oocyte complexes from 2-8mm follicles were aspirated using an 18G needle aided with a suction pump into a medium of 0.3% BSA (Sigma –Aldrich Canada) in Dulbecco's phosphate buffered saline (GIBCO BRL, Canada Life Technologies, Burlington, ON) supplemented with 50 μgmL^{-1} gentamycin (Sigma –Aldrich Canada). Good quality oocytes with an evenly granulated cytoplasm and surrounded by more than three layers of cumulus cells were selected for maturation for 24 h at 38.5^0 C in a humidified atmosphere of 5% CO_2 in air. Maturation medium consisted of TCM 199 (Sigma –Aldrich Canada), 10 μgmL^{-1} follicle stimulating hormone (Folltropin: Belleville, ON), 5% super ovulated cow serum and 50 μgmL^{-1} gentamycin (Sigma –Aldrich Canada).

3.2.2 Assessment of *in vitro* maturation rate

The selected oocytes were incubated in groups of 20 (per treatment group) in maturation medium consisting TCM 199 (Sigma–Aldrich Canada), 5% super ovulated cow serum and 50 μgmL^{-1} gentamycin (Sigma –Aldrich Canada). One group was treated with 0.8 μgmL^{-1} buserelin (the concentration which induced sperm functions) and the other with 10 μgmL^{-1} FSH as a positive control (Folltropin: Belleville, ON) while a third group was kept as a negative control. After 24 h of incubation at 38.5^0 C in a humidified atmosphere of 5% CO_2 in the air, oocytes in each group were denuded by vortexing for 3 min in 0.1% Hyaluronidase in DPBS and fixed in 2.5% glutaraldehyde solution for 10 min followed by washing three times in 0.3% BSA in PBS to remove glutaraldehyde. Afterwards, the denuded and fixed oocytes were stained with 10 μgmL^{-1} bisbenzamide stain by incubating for 10 min in a dark humidified chamber at 38.5^0 C, followed by

washing in 0.3% BSA in PBS before being mounted on glass slides (Giritharan, 2004). Stained oocytes were examined under a fluorescent microscope using UV 2A filter (excitation filter 320-380nm & barrier filter 420nm) at 100X to 400X magnification. The percentage of matured oocytes showing a bright blue polar body in the perivitelline space were counted for each treatment group and the percentage of oocytes under gone maturation was calculated.

3.2.3 Preparation of sperm for insemination

Frozen semen from a Holstein AI sire was obtained from Westgen, Milner, British Columbia, Canada. Frozen semen straws were thawed in 37^0C water bath for 14 s followed by swim-up separation in modified Tyrode's medium (Sp/Hepes-TALP) to obtain the motile fraction of the sperm and then washed twice by centrifugation at 500g for 5 min. The resulted sperm pellet was diluted in modified Tyrode's medium with 10 μgmL^{-1} heparin and no glucose or hypotaurine (IVF-TALP) to a concentration of 1x $10^6 mL^{-1}$ for insemination (Parrish et al., 1988).

3.2.4 *In vitro* fertilization and embryo production

In vitro, matured oocytes were washed three times in IVF-TALP before placing them in groups of 20 per IVF droplet (100 µL final volume) under mineral oil and inseminated with 1x $10^6 mL^{-1}$ motile sperm and co-incubated for 18-22 h at 38.5^0 C in a humidified atmosphere of 5% CO_2 in air. Presumptive zygotes were gently vortexed for 40 s to remove cumulus cells and cultured under mineral oil in media consisting TCM 199, 5% SCS, 5 μgmL^{-1} insulin (Sigma–Aldrich Canada) and 50 μgmL^{-1} gentamycin (20

presumptive zygotes/20 µL) at 38.5^0 C in a humidified atmosphere of 5% CO_2 in air. Uncleaved oocytes were removed and culture media was changed every 72 h. Two cell, 4 cell, 8 cell, and blastocysts were collected during 168 h of culture.

3.2.5 *In vitro* fertilization rate

For *in vitro* fertilization assays, one group of *in vitro* matured oocytes were treated with 0.8 µg m L^{-1} buserelin(the concentration which induced sperm functions) while the other was not treated and served as a control group. After 24 h of co-incubation with 1x $10^6 mL^{-1}$ motile sperm at 38.5^0 C, in a humidified atmosphere of 5% CO_2 in air presumptive zygotes were gently vortexed for 40 seconds to remove cumulus cells and they were fixed in 2.5% glutaraldehyde solution for 10 min followed by washing three times in 0.3% BSA in PBS to remove glutaraldehyde. The fixed presumptive zygotes were stained with 10 µgmL^{-1} bisbenzamide stain by incubating for 10 min in a dark humidified chamber at 38.5^0 C followed by washing in 0.3% BSA in PBS before being mounted on glass slides for fluorescent microscopy using UV 2A filter combination (Giritharan, 2007). Oocytes containing 2 bright blue polar bodies and/or 2/more misty blue pronuclei were considered fertilized.

3.2.6 Investigation of GnRH receptors in oocytes, cumulus cells, cumulus oocyte complexes and early embryonic stages

3.2.6.1 Semi-quantitative RT-PCR

Total RNA from pools of embryos, denuded oocytes, cumulus cells and cumulus oocyte complexes were extracted using the PicoPure RNA Isolation Kit (Arcturus

Bioscience, Inc., CA., USA) according to the manufacturer's instructions. Samples were briefly lysed using 10 µL of lysis buffer at 42 ºC for 15 min. Extractions were centrifuged (3000g for 2 min) and the supernatants were transferred into a new microcentrifuge tubes. Equal volume of ethanol was added and the mixture pipetted in to the preconditioned purified column. Columns were then centrifuged (100g for 2 min and then 16000g for 30 s) washed with wash buffers twice and the RNA was extracted by centrifuging (1000g for 1 min) with elution buffer (11µL). The quality and the quantity of the RNA were assessed by measuring optical densities using Nanodrop ND-1000 spectrophotometer and by observing clear bands for 28s and 18s, ribosomal RNA species on ethidium bromide stained agarose gel (1%) respectively. Extracted RNA was stored at -80ºC until processing.

Reverse transcription (RT) was accomplished by using the commercially available first strand cDNA synthesis kit (Cells to cDNA II kit, Ambion Inc., Austin, TX, USA). RNA samples were DNAse-treated and first-strand cDNA was synthesized by incubation of a 20 µL reaction mixture containing 5-10 µL of cell lysate, 2 µL of random decamers, 4 µL deoxyribonucleoside triphosphate mixture, 2 µL of X10 RT buffer pH 7.4, RNase inhibitor (0.5 $U\mu L^{-1}$), M-MLV reverse transcriptase (0.5 $U\mu L^{-1}$) and nuclease free water at 42 °C for 1 h. The reverse transcriptase was inactivated by incubation of reaction mixture at 94 °C for 10 min (Giritharan et al. 2007). Mature bovine cumulus oocyte complexes served as positive controls for this experiment.

The polymerase chain reaction (PCR) was performed by using Jumpstart RED Taq Ready Mix PCR reaction mix (Jumpstart; Sigma-Aldrich Canada Ltd.) and two sets of gene specific primers for bovine GnRH-R (Funston & Seidel 1995,

Ramakrishnappa et al. 2003) and GAPD was used as house keeping gene and internal control during the experiment(Giritharan et al. 2007). The primer sequences, annealing temperature, number of cycles and fragment size are the same as in given in Table 1. Gene specific primers, 8.5 µL PCR water and 2 µL of cDNA template were added to 12.5 µL of Jumpstart to make a 25 µL reaction mixture. The PCR products were fractionated on a 2% agarose gel with 0.2µg μL^{-1}ethidium bromide and visualized under ultraviolet illumination. To compare the GnRH-R mRNA expression in immature and mature COC the optical density of individual bands was analyzed using Scion Image Beta 4.02 software (http://www.scioncorp.com/) and relative optical density was normalized to the house keeping gene, GAPDH.

3.2.6.2 Immunostaining

Cumulus oocyte complexes, oocytes denuded by vortexing for 3 min in 0.1% Hyaluronidase in DPBS and cumulus cells were examined through immunohistochemical examination employing DakoCytomation EnVision+System-HRP (DAB) kit (Dako North America, Carpinteria, Ca, USA). Oocytes, COCs and cumulus cells were fixed in 2% paraformaldehyde (30 min at room temperature) in phosphate buffered saline and washed three times in PBS. Endogenous peroxidase activity was quenched by incubating for 10 min in peroxidase block and then was rinsed three times in PBS followed by incubating in 5% normal goat serum (NGS) with 0.1% TritonX100 in PBS for 60 min at room temperature to block non specific binding. After washing them three times in wash buffer (0.005% TRITON X100 and 0.5% NGS in PBS) oocytes, COCs and cumulus cells were incubated with GnRH receptor mouse monoclonal antibody (Thermo Fisher

Scientific, USA) at 4^0C over night. The negative controls were incubated in 1:100 mouse IgG (Millipore, Australia). Sections of pituitary (Thermo Fisher Scientific, USA) served as positive controls. Afterwards they were washed in PBS (3 X 5 min) followed by incubating with polymer-HRP for 30 min at room temperature in a humidified chamber, washed in PBS (3 X 5 min) and incubated for 7min in DAB-chromogen and rinsed in distilled water, mounted and examined under a light microscope for the development of a brown colored precipitate (Raga et al.1999).

Similarly, cumulus oocyte complexes, oocytes denuded by vortexing for 3 min in 0.1% Hyaluronidase in DPBS and cumulus cells were examined by immunofluorescence staining technique. Oocytes, cumulus cells and COCs were fixed in 2% paraformaldehyde (30 min) in phosphate buffered saline and washed twice in PBS. To block non specific binding, 5% normal goat serum (NGS) with 0.1% TRITONX100 in PBS was applied to these for 30-60 min at room temperature. Then, they were washed 3 times in wash buffer (0.005% TRITON X100 and 0.5% NGS in PBS) followed by incubation with primary antibody specific for GnRH receptor at 4 ^{0}C over night. For control, oocytes/COC/cumulus cells were incubated with mouse IgG. After rinsing twice with PBS, they were incubated with FITC labeled secondary antibody (37^0C for 90 min in a dark chamber). Stained oocytes/ COCs/cumulus were washed in PBS, mounted on glass slides and examined under fluorescent microscope (excitation filter 450-590 and barrier filter 520).

3.2.7 Statistical analysis

Data analysis was done by one way ANOVA using JMP statistical software (SAS Institute Inc., USA). Mean separation was done using the Turkey-Kramer HSD test.

Optical densities of individual bands were analyzed by using computerized densitometry software, Scion Image Beta 4.02 (Scion Corporation, Fredrick, Maryland, USA; http: www. scioncorp.com). Mean levels of relative mRNA expression in immature and mature COCs were compared by student t- test. Differences were considered significant for $P \leq 0.05$.

3.3 RESULTS

3.3.1 Effect of buserelin on maturation of oocytes *in vitro*

The percentage of oocytes that had undergone nuclear maturation after 24 h of incubation in appropriate conditions, were not significantly different ($p > 0.05$) between the different treatment groups. The mean maturation rate of oocytes were 55.14 ± 0.39, 50.69 ± 0.42 and 43.5 ± 0.46 for oocytes treated with FSH, buserelin and negative control respectively (Fig. 4.1).

3.3.2 Effect of buserelin on *in vitro* fertilization

The percentage of oocytes fertilized was not significantly different ($p > 0.05$) between the two groups examined. The mean number of matured oocytes that were successfully fertilized *in vitro* in the buserelin treated group was 55.25 ± 0.89 against 51.25 ± 0. 51 for the control group (Fig. 4.2).

3.3.3 GnRH-R mRNA and protein expression

RT-PCR procedure revealed that bovine cumulus cells and immature and mature cumulus oocyte complexes express GnRH-R mRNA. GnRH-R mRNA expression was not observed (Fig. 4.4) in oocytes alone or early embryonic stages (2/4/8 cell and blastocyst). GnRH receptor mRNA expression by mature COC(2.01±0.12) was observed to be significantly higher ($P < 0.05$) than immature COC (0.89±0.2) (Fig. 4.3).

Immunofluorescence staining and immunocytochemical techniques also gave similar observations of GnRH-R protein expression by cumulus cells, oocytes and COC. Immunocytochemical staining produced brown colored precipitate in cumulus cells and cumulus enclosure of the oocytes in COC, but not on denuded oocytes. Upon immunofluorescence staining pin point green fluorescence was evident on cumulus cells but not on oocytes.

3.4 DISCUSSION

The role of GnRH on maturation of bovine oocytes, on *in vitro* fertilization, and the presence of GnRH receptors on oocytes and early embryos were investigated in this study. Previous studies in bovine are suggestive of the presence of GnRH receptors on bovine oocytes and early embryos and a possible positive role in reproduction (Funston and Seidel 1995). While it was reported to stimulate meiotic maturation of follicle enclosed rat oocytes *in vitro* (Hillensjo & LeMaire 1980), in this study I did not observe a positive effect by GnRH agonist buserelin on *in vitro* maturation of cumulus intact bovine oocytes. While GnRH receptors have been shown to be present in rat oocytes

(Dekel et at 1988), I could not detect receptors for GnRH on cumulus free bovine oocytes through immunostaining, nor could I detect GnRH-R mRNA expression.

My study supported the observation of the presence of GnRH-R mRNA in bovine cumulus oocyte complexes reported by Funston and Seidel (1995). It is well known that cumulus cells communicate metabolically with the oocyte by means of gap junctions and exert a regulatory role on oocyte development (Speroff 2005). It has also been suggested that GnRH may have acted through its receptors on cumulus oocyte complexes to stimulate the cleavage rate they observed in bovine oocytes fertilized *in vitro* (Funston and Seidel 1995). According to this study however, GnRH receptors on cumulus cells do not appear to transduct signals to stimulate the meiotic maturation of the associated oocytes. The increase in GnRH-R mRNA expression in mature COC more than two fold compared to the immature COC does indicate that GnRH may be playing an important role in the fertilization of oocytes. Nevertheless, I did not observe a significant increase in the *in vitro* fertilization rate at 24h post insemination with GnRH agonist treatment. Previous studies on bovine *in vitro* embryo production by Funston and Seidel (1995) also did not demonstrate a positive effect on blastocyst development rate although they did observe an increase in the cleavage rate at 72h post insemination. In contrast, a similar study on porcine *in vitro* embryo production supplementing GnRH agonists reported that it enhances both blastocysts development rate as well as the quality of the resulted blastocysts (Nam et al 2005). Preimplantation embryonic development was also shown to be enhanced by incubation with GnRH agonists in mice (Raga et al 1999). In the above species presence of GnRH-R mRNA has also been detected using RT-PCR. Studies by the same group (Raga & co workers) on human preimplantation embryos have

demonstrated that human preimplantation embryos express GnRH receptor at both the mRNA and protein level (Casan et al 1999). However, we did not detect expression of GnRH receptor mRNA by bovine preimplantation stage embryos. The question of whether the amount of mRNA was not sufficient to be detected does not seem to apply as 18-20 oocytes, 2/4 and 8 cell embryos and 5 blastocyst were used to extract total RNA per experiment and in the study on human (Casan et al 1999) and mouse (Raga et al 1999) preimplantation embryos, GnRH-R mRNA have been demonstrated using single embryos from different developmental stages. However, we can not totally reject the possibility of the presence of a lower affinity binding site or a different isoform, than the typical bovine pituitary GnRH receptor eluded it from being detected as both primers for RT-PCR and primary antibodies for immunostaining were based on the pituitary GnRH receptors. Although we failed to observe a direct positive effect by GnRH agonist on in vitro fertilization in bovine, to rule out the expression of GnRH receptor by bovine oocytes and early embryos beyond all reasonable doubt, it would be appropriate to carry out more sensitive investigations using real time PCR.

3.6 CONCLUSIONS

I conclude that GnRH does not exert a direct effect on the maturation of bovine oocytes *in vitro,* nor does it have a significant positive effect on *in vitro* fertilization of bovine oocytes. This study did not reveal the expression of GnRH-R mRNA by bovine oocytes or preimplantation stage embryos and also presence of GnRH receptor protein was not detected on bovine oocytes.

ACKNOWLEDGEMENTS

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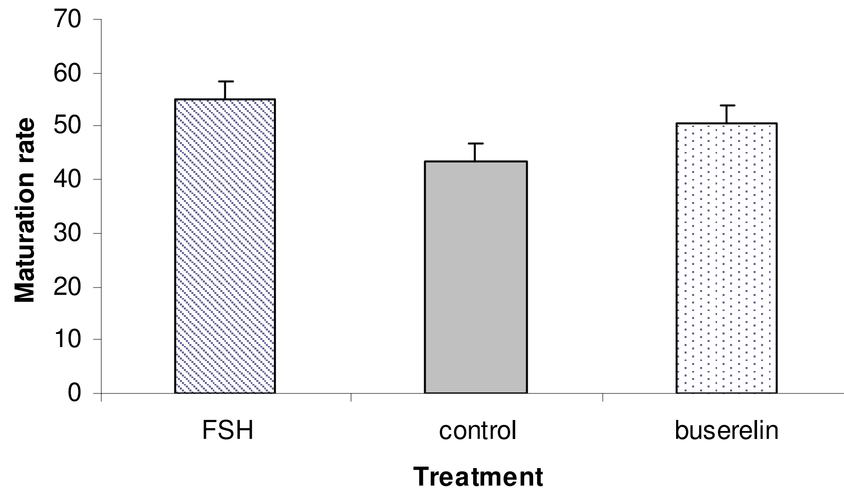


Fig. 3.1. Effect of buserelin (GnRHa) on maturation rate of cumulus enclosed bovine oocytes *in vitro*. Data expressed as mean ± SEM.

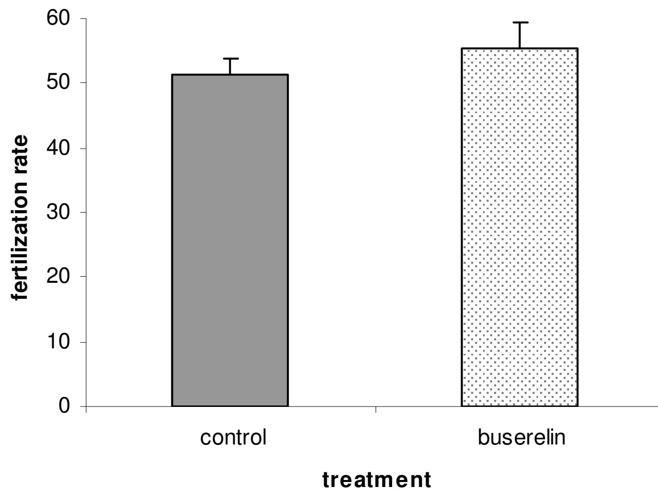


Fig. 3.2. Effect of buserelin (GnRHa) on *in vitro* fertilization rate in bovine. Data expressed as mean ± SEM.

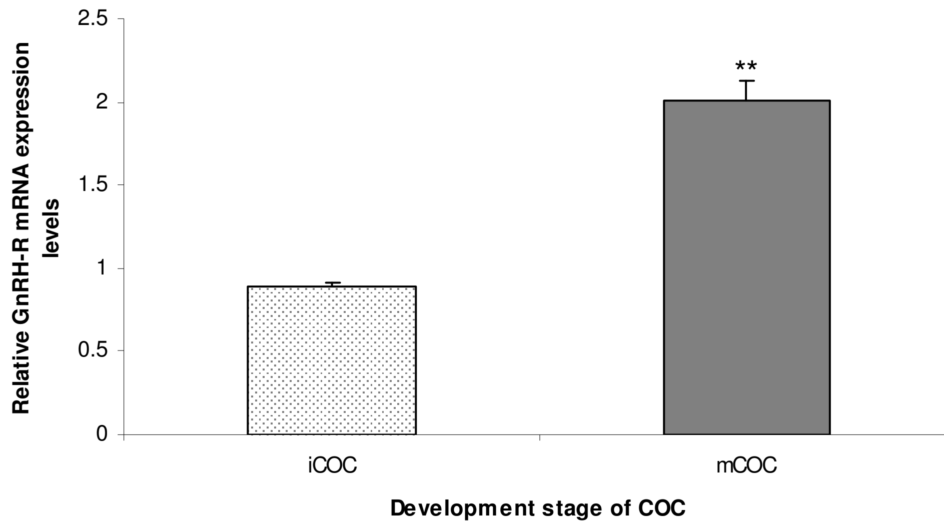


Fig. 3.3. Relative GnRH-R mRNA expression by cumulus oocyte complexes. The mRNA levels were normalized to G3PDH, used as housekeeping gene in the experiments. Value with ** shows statistically significant difference (P≤ 0.01).

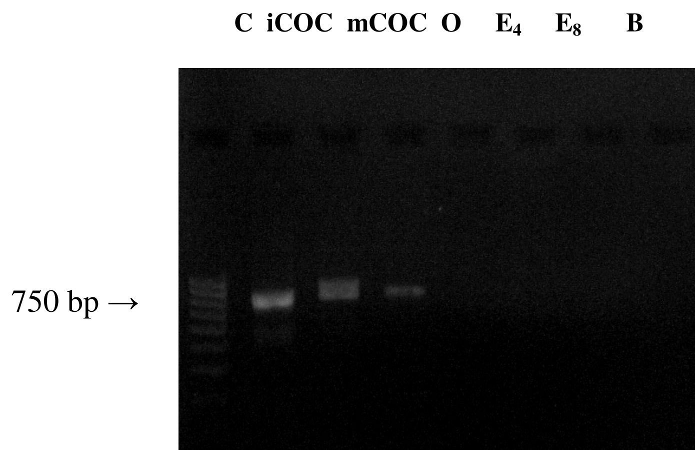
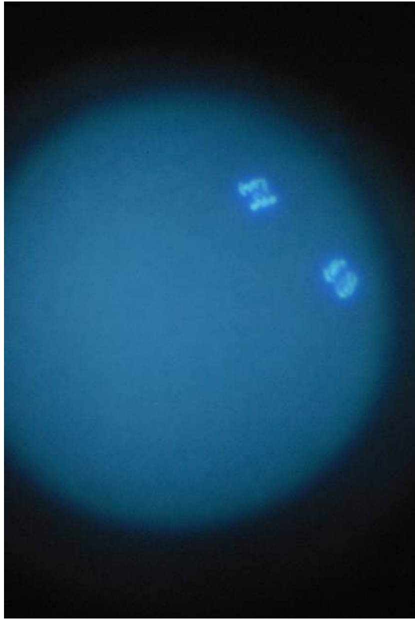
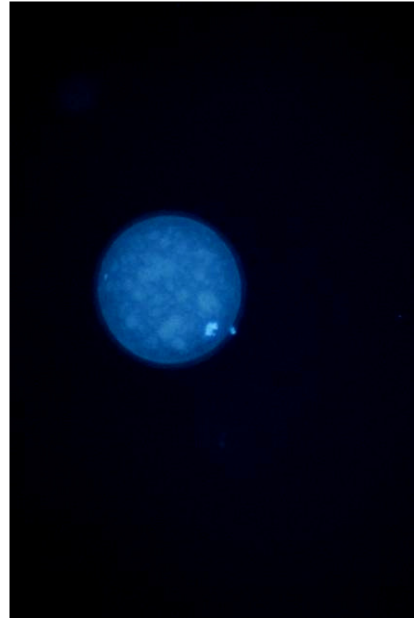


Fig. 3.4. Reverse transcription–polymerase chain reaction (RT-PCR) amplified products of total RNA isolated from cumulus cells (C),immature cumulus oocyte complexes (iCOC) mature cumulus oocyte complexes (mCOC),oocytes(O) and early embryos of 2/4cells (E_4),8 cells (E_8) and blastocyst (B) resolved on ethidium bromide stained 2% agarose gel.



A



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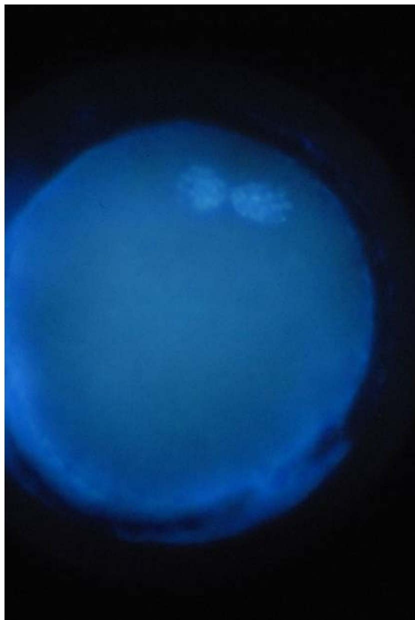

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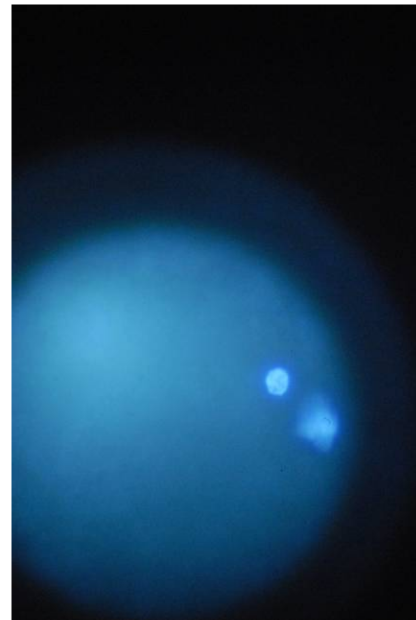
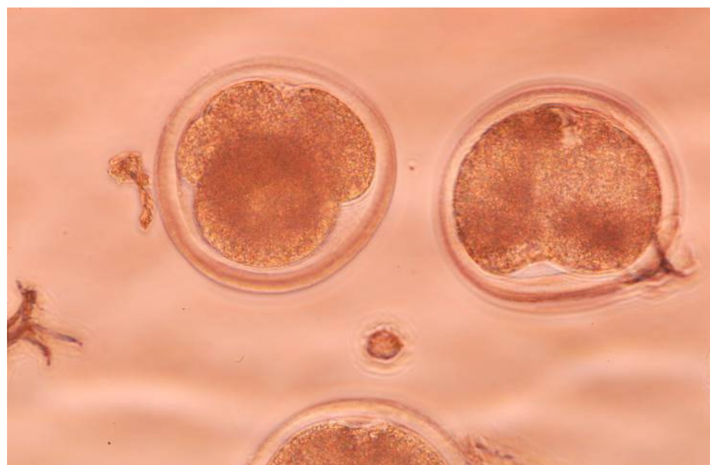
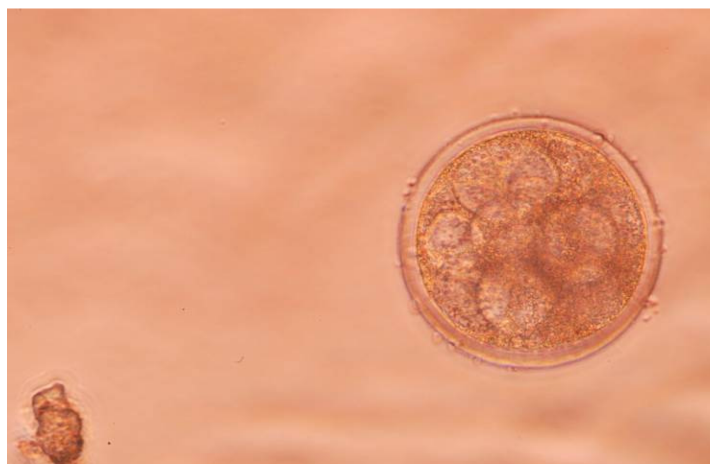


D

Plate 3.1. Fluorescence microscopic images of bovine oocytes and zygotes after staining with HOECHST 33342 (using UV-2A filter combination). **A)** maturing oocyte at anaphase I (X400), **B)** mature oocyte with one polar body (X100), **C)** zygote with 2 pronuclei (X400) and **D)** zygote with larger group of paternal chromosomes and smaller group of maternal chromosomes (X400)

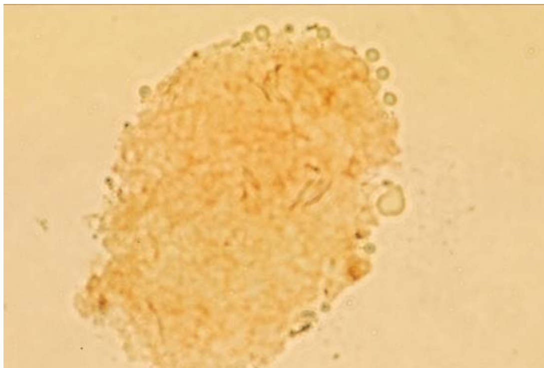


A

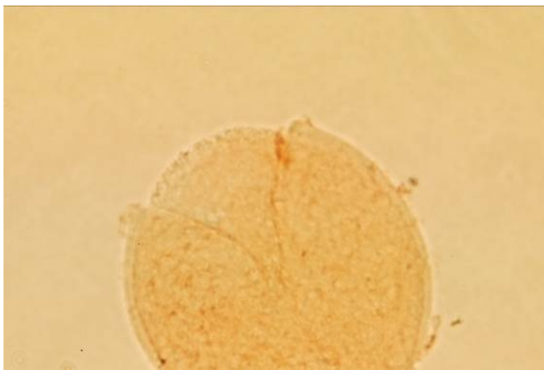


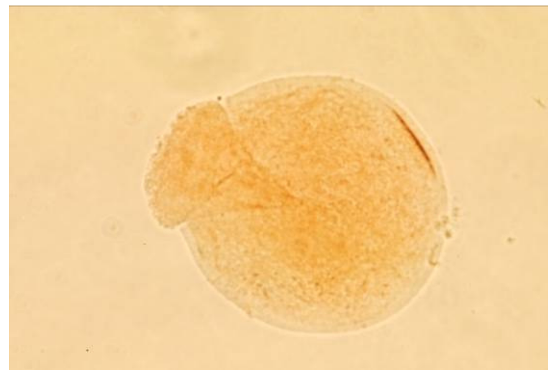
B

Plate 3.2. Light microscopic images of bovine early embryos (X400). **A)** Dividing zygote and a 4 cell stage embryo, **B)** an 8 cell stage embryo.



A **B**





C **D**

Plate 3.3. Immunocytochemical staining of bovine oocytes (X400) and cumulus cells (X100). **(A),** negative control (without antibody) of cumulus cells showing no brown colored precipitate while **(B),** cumulus cells showed positive staining. **(C)** negative control without antibody and **(D)** positive control of oocytes did not show brown colored precipitate.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

4.1 GENERAL DISCUSSION

Gonadotropin releasing hormone is a neurodecapeptide secreted from the hypothalamus, which orchestrates mammalian reproduction through binding to the specific receptors on the anterior pituitary gland to stimulate gonadotropin synthesis and secretion. The specific receptors for GnRH have been identified to belong to the family of G protein-coupled receptors. Since the elucidation of the structure of GnRH in 1971, (Matsuo et al. 1971) numerous analogues have been developed to be used in the field of reproductive endocrinology. The pharmacological basis for the use of GnRH and its analogues derives from its physiological role of stimulating LH and FSH from the pituitary gland (Peters 2005). In addition to this central role, GnRH is shown to play an important role in the regulation of extrapituitary physiological roles (Cheng and Leung 2005; Ramakrishnappa et al. 2005). With more information regarding the extrapituitary expression of GnRH and its receptors in reproductive tissues forthcoming, the quest to elucidate their local modulatory roles in reproductive physiology has become a necessasity and a matter of utmost interest.

The aim of this thesis was to find out the direct effects of GnRH sperm function, namely, sperm zona-binding and acrosome reaction, maturation of oocytes and *in vitro* fertilization in bovine, and also to find out whether bovine gametes and early embryonic stages express receptors for GnRH.

In the first sets of experiments (Chapter 3), direct effects of GnRH on sperm acrosome reaction and sperm zona-binding was investigated using GnRH agonists used most extensively in veterinary medicine, namely buserelin. The expression of GnRH-R by bovine sperm was examined using RT-PCR and immunostaining to detect GnRH mRNA and protein. Buserelin was observed to increase both acrosome reaction and Sperm zona-binding *in vitro*. It was also revealed that GnRH antagonist, antide was able to block the stimulatory effect of GnRH agonist effectively. Similar observations by GnRH on sperm zona-binding to human zonae pellucidae is reported, but no significant effect on acrosome reaction of human sperm by GnRH has been observed (Morales 1998). Although above observations indicated that GnRHa must have acted through a specific receptor, results of RT-PCR and immunostaining of bovine sperm were not able to confirm the presence of GnRH receptor transcript or the presence of GnRH receptor protein in bovine sperm. In contrast, immunohistochemical staining of human sperm (Lee et al 2000) had been able to demonstrate the presence of GnRH-R in the acrosomal region. In this study, we used primers and primary antibodies synthesized based on the pituitary GnRH receptor (GnRH-R type I) as type II GnRH receptor gene is reported to be inactivated in many mammalian species, including bovine and human (Morgan et al 2006; Morgan et al 2003). However, another study investigating the expression of type II GnRH-R by different human tissues reveals the detection of transcripts in post meiotic testicular cells and mature sperm by in situ hybridization and RT-PCR screening (van Biljon et al. 2002), and van Biljon and co-workers conclude that it is possible that GnRH receptor gene (in chromosome 1) is functional. It is also reported that GnRH binding sites in the human placenta showing low affinity to native

GnRH and its agonists, are distinct from the typical GnRH receptors found in the pituitary (Rama and Rao 2001). GnRH II receptor mRNA expression in human endometrial and ovarian cancer cells has also been elucidated by Grundker and co-workers (2002). Another important fact that we have to bear in mind is that sperm are both transcriptionally and translationally quiescent and the hormone receptors which have been detected in sperm so far are all non genomic receptors (Naz and Sellamuthu 2006). Thus, the amount of GnRH-R mRNA might have been not sufficient to be detected even it was present. Considering this and the conflicting information regarding the expression of GnRH receptors in human tissues, we do not want to conclude beyond all reasonable doubt, that bovine sperm do not express receptors for GnRH. Therefore, the mechanism by which GnRH agonist increased sperm function should be further explored.

In the second set of experiments (Chapter 4) a nuclear staining technique using bisbenzamide stain was employed to determine the effect of GnRHa on meiotic maturation of *in vitro* matured bovine oocytes and on *in vitro* fertilization of bovine oocytes. The presence of GnRH receptor mRNA and protein in bovine oocytes and early embryonic stages were investigated using RT-PCR and immunostaining. GnRH agonist, buserelin did not have a significant influence on the *in vitro* maturation of cumulus intact bovine oocytes. Both RT-PCR screening and immunostaining confirmed the expression of GnRH receptor transcripts and protein by cumulus cells. Interestingly, we observed an increase in GnRH-R mRNA expression by mature COCs compared to immature COCs. However, we did not observe a significant increase in maturation or fertilization rate in GnRHa treated group compared to controls as anticipated, although

it's well documented that surrounding cumulus cells act as supportive cells to the oocytes and can transduct signals to regulate oocyte development (Speroff 2005). GnRHa is reported in meiotic maturation in follicle enclosed rat oocytes (Dekel et at 1988) but clinical studies in women under gone fertility treatment have not shown a significant difference in the number of MII stage oocytes collected in women treated with GnRHa compared to GnRH antagonists (Hernandez 2000).

Molecules that influence the sperm zona-binding process are believed to alter the fertilization out come. (Yanagimachi 1994) In humans, it is reported that binding of sperm to hemizona has been correlated to the fertilizing potential of sperm (Burkman et al 1988). In cattle, contrasting observations have been reported. Fazeli and co-workers (1997) observed a significant correlation between the number of bovine sperm binding to hemizona and on 56 day nonreturn rate, while Braundmeier et al (2002) and Giritharan et al (2005) did not observe a correlation between zona-binding ability and 60-90 day nonreturn rate. According to our observations in this study, we did not see a positive influence by GnRHa on fertilization rate, although we observed a significant increase in sperm function upon GnRHa treatment (Chapter 3). Peters (2005) analysis of cattle fertility studies points out that the percentage of improvement with GnRH treatment at AI is higher, when lower the background fertility in the herd. Thus, this provides evidence that GnRH treatment is more effective where the background fertility is poor. The semen used in this study was from a proven AI sire with top quality semen and after thawing the motile fraction of the sperm is used for insemination in *in vitro* fertilization. Also, capacitated sperm are inseminated in to the immediate vicinity of the oocyte confined to a minute area of 100 µL, eliminating all the barriers and hostile

environments which sperm would have to conquer in, *in vivo* conditions. On the other hand cumulus intact oocytes with evenly granulated cytoplasm and more than 3 layers of cumulus cells ere selected for *in vitro* maturation and then oocytes with extensive cumulus expansion among those were selected for insemination at the end of maturation. Therefore, this may also be a reason why we did not observe a significant difference in fertilization rate in this study.

RT-PCR screening and immunostaining did not reveal GnRH receptor expression by bovine oocytes, 2cell, 4 cell, 8 cell and blastocyst stage embryos. GnRH receptor expression is reported in human (Casan et al 1999), mouse (Raga et al 1999) and pig (Nam et al 2005) preimplantation stage embryos. Furthermore, GnRH agonists are reported to enhance preimplantation embryonic development in mouse and pig (Raga et al 1999; Nam et al 2005). In contrast, Funston and Seidel (1995) did not observe a significant effect on bovine blastocyst development rate by GnRH. The improvement of the pregnancy rate, with routine treatment of GnRHa 11-14 days after insemination, (which coincides with maternal recognition of pregnancy) is justified pharmaco-physiologically as it is believed to enhance luteinisation stimulating progesterone secretion and a reduction in estradiol secretion (Peters 2005). Although, according to our observations and previous *in vitro* informations it is difficult to speculate that GnRH may be having a direct beneficial role contributing to the development and implantation of bovine preimplantation embryos, we suggest bovine oocytes and early embryos should be further explored using more sensitive methods such as real time PCR before ruling out the possibility of the presence of GnRH receptors in them.

4.2 GENERAL CONCLUSIONS

This study concludes that GnRHa enhances bovine sperm function *in vitro* but does not seem to have a significant effect on *in vitro* maturation of bovine oocytes or *in vitro* fertilization of bovine oocytes. Also, observations of this study did not reveal the expression of GnRH receptors by bovine sperm, oocytes and early embryonic stages. GnRH receptors are expressed by bovine cumulus cells which increased with maturation.

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