CHAPTER 2

Literature review

This chapter summarizes the background and biological studies performed on GnRH agonists in dogs. In the scope of this thesis only the contraceptive effects of GnRH agonist, deslorelin, in dogs along with the solutions to the problems mentioned in chapter 1 and its possible practical application under the current knowledge will be discussed under the following heading.

2.1 Fertility in female dogs

2.1.1 Hormonal control

Dog is non-seasonal monoestrus and the period of oestrus cycle extended due to a long anoestrus. In this respect, the ovarian activity of canine species found to be different from this in other domestic species. However, the oestrous cycle of dog is controlled by Hypothalamic-gonadal axis as shown in other species:

a. The hypothalamus and pituitary gland, where primary control resides.
b. The ovary, the source of the steroid hormones oestrogen and progesterone.
c. The reproductive tract, response to sex steroid hormones.

In conclusion, the reproductive cycle of female dog is under the control of neuroendocrine system especially in gonadotrophin secretion. Both the gonadotrophins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), are necessary ovarian folliculogenesis (Concannon et al., 1989; Concannon et al., 1993).

2.1.2 Role of gonadotrophins

In the female, FSH promotes follicle growth and estrogen production by granulosa cells in the ovarian follicle. The action of FSH on follicular development and steroidogenesis are mediated through its binding to FSH receptors located exclusively on granulosa cells. Also, LH causes ovulation (rupture of follicle with release of oocyte) and stimulates formation of the corpus luteum and production of progesterone call "luteotropic effect" (Wingard et al., 1991; Lincoln, 1992; Bearden and Fuquay, 1997). During luteinization, there is a major increase in LH receptors (LaPolt et al., 1990).

Ovaries of female dogs are relatively in active during anoestrus despite apparently adequate circulating FSH concentrations (McBride et al., 2001). Endocrine changes that lead to the termination of anoestrus and consequently the start of a new follicular phase are poorly understood in female dogs. In particular, information concerning the role of the changes in the secretion patterns of FSH and LH in the initiation of ovarian folliculogenesis is limited. An increase in pituitary secretion of gonadotrophins has been reported to occur during the progression of anoestrus. An increase in circulating FSH should be considered to be a critical event required for ovarian folliculogenesis. In addition, an

increase in ovarian responsiveness to gonadotrophins, an increase in circulating basal LH concentrations at the end of anoestrus and a brief period of increased LH pulsatility have been reported to be important determinants in the start of a new follicular phase (Kooistra and Okkens, 2001).

2.2 Control of Gonadotrophin

2.2.1 GnRH

Controlling both FSH and LH is by gonadotrophin-releasing hormone (GnRH), also known as luteinizing hormone-releasing hormone (LHRH) or luteinizing hormone releasing factor (LRF). GnRH is known to regulate and modulate the biosynthesis and release of gonadotrophins from the anterior pituitary gland. It also a key hormone involved in sexual behavior. It acts as the humoral link between the neural and endocrine system.

2.2.2 Structure of GnRH

GnRH is a hypothalamic decapeptide hormone with the following structure in mammals (Matsuo et al, 1971) :

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

The structure is conserved in all mammalian species that have been studied so far. The key to intrinsic activity on GnRH peptides is the His residue at position 2. All vertebrate GnRH peptides are active in other vertebrates although there is a wide range of response patterns; mammals respond preferentially to mammalian GnRH (Millar and King, 1988 cited by Cain et al., 1989). The elucidation of the structure has led to the development of agonists and antagonists which have been used for fertility regulation (Matsuo et al., 1971).

2.2.3 Source of GnRH

GnRH is the central neuropoptide which synthesized by neurosecretory cells, name GnRH neurons, and stored in the medial basal hypothalamus as a form of secretory vesicles or secretory granules (Guyton and Hall, 1996). In response to neural signals, GnRH is released by hypothalamic nerve endings into the portal capillaries, and is delivered to the anterior pituitary gland by the hypophyseal portal veins, also call hypothalamic-hypophyseal portal circulation. These capillaries allow GnRH to be delivered into the circulation without passing through a blood-brain barrier to the target cells which are gonadotropes in anterior pituitary gland, initiated the releasing of two gonadotrophins, FSH and LH (Wingard et al, 1991). GnRH release into the hypophyseal portal vessel is characteristically episodic (Carmel et al., 1976). Pulsatile mode of GnRH release is essential to drive the secretion of gonadotrophins (Kalra, 1993). There are many factors involved in the pulsatile release of GnRH, for examples, catecholamines, serotonin, gamma amino butyric acid (GABA), histamine, glutamate, and other neuroactive substances, which are

substance P, atrial natriuretic peptide (ANP), neuropeptide Y, prolactin and endothelin. (Krieg and Sawyer, 1976; Coen and Mackinnon, 1979; Vijayan and McCann, 1979; Fuchs et al., 1984; Van Hirk et al., 1989; Azad et al., 1990; Bauer et al., 1991; Bram and Mahesh, 1991; Krsmanovic et al., 1991). Furthermore, GnRH may also modulate its own secretion by an ultra-short loop feed back mechanism (Krsmanovic et al., 1993).

2.2.4 Control of Gonadotrophin synthesis and release

Understanding the basic mechanisms by which GnRH alters gonadotropic function has been a valuable resource for development of clinical and veterinary strategies. The GnRH neuronal network is extremely complex and is influenced by several different areas of the brain. Both the frequency and the amplitude of GnRH release are important features of its function in stimulating the release of FSH and LH. With a neuroendocrine mechanism, GnRH controls gonadotrophin synthesis by acting on pituitary receptors in a pulsatile manner exerting a physiological effect of activating and maintaining the reproductive status from gonadotrophin synthesis, and releasing to gametogenesis and gonadal steroidogenesis (Miller, 1993). The control of gonadotrophin synthesis and secretion is complex. These controls influence the pulsatile output of GnRH from the hypothalamus, GnRH receptor number and their sensitivity to GnRH and the regulation of gene expression within the pituitary gland (Miller, 1993).

2.3 GnRH receptors

2.3.1 Role of the receptors

GnRH receptors are found in the anterior pituitary and brain. They belong to the family of G protein coupled receptors, as all G protein coupled receptors, the GnRH receptor is characterized by seven putative transmembrane segments (Figure 6). It has a short N-terminal extracellular extension. A unique feature of this receptor is the absence of a C-terminal intracellular tail. The receptor protein is 328 amino acids long and contains two putative N-glycosylation sites, which are localized in the N-terminus and in the first extracellular loop but the only sites at the N-terminus are glycosylated.

The number of GnRH receptors on the pituitary gonadotropes usually does not remain constant, because of the receptor proteins themselves are often inactivated or destroyed during the course of their function, and at other times, they either reactivated or new ones manufactured.

On binding to GnRH receptor, the GnRH stimulates gene expression, synthesis, and release of the gonadotrophins, LH, and FSH, from the anterior pituitary gland. Stimulation of receptor to its ligand induces signaling via a G-protein-coupled secondary messenger cascade. An increase in phospholipase C (PLC) activity results in mobilization of calcium from intra- and extracellular sources and activation of the PKC and MAPK pathways (Marian and Conn, 1983). Otherwise, the GnRH receptor stimulates phospholipase C via G_q/G_{11} proteins and stimulation of phospholipase A_2 and D (Davidson et al., 1995; Roux

and Milgrom, 2001). Sharaga-Levine et al. (1996) demonstrated the cross-talk between arachidonic acid (AA), leukotrient C_4 (LTC_4), 5-hydorxyeicosatetraenoic acid (5 HETE) and protein kinase C_β (PKC_β) mRNA levels appears to be physiologically relevant as it play in a role during the action of GnRH.

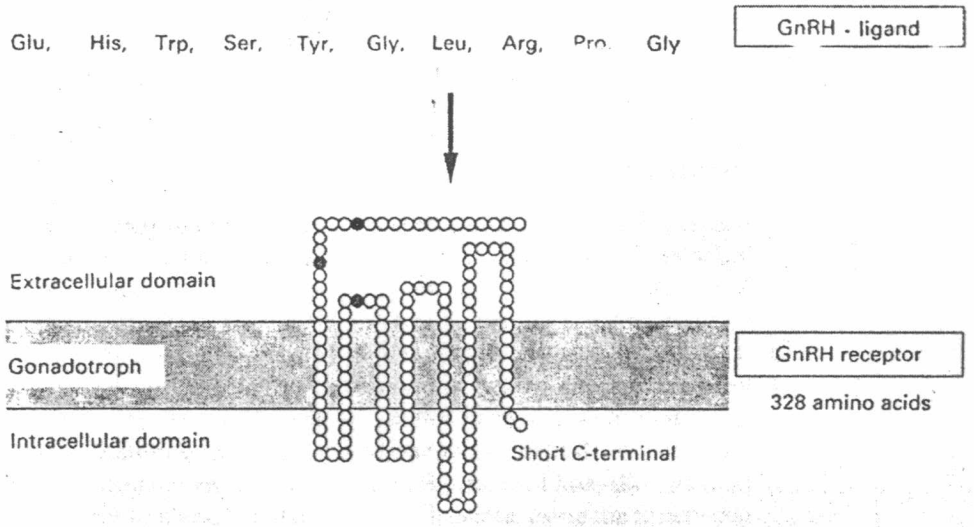

Figure 6. Diagrammatic illustration of the interaction between GnRH and the GnRH receptor. The GnRH receptor is one of the smallest seven-transmembrane receptors out of more than seventy that have been sequenced. (modified from Lyncoln, 1992).

2.3.2 Factor affecting receptor activity

The GnRH receptors are regulated according to the physiological conditions. It is seen to increase during oestrus and get maximal level before preovulatory surge of LH and decrease following the surge. The number also decreases during pregnancy and lactation (Clayton et al., 1980; Conn, 1994). The number of GnRH receptors are not only regulated by hormone like GnRH, LH and FSH but also by steroids and protein products from the gonads (Wise et al., 1984; Young et al., 1984; Greg and Nett, 1989; Wang et al., 1989).

2.3.2.1 Up-regulation

In general, hormones cause hypersensitization of receptors; that is, the stimulating hormone induces the formation of more receptor molecules than normal. In this instance, the target tissue becomes progressively more sensitive to the stimulating effects of the hormone (Guyton and Hall, 1996).

2.3.2.2 Down-regulation

Desensitization of G protein coupled receptors may divide in two categories, short-term desensitization and long-term desensitization. Short-term desensitization of receptors due to

phosphorylation and internalization (sequestration). The phosphorylation of amino acid residues (Ser, Thr) leads to conformation changes of the receptor, thereby impairing the coupling to a G protein. Long-term desensitization of receptors includes changes in the rate of receptor turnover, receptor gene transcription, and receptor mRNA turnover. A decrease of receptor population due to a decrease in mRNA synthesis, an increase in inactivation (instability) of mRNA or an increase in breakdown of receptor protein. In either event, this is also called "Down-regulation" of the receptor numbers as it decreases the responsiveness of the target tissue to the hormone.

2.4 GnRH agonists

2.4.1 Structure and preparation

Because of the low potency and short half-life of native GnRH, long-acting, potent analogues have been developed (Pace et al., 1991). It was forecast that chemical substitutions in the molecule would lead to analogues possessing antagonist or increased agonist activity useful as anti- and pro-fertility agents. The isolation and structure determination of GnRH have led to the synthesis of an extensive series of analogues, some of which are agonists and others have antagonistic properties. The replacement or substitution or deletion of one or more amino acids results in analogues possessing features requisite for a functional state i.e. the stimulation of LH and FSH release with up to 200 times the potency of the native molecule (Nester, 1984 cited by Vickery et al., 1989). Continuous exposure to an agonist leads to desensitization of the GnRH receptors.

In the past, the administration of GnRH in a pulsatile mode required the use of a specialized infusion device, the limitation of this method is the high cost of pulsatile pump systems and not practically to use in clinic due to the need to use intravenous catheterization (Cain et al, 1989). An alternative approach to pulsatile infusion of GnRH has become available with the introduction of a depot-injection form of a potent GnRH agonist such as leuprolide acetate, nafarelin, gonadorelin, lutrelin-like agonist, buserelin and deslorelin, These analogues can be injected subcutaneously by several techniques such as using osmotic mini-pump, combination with silastic polymer and microencapsulated form (Concannon, 1989; Vickery et al., 1989; Cain et al., 1990; Cinone et al., 1996; Concannon, 1998; Inaba et al., 1998).

2.4.2 Products

Approximately 1,000 compounds have been synthesized and evaluated for agonist and antagonist activity for potential use as investigative tools or contraceptive agents in human. In veterinary practice, very potent agonistic analogues of GnRH are undergoing clinical evaluation in both sexes of many species. Synthetic agonists frequently share the presence of a d-amino acid which enhances its affinity to bind receptors (Conn, 1994). Their increased potency over GnRH itself

results from increased receptor binding affinity and/or resistance to metabolism (Vickery, 1985). Several synthetic GnRH agonists are available (Table 3) although none is licensed for use in the dog. Mostly these agents use by subcutaneous injection everyday so it is limitation for using as contraceptive agent and other applications. For now, the development of delivery system, slow release and long acting GnRH agonist is initiated in "delorelin".

Table 3 Commercially available GnRH agonists that may be useful for the manipulation of reproduction in the dog and bitch. (modified from England, 1998).

GnRH agonist	Trade name	Formulation	Distributor
Buserelin	Receptal	0.1 mg/ml (injection)	Hoechst Rousell Vet Ltd
Fertirelin	Ovalyse	50 microgram/ml (injection)	Pharmacia & Upjohn Ltd
Gonadorelin	Fertagyl	0.1 mg/ml (injection)	Intervet UK Ltd
Buserelin	Suprefact	1.0 mg/ml (injection)	Hoechst UK Ltd
Goserelin	Zoladex	3.6 mg(implant for 1 month)	Zeneca Phamaceuticals
	Zoladex LA	10.8 mg(implant for 3 month)	Zeneca Pharmaceuticals
Nafarelin	Synarel	Nasal spray	Searle DG and Co. Ltd
Deslorelin	Ovuplant	2.1 mg (implant)	Peptech Animal Health Ltd

2.4.3 Effect of agonists and comparison with effect of natural hormone

Natural GnRH actions as stated before leads to the release of LH and FSH. The concentration of circulating gonadotrophins are known to depend on the frequency of GnRH pulses discharged into the pituitary portal vessels. The response of pituitary gonadotropes to GnRH correlates directly with the concentration of GnRH receptors on the cell surface, which is mediated in part at the level of GnRH receptor gene expression. Because GnRH itself regulates the expression of its own receptor (Kaiser et al., 1995), failure of GnRH to stimulate GnRH receptor gene promoter activity would be predicted to result in a lack of homologous up-regulation of the receptor by pulsatile GnRH, contributing to a reduced number of GnRH receptros on the cell surface of gonadotropes. On the other hand, chronic treatment with agonists of GnRH elicits first an increase in gonadotrophins followed by desensitization of the pituitary receptors to further agonist stimulation.

2.4.4 Use in human medicine

The ability of GnRH to stimulate reproductive functions (at pulsatile doses) or suppress them (at high dose and chronic treatment) has been clinically applied for different purposes, including the induction of ovulation and spermatogenesis, and for contraception. The phenomena of desensitization and inhibition of sex steroid levels by GnRH

agonists are being used for treatment of true idiopathic precocious puberty, endometriosis, and steroid dependent tumors (i.e. prostate and breast cancer) as well as a range of veterinary purposes (Leschek et al., 1999 ; Municchi et al., 1993 ; Reichler et al., 2003).

2.4.5 Potential use in dogs

2.4.5.1 Male dogs

2.4.5.1.1 Contraception

The use of GnRH agonists in dog have been reported to suppress of LH, FSH and testicular function, causing reduced libido and infertility. These effects appear to be mediated solely through pituitary down regulation (Vickery et al., 1984). Inhibition of spermatogenesis is rapid at higher dose (2 or 10 μg/kg/day) of nafarelin acetate, a potent GnRH agonist, noticeable histologically by 10 days of treatment. At all of dose levels, total inhibition of spermatogenesis to presence of spermatogonia and Sertoli cells is achieved (Vickery et al., 1985). The initial influence on serum testosterone after GnRH agonist treatment in dog was demonstrated by Lacoste et al., 1988. Daily subcutaneous administration of GnRH agonist in adult dog caused a transient increase in the serum testosterone concentration on days 2 to 4 of treatment and subsequent progressively decreases. In particular, depot preparations appear to be useful as reversible methods of contraception (Trigg et al., 2001).

2.4.5.1.2 Treatment of benign prostatic hyperplasia (BPH)

The prostate is the only accessory sex gland in male dog. It is an encapsulated, bilobed and bilaterally symmetrical ovoid gland, located caudal to the bladder, encircling the proximal urethra (Garraway et al., 1991). Recently, dogs have been living longer, and canine benign prostatic hyperplasia has become an important age-related disease (Kawakami et al., 1998). More than 80% of sexually intact male dogs, the prostate hyperplasia were reported to develop by > 5 years of age with gross or microscopic evidence of BPH (Sirinarumithr et al., 2001). To maintain prostatic size, dihydrotestosterone (DHT) is well accepted as a key hormone in men and dogs by enhancing growth in the stromal and glandular compartment (Lee, 1996). Testosterone is metabolized by the enzyme 5α-reductase into DHT. DHT has a receptor binding affinity about twice that of testosterone, and the rate of dissociation of DHT from its receptor is a fifth of the rate of dissociation of testosterone (Grino et al., 1990). The use of chemicals including GnRH agonists to induce prostatic regression in dogs has been investigated extensively and has revealed great potential (Lacoste et al., 1989; Iguer-Ouada and Verstegen, 1997; Kawakami et al., 1998; Tsutsui et al., 2001; Murakoshi et al., 2000; Sirinarumitr et al., 2001). In addition, Ponglowhapan et al. (2002) revealed that subcutaneous GnRH agonist (deslorelin) implantation can be used to reduce prostatic

volume in dogs. From the clinical point of view, the further investigation in the effects of deslorelin implantation on benign prostatic hyperplasia (BPH) dogs is needed to be explored.

2.4.5.2 Female dogs

The clinical use of GnRH agonists female dogs is control of oestrus, induction of oestrus, identification of ovarian tissue, hastening of ovulation, unwanted mating and treatment of mammary gland tumors. However, the effectiveness of GnRH agonists should be considered several factor; duration of drug usage, quantity of drugs, potential of drugs and route of drug administration such as intravenously, subcutaneously and more convenient method of intranasal delivery (in human) is therefore necessary (Chrip and Goa, 1990).

2.4.5.2.1 Control of oestrus

GnRH agonists have been shown to suppression female reproductive cyclicity and male gonadal function. Use of these analogues to suppress sexual function in bitches therefore had a high probability of success. The first studies were started in 10-11-month-old, prepubertal (before first heat) beagle bitches (Vickery et al., 1987 cited by Vickery et al., 1989). The study using repetitive monthly subcutaneous implantation of GnRH agonist (Nester, 1984 cited by Vickery et al., 1989) demonstrated both the stimulatory and suppressive effects of the GnRH agonist in that the 3 bitches came into oestrus within 6 days of starting treatment, but oestrus was not expressed again during the year of treatment. Termination of treatment after 1 year was followed by oestrus within 8 months and there were no long-lasting effects on fertility as all animals conceived at the 1^{st} and 2^{nd} return oestrus. Further studies were initiated in the beagle bitch (McRae et al., 1985) which performed with nafarelin. These studies established a dose requirement of 32 μg nafarelin/day and showed that stimulation of oestrus could be avoided if treatment was started at or before 4 months of age, within a period of 60 days following an ovulatory heat, or within 7 days after parturition. If treatment was initiated at the time of a spontaneous heat and mating took place, the outcome of pregnancy was not affected. However, if mating occurred at estrous induced by treatment, then fertility appeared to be low (Vickery et al., 1989). The pregnancy may be occurred after estrous induced by GnRH agonist but the progesterone level was not high enough to support pregnancy along two months (Olson et al., 1989; Onclin et al., 2000). The oestrous induction during 5-28 days after the first usage of GnRH agonists is contraindication to use this hormone for contraceptive application. To solve this problem, Wright et al. (2001) demonstrate that the initial oestrus induced in anestrous bitches by deslorelin treatment may be suppressed by progestin treatment, Trigg et al. (2001) found that no bitch showed an induced oestrus when the minimum plasma progesterone

concentration at the time of implantation circulation was more than 5 ng/ml and Vickery et al. (1989) suggested to use this hormone at the time of diestrous period for avoid estrous induction effect.

2.4.5.2.2 The other applications

2.4.5.2.2.1 Induction of oestrus

The potential utility of method for induction of a fertile estrus in bitch using several compounds including GnRH agonists. Up to now, the using of GnRH agonist to induce estrus has been continuously developed because its process likes natural stimulation of pituitary gland, causes a releasing of FSH and LH, results in follicular development sufficient to produce proestrus, estrus and spontaneous ovulation (Cain et al., 1988; Concannon et al., 1997; Vanderlip et al., 1987). The study of preovulatory LH surge in the bitch induced estrus by administration of Lutrelin reported the LH level in pregnancy groups was 4.7±0.9 ng/ml, in non-pregnant but ovulation group was 2.7±0.6 ng/ml and non-ovulation group was 1.9±0.3 ng/ml. The deficient preovulatory LH release in many bitch compared with normal level (16±3 ng/ml) may have been due to down-regulation of pituitary responsiveness to endogenous GnRH as a result of continued administration of high doses of the GnRH agonist (Concannon, 1989). Recently, Kutzler et al. (2002) reported the success of 5 pregnant from 8 bitches induced by vulvar mucosa implantation of Deslorelin.

2.4.5.2.2.2 Identification of ovarian tissues

The intravascular administration of GnRH agonist to intact bitches produces an increase in LH and subsequent rise in plasma estrogen concentration. This test may be useful for detection of bitches that have been ovariectomized or ovariohysterectomized where no rise in oestrogen will occur (England, 1998).

2.4. 5.2.2.3 Hastening of ovulation

Single intravascular does of GnRH may be useful to hasten ovulation in bitches in oestrus (England, 1998).

2.4. 5.2.2.4 Unwanted mating

The down-regulation effects of continuous GnRH administration may be used in attempts to withdraw gonadotrophin support of the corpus luteum (England, 1998).

2.4. 5.2.2.5 Treatment of mammary gland tumor
Mammary neoplasm account for about half of all tumors in bitches (Johnson, 1993). The major treatment of mammary neoplasia is surgical excision of all abnormal tissue. The alternative treatment is using GnRH agonist to decrease the size of tumor and increase survival time of hormone dependent canine mammary cancer bitches (Lombardi et al., 1999)

2.4.6 Deslorelin

2.4.6.1 Structure
Deslorelin is a nona-peptide, potent, synthetic agonist of GnRH. Its potency is 100-130 times than natural hormone with the following structure:

pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro- NHEt

Average plasma levels of deslorelin following subcutaneous implantations to the dogs, these implants released doses of > 1 µg / day for periods of > 1 year (Peptech Animal Health, Sydney, personal communication).

2.4.6.2 Use in animals
In 1995, deslorelin was licensed for use in veterinary medicine (equine practice) by FDA. Following that time, many research in mammal reproduction scope in using this agent for initiate follicular, oocyte maturation and accelerate ovulation in equine oocyte transfer techinque (Carnevale et al., 2001; Mayers et al., 1997), provide a model for studying the hormonal requirements for follicle growth and in vivo oocyte maturation in bovine (Maclellan et al., 1997; Padula et al., 2002a; Padula et al., 2002b), stimulate a LH surge capable of reducing the time span of ovulations in swine (Kraeling et al., 2000), suppress of oestrous cycle in feline (Munson et al., 2001).

2.4.6.3 Use in dogs
In canine practice, deslorelin was reported for control fertility on both male and female canine (Trigg et al., 2001 ; Wright et al., 2001), otherwise it can control of reproduction and sex related behaviour in exotic wild carnivores (Bertschinger et al., 2001) The suppression of fertility by deslorelin is over 12 months by 2, 3, 6 and 12 mg/bitch subcutaneous implantation but the efficiency was depended on dosage per bitch, the same as the studying of Vickery et al (1989) reported that the dosages affect the responsiveness of reproductive suppression. In our opinion, not only dosage of deslorelin but also other factors relate with the effective of this agent.