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Ovarian function in dairy cattle after gonadotropin-releasing hormone treatments during perioestrus

by

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ACADEMIC DISSERTATION

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Hedelmättömyys.

Usein kuulee eläinten omistajain valittavan sitä, että on vaikea saada eläimiä tiineiksi. Toisinaan ilmautuu hedelmättömyys liiallisen, toisinaan vähentyneen siitoshimon ohella. Hedelmättömyys riippuu välistä myös koiraksien kykenemättömyydestä. Mutta tavallisesti tulee se siitä, että naaras-eläin on liian lihava tahi sen siitos-elimet virheellisiä.

Liiallisen siitoshimon vähentämiseksi käytettäköön kanverttiä, 4 – 6 grammaa kahdesti päivään ja useampien päivien kuluessa. Liian laimeaa himoa voidaan joskus virkistää Espanjan kärpäsillä, mutta nämä ovat myrkyllisiä ja sentähden vaarallisia käyttää. Suonenisku vähää ennen astuttamista vaikuttaa toisinaan edullisesti lihaviin eläimiin. Jos hedelmättömyyden syynä on epämuodostuksia naaraksen siitos-elimissä, tulee käyttää eläinlääkärin apua.

Kirjasta Suomen maataloudessa käytettävien kotieläinten taudit, toim. läänineläinlääkäri O.V. Löfman, G.L. Söderström 1896

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ABSTRACT

In tie-stall conditions, especially during the long wintertime in Finland and in other northern countries, oestrus detection is difficult because of the common occurrence of silent oestrus. It may be very difficult to decide when to call the artificial insemination (AI) technician to inseminate the cow. Because of this, gonadotropin-releasing hormone or its agonists (GnRH) are often used by practitioners to induce ovulation close enough to insemination in hope of improving the pregnancy rate. Under these circumstances, GnRH may be given even as long as one or two days before, or even after the physiological luteinising hormone (LH) surge.

Although the effect of GnRH treatment given at the time of AI on pregnancy rate has been mainly positive, several trials have shown lower pregnancy rates in treated animals than in control animals. Although the decrease has generally been small, two studies have shown a significant negative effect of GnRH on pregnancy rate. GnRH treatment in combination with induction of oestrus using prostaglandin $F_{2\alpha}$ or its agonists (PG) have shown some evidence of possible negative effects of GnRH on subsequent luteal function or fertility. In addition, some studies have shown a negative effect of GnRH when given during metoestrus.

The overall aim of the present study was to find and characterise possible negative effects of GnRH treatments given during perioestrus on reproductive functions, with special focus on the prostaglandin-induced oestrus period and effects on the ovulatory follicle, ovulation, subsequent follicular growth, and luteal function.

The first experiment studied the influence of exogenous GnRH (gonadorelin) given before the expected physiological LH surge on oestradiol secretion of the ovulatory follicle, on occurrence of ovulation, development and function of the corpus luteum (CL) and growth of a dominant follicle after ovulation. Six heifers and three cows were assigned once to each of the following treatment or control manipulations, receiving either a single dose (100 μg) of gonadorelin at 24 h, 48 h, or 72 h after PG, or no gonadorelin (control). In two animals, when GnRH was given 24 hours after PG, luteolysis took place on day 5 or 6 after ovulation, and animals ovulated on day 9 or 10. It is suggested that early induction of ovulation with GnRH can cause shortened luteal function in cattle and, ultimately, reduced fertility.

The influence of exogenous GnRH given shortly post-ovulation on the development and function of the CL and the length of the subsequent oestrous cycle was studied in six heifers and three cows. The oestrous cycles were synchronised using PG. After detection of ovulation, one of the following treatments was administered: gonadorelin (250 µg) at either 0-24 hours or 24-48 hours post-ovulation, or no gonadorelin (control). Every animal was assigned once to each of these three manipulations. GnRH treatment during metoestrus did not seem to alter subsequent luteal function, thus failing to confirm previous reports of reduced fertility post-treatment.

To confirm earlier findings, obtained with a small number of animals, that GnRH can shorten CL functional life when it is administered 24 h after PG treatments given 7 to 9 days after oestrus, sixty lactating commercial dairy cows were selected. In addition, the effects of two treatments, PG alone or PG plus GnRH given before mid-dioestrus, on signs of oestrus were studied. Eight days after spontaneous oestrus, animals were given PG. They were then divided into two groups. One group (n=25) received gonadorelin (100 µg) 24 h after the PG treatment, while the other group (n=35) served as controls without any further treatment. No short cycles were observed in the control group, whereas 33% of the cows in the treatment group exhibited premature luteal regression. PG treatment on day 8 had no effect on the intensity of the oestrous signs. In contrast, GnRH treatment 24 h after PG treatment weakened the oestrous signs significantly. Thus, GnRH administration 24 h after PG treatment given 8 days after oestrus can cause short oestrous cycles in some cows.

The fourth study explores the mechanisms behind the observed short oestrous cycles. The aim was to confirm that the luteolysis seen during short oestrous cycles is caused by a premature release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Further, the aim was to study the $PGF_{2\alpha}$ release pattern more closely to determine whether it resembles the spontaneous release occurring during normal CL regression or whether $PGF_{2\alpha}$ is continuously secreted after the induced ovulations leading to short oestrous cycles. Twenty-four heifers were allotted to four equal groups. After oestrus synchronisation, premature luteolysis was induced with PG on day 6 or day 7 after ovulation. Gonadorelin (100 µg) was given to treatment groups to induce premature ovulation 24 h later. Seven of twelve treated heifers showed a short oestrous cycle of 8 to 12 days duration, while all others had an oestrous cycle of normal length. Significant elevations in 15-ketodihydro-$PGF_{2\alpha}$ (a metabolite of $PGF_{2\alpha}$) concentrations with recurrent high peaks coincided with a decrease in progesterone concentration and were detected in all heifers that

showed a short oestrous cycle, but not in any heifers with normal oestrous cycles. Premature release of $PGF_{2\alpha}$, which closely resembles release during spontaneous luteolysis, caused luteal regression in these short cycles.

In conclusion, GnRH treatments given during prooestrus, clearly before the physiological release of LH, can shorten luteal function and, thus, reduce fertility. This disturbance can be caused at least when the GnRH treatment has been given in combination with PG treatment. A premature release of $PGF_{2\alpha}$ causes luteal regression in these short cycles.

ORIGINAL ARTICLES

The dissertation is based on the following original articles, which will be referred to in the text by their Roman numerals:

I Taponen J., Katila T. & Rodríguez-Martínez H. (1999) Induction of ovulation with gonadotropin-releasing hormone during proestrus in cattle: influence on subsequent follicular growth and luteal function. *Animal Reproduction Science,* 55: 91 - 105.

II Taponen J., Rodríguez-Martínez H. & Katila T. (2000) Administration of gonadotropin-releasing hormone during metoestrus in cattle: influence on luteal function and cycle length. *Animal Reproduction Science,* 64: 161 - 169.

III Taponen J., Kulcsár M., Katila T., Kátai L., Huszenicza G. & Rodríguez-Martínez H. (2002) Short estrous cycles and estrous signs after premature ovulations induced with cloprostenol and gonadotropin-releasing hormone in cyclic dairy cows. *Theriogenology,* 58: 1291 - 1302.

IV Taponen J., Hjerppe P., Kopra E., Rodríguez-Martínez H., Katila T. & Kindahl H. (2003) Premature prostaglandin $F_{2\alpha}$ secretion causes luteal regression in GnRH-induced short estrous cycles in cyclic dairy heifers. *Theriogenology,* 60: 379 - 393.

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ABBREVIATIONS

AI	artificial insemination
ARG	arginine
Ay	Ayrshire
CL	corpus luteum, corpora lutea
E-17β	oestradiol-17β
ELISA	enzyme-linked immunosorbent assay
FSH	follicle stimulating hormone
GLY	glycine
GnRH	native gonadotropin-releasing hormone and its agonistic analogues
hCG	human chorionic gonadotropin
HFr	Holstein-Friesian
HIS	histidine
i.m.	intramuscular
LEU	leucine
LH	luteinising hormone
P_4	progesterone
PG	prostaglandin $F_{2\alpha}$ and its agonistic analogues
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PRO	proline
PYROGLU	pyroglutamic acid
RIA	radioimmunoassay
SD	standard deviation
SEM	standard error of mean
SER	serine
TRP	tryptophan
TYR	tyrosine

INTRODUCTION

Gonadotropin-releasing hormone or its agonists (GnRH) have been used to treat reproductive disorders in cattle in Finland since the early 1980s. According to generally accepted principles in veterinary medicine in Finland, all medical treatments should be based on clinical examination of the animal and diagnosis of the disorder. Hence, routine hormonal therapies in reproductive management, e.g. oestrus synchronisation programs, are not used as a rule. In general, cattle are treated with GnRH for the following four reasons: (1) close to the time of artificial insemination (AI) in order to enhance pregnancy rates especially in suspected cases of ovulation failures, (2) treatment of cystic ovarian disease, (3) treatment of prolonged puerperal anoestrus, (4) to some extent as a post-insemination treatment to enhance embryo survival in repeat breeder cows.

Finnish statistics on herd health control (H. Rautala, personal communication) indicate that approximately 2% of cows are treated annually with luteotropic injections at the time of AI, mainly with GnRH. This corresponds to 6000 to 7000 treatments. The rate of treatment has decreased during the last ten years from about 3.5% to the above-mentioned 2%. The rate of treatment of cystic ovarian disease was 2.3% in year 2001, corresponding to about 8000 treatments. However, it is impossible to determine, how many of these were done with GnRH, human chorionic gonadotropin (hCG) or progesterone-releasing devices.

In tie-stall conditions, especially during the long wintertime in Finland and in other northern countries, silent oestrus is one of the most important causes of subfertility. Often the only sign of oestrus is a slight vaginal mucous discharge for 1 to 4 days. Hence, it may be very difficult to decide when to call the AI technician to inseminate the cow. Because of this, GnRH treatment is often employed to induce ovulation close enough to insemination in hopes of enhancing the pregnancy rate. Under these circumstances, GnRH treatment may be given even as long as one or two days before, or even after the physiological luteinising hormone (LH) surge. It has been suspected that incorrectly timed GnRH injections may reduce fertility.

The original idea of the present dissertation was to find and characterise possible negative effects of incorrectly timed GnRH treatments on reproductive functions. To test the hypothesis of negative effects, two models were established. In the first model, GnRH treatment was given 24 to 72 h after administration of prostaglandin $F_{2\alpha}$ or its agonists (PG), in other words approximately 0 to 2 days before the expected physiological LH surge. In the second model, GnRH

treatment was administered 0 to 48 h after ovulation. Ovulation, subsequent follicular growth, luteal function and cycle lengths etc. were investigated in order to detect changes in reproductive functions. In the subsequent experiments, the changes in reproductive functions caused by incorrectly timed GnRH treatments were studied more closely.

REVIEW OF LITERATURE

1. Normal oestrous cycle of cattle

The cow is polyoestrous, i.e. oestrous cycles continue throughout the year, unless the cow becomes pregnant. The length of the normal oestrous cycle is 21±3 days in cows and 20±3 days in heifers. The oestrous cycle is commonly divided into four periods: prooestrus, oestrus, metoestrus and dioestrus. Sometimes it is also divided into two periods based on ovarian findings: the follicular phase, comprising prooestrus, oestrus and the beginning of metoestrus, and the luteal phase comprising the rest of the oestrous cycle. For many breeds of cows the duration of oestrus is on average 15 hours, although there is good evidence for modern Holstein that it is much less. The normal variation is, however, wide, viz. 2 to 30 hours. The lengths of the other periods are more difficult to define. LH surge takes place during the first 6 to 12 hours of oestrus, initiating the processes leading to ovulation, which normally occurs during the first hours of the metoestrus, about 24 to 30 hours after the onset of oestrus. Corpus luteum (CL) begins to develop from the cells originating from the walls of the ovulated follicle, and after a couple of days it starts gradually produce more and more progesterone (P_4). At the end of dioestrus, the endometrium begins to secrete prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which causes CL regression, luteolysis. During prooestrus, the maturing dominant follicle produces gradually increasing oestradiol-17β, which causes, with simultaneous absence of P_4, behavioural and other signs of oestrus. Follicular growth during the oestrous cycle is recurrent, i.e. it occurs as so-called follicular waves. During a normal cycle, two or three (sometimes four) waves can be detected. Thus, smaller and larger, growing and regressing, follicles can be seen practically at any time during the cycle. The main endocrinological events during an oestrous cycle are illustrated in Figure 1. To achieve optimal pregnancy rates, AI should occur 6 to 24 hours before ovulation (Trimberger, 1948).

2. Gonadotropin-releasing hormone

Isolation and chemical characterisation of gonadotropin-releasing hormone in 1971 by Schally and Guillemin, who were awarded the Nobel Prize for these accomplishments, was probably one of the most significant occurrences in the research into the neuroendocrine control of reproduction. After this, the translation from basic discovery to clinical usefulness with various indications was swift in human medicine (Conn and Crowley, 1991).

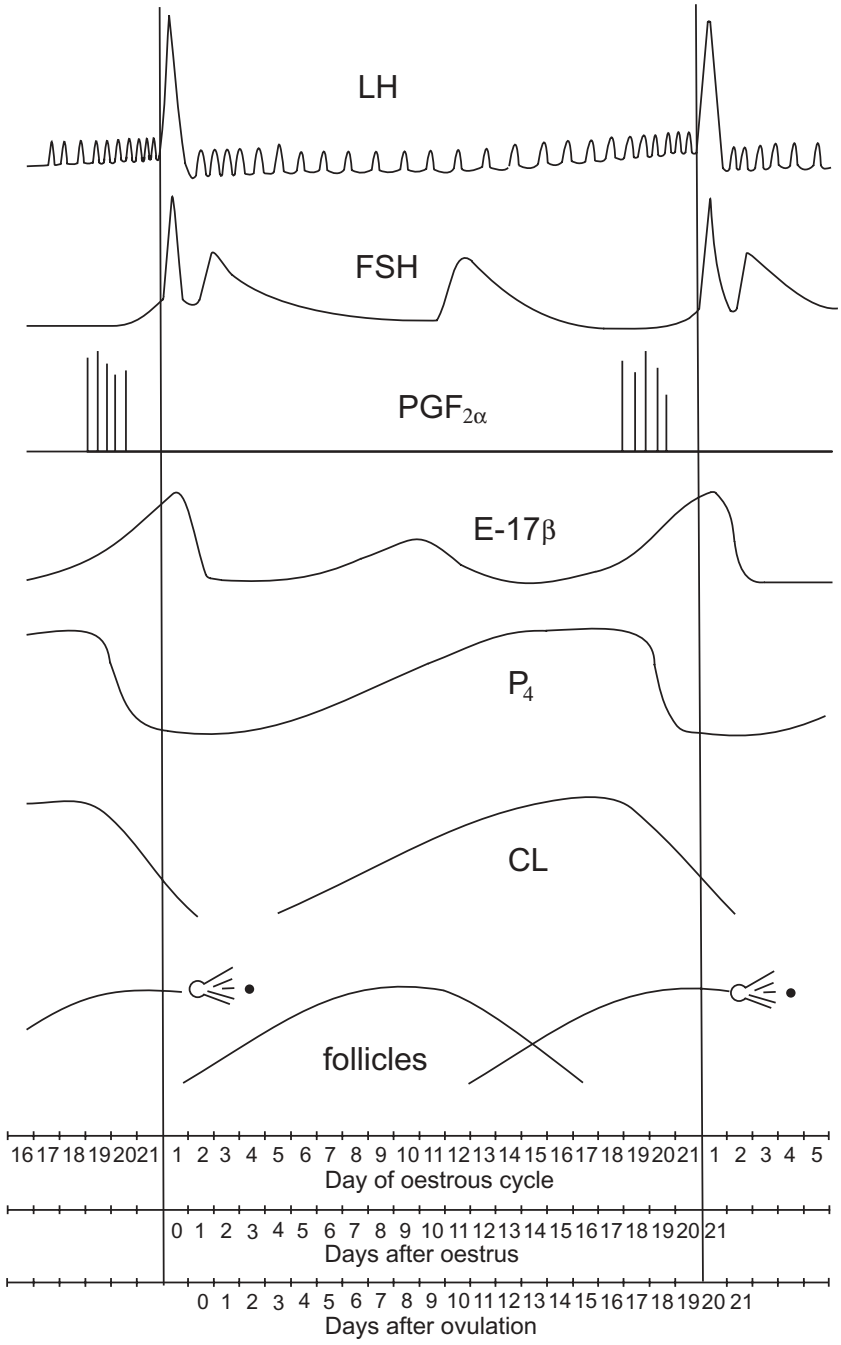

Figure 1. Schematic presentation of main endocrinological events during an oestrous cycle of cattle.

Basic research and clinical applications of gonadotropin-releasing hormone in veterinary medicine developed soon thereafter. Synthesis of a decapeptide having gonadotropin-releasing hormone activity (Geiger et al., 1971) made the hormone readily available for research on its use in animal reproduction. Since the release of pituitary gonadotropins after administration of GnRH was reported in cattle (Kaltenbach et al., 1974; Kinder et al., 1975), studies have been undertaken to evaluate its effect on ovarian function and on the oestrous cycle, e.g. on the luteinisation of ovarian cysts (Bierschwal et al., 1975; Cantley et al., 1975; Bentele and Humke, 1976; Christl, 1976; Garverick et al., 1976; Saalfeld and Hollmann, 1976; Seguin et al., 1976), on stimulation of follicular development (Mellin et al., 1975; Shareha et al., 1976), and on treatment of delayed ovulation (Bentele and Humke, 1976). The finding that ovulation could be induced by GnRH (Baker et al., 1973; Seeger and Humke, 1975) in a way similar to hCG (Puschel, 1974) suggested the feasibility of studying its effects on the fertility of artificially inseminated cows, since one of the major factors determining the success of artificial breeding is the proper timing of insemination in relation to ovulation.

GnRH is a straight-chain decapeptide (Figure 2) synthesised in cell bodies of neurosecretory neurons located in the mediobasal hypothalamus and transported by their axons to circumscribed areas of the median eminence. Here they are released into the capillary plexus of the portal vascular system and transported to the anterior pituitary gland (Beattie, 1982; Thatcher et al., 1993). GnRH has been found in the central nervous system of all species of vertebrates examined. Both the natural and synthetic decapeptides release LH and follicle stimulating hormone (FSH) and induce ovulation in all mammalian species so far examined. Passive immunization of a variety of mammalian species with an antiserum to GnRH reduces blood levels of both FSH and LH and blocks ovulation. Collectively, these observations indicate that the decapeptide represents the moiety responsible for the release of LH and FSH (Beattie, 1982). Plasma half-life of GnRH is short, two to four minutes (Bennett and McMartin, 1978; Handelsman and Swerdloff, 1986).

GnRH is a pyroglutamic acid peptide. The amino-terminal tripeptide and tetrapeptide fragments of GnRH do not stimulate gonadotropin release. The carboxy-terminal octapeptide and nonapeptide also have negligible gonadotropin-releasing activity. Individual amino acid substitutions drastically alter the gonadotropin-releasing activity of the native molecule. In general, the amino acids in positions 1 and 2 and from positions 4 to 10 appear to be involved only in the binding of GnRH to its target-tissue receptors and in exerting conformational effects. However, histidine-2 and tryptophan-3 exert an important functional

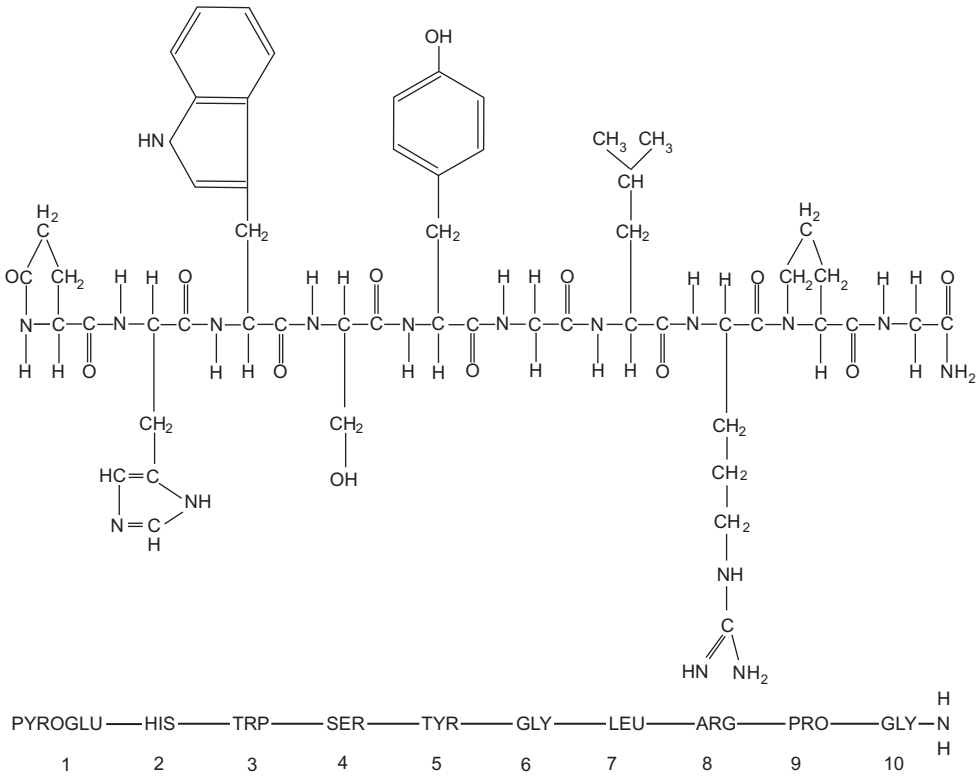

Figure 2. Chemical structure (amino acid sequence) of gonadotropin-releasing hormone (adapted from Beattie, 1982).

effect, since substitution of these L-amino acids with other D-amino acids produces analogues of GnRH with a significant loss in gonadotropin-releasing activity and even potent antagonistic properties. Deletion of positions 2 and 3 also leads to dramatic losses in gonadotropin-releasing activity (Beattie, 1982).

Alterations in the chemical structure of the native GnRH molecule have led to the synthesis of potent GnRH agonists. Substitutions usually involve replacement of the glycine molecules at positions 6 and 10 with a D-amino acid at position 6 and/or an N-ethylamide group at position 10 (Thatcher et al., 1993). The design of GnRH agonists has been directed toward stabilisation of the molecule against enzymatic attack, increasing binding to plasma proteins and membranes, and increasing the affinity of the agonist for the GnRH receptor (Conn and Crowley, 1991). At least the following GnRH analogues and GnRH agonists are or have been available commercially: gonadorelin (native-like GnRH; gonadorelin diacetate tetrahydrate or gonadorelin hydrochloride), buserelin (D-serine at

position 6 and ethylamide at position 10), fertirelin acetate (ethylamide at position 10) and deslorelin (D-tryptophane at position 6 and ethylamide at position 10). In Finland, two preparations are available at the moment: Fertagyl (gonadorelin 0.1 mg/ml; Intervet International B.V., The Netherlands) and Receptal (buserelin 4 µg/ml; Intervet International B.V., The Netherlands).

Owing to alterations in chemical structure, marked differences exist between the various GnRH analogues in their relative potencies to release LH and FSH in cattle (Nawito et al., 1977; Chenault et al., 1990). For example, fertirelin acetate was approximately four to ten times more potent than gonadorelin as measured by LH and FSH release during the luteal phase of the bovine oestrous cycle; buserelin was 50 times more potent than gonadorelin (Chenault et al., 1990). Based upon a carryover effect of a moderate dose followed by a low dose of these products, the pituitary's gonadotropes apparently become refractory to GnRH for up to 48 h after injection of doses greater than or equal to 50 µg fertirelin acetate, 500 µg gonadorelin and 10 µg buserelin. A single intramuscular (i.m.) injection of native GnRH and GnRH agonists gives a predictable release of both LH and FSH into the peripheral circulation over a 3 h period (Thatcher et al., 1993).

3. Applications of GnRH for reproductive management

Control of ovarian function and reproductive efficiency in cattle with GnRH has been studied intensively (review by Thatcher et al., 1993). The main clinical applications of GnRH treatments are as follows: treatment at the time of AI in normal and repeat breeder cows to improve fertility, treatment of cystic ovarian disease, treatment of anoestrus, oestrus synchronisation programmes, post-insemination treatments to improve fertility, postpartum reproductive management to improve fertility later at the breeding period and treatment of embryo transfer recipients to enhance embryo survival. In the present review, only the treatments during oestrus, at the time of AI, are discussed in detail.

4. GnRH treatments during perioestrus and at the time of AI to improve fertility

4.1. The effect of GnRH treatments on pregnancy rate

A considerable number of studies have examined whether GnRH treatments given at the time of AI are able to improve pregnancy rates in cattle. Approximately 49 experiments from 36 published research articles that reported clinical trials in which GnRH was used to treat dairy cattle (one trial with beef cattle) at the time of AI are summarised in Tables 1 and 2. The studies were collected from scientific journals published from 1974 on, thus covering practically the entire history of the use of GnRH in veterinary clinical trials. Probably the majority of the studies dealing with this topic are reviewed here, with exception of perhaps some in national journals and papers published in languages other than English. Because it is obvious that the serial number of AI modifies the effect of the treatment (Mee et al., 1990; Stevenson et al., 1990), the studies have been divided into those where the treatment was given at the first or second AI postpartum (Table 1) and in repeat breeders (cows not conceiving after at least two previous inseminations; Table 2).

In 19 of 31 experiments where GnRH treatment was given at the time of first or second AI, native GnRH (or gonadorelin) was used, while the remaining 12 experiments utilised agonistic analogues, buserelin, fertirelin acetate and sulfagon. The doses generally varied around 100 μg of native GnRH or corresponding agonistic analogues. Only in a few experiments were remarkably larger doses used. Generally, the experimental animals were parous cows; only in five trials were some, or all, animals heifers. The treatment was usually given at the time of AI; in five experiments, the treatment was given before AI, but never more than 15 hours before. In most experiments, a numerically increased pregnancy rate after GnRH treatment was detected, but the difference was statistically significant only in six trials (Table 1). Each of these six trials involved more than 200 animals. In one trial, a negative effect of GnRH on pregnancy rate was found when the statistical significance was calculated separately between each treatment and the control group (Chenault, 1990; Table 1).

Mee et al. (1990) reviewed 16 studies (17 experiments; all of these included in Table 1) reporting the administration of various doses of GnRH to dairy cattle at the time of AI or 0 up to 6 hours preceding AI. A significant (6 trials) or nonsignificant (5 trials) advantage in pregnancy rate was reported, whereas others (6 trials) reported no benefit of GnRH administration on pregnancy rates at first

service. Overall, the studies demonstrated an advantage in pregnancy rate of 6 percentage points (53 vs. 59%) or an 11% improvement for cows treated with GnRH at the time of first AI compared with untreated controls. Based on these studies and their own data, Mee et al. (1990) concluded that GnRH administration to dairy cattle at first services postpartum fails to improve pregnancy rates and that it is inadvisable to recommend the use of GnRH at first services. Based on the review by Mee et al. (1990), Thatcher et al. (1993) came to the conclusion in their review article that, as changes in pregnancy rates varied among studies in first service dairy cows from -7 to +17%, it is difficult to accept that GnRH is eliciting a predictable increase in fertility that can be reliably applied from herd to herd.

Repeat breeding has considerable impact on the economy of dairy farmers (Bartlett et al., 1986; Lafi and Kaneene, 1992; Lafi et al., 1992) and has been the subject of a number of reviews (Casida, 1961; Ayalon, 1978; Hawk, 1979; Ayalon, 1984; Levine, 1999) presenting diverse explanations for the syndrome. In a review by Ayalon (1984), incidence of repeat breeding problems in various countries ranged from 10 to 18%. In Finland, however, according to the strict definition that follows, the incidence was only 1.0%, but when the criterion was only more than three AIs, the incidence was 7.5% (Rautala, 1991). The current definition of repeat breeding includes pregnancy failures occurring after three or more AIs performed at oestrus with normal inter-oestrus intervals in the absence of detectable abnormalities (Zemjanis, 1980). However, in most studies dealing with the effect of GnRH given at the time of AI in repeat breeders, they were classified as cows that return to oestrus for a third or further service, and without any other definitions.

Altogether the results of 18 experiments that studied the effect of GnRH given at the time of AI in repeat breeders are shown in Table 2. In all of these, with two exceptions, native GnRH (or gonadorelin) was used. The dosages used were also consistent, viz. 100 µg of native GnRH or agonistic analogue corresponding to this, again with only two exceptions. The treatment was usually given at the time of AI, in three experiments 0 up to 15 hours before AI. In 14 experiments, a numerically increased pregnancy rate after GnRH treatment was detected; in five of these the increase was significant. All five of these were among those eight trials using more than 300 AIs. In three experiments, a numerical, but not significant, decrease in pregnancy rate, 9 to 15 percentage points, was detected.

Stevenson et al. (1990) reviewed eight studies (nine experiments) where GnRH was used as a treatment for repeat breeding at the time of, or 0 to 12 hours preceding, AI (all of these are included in Table 2). Collectively, these studies

Table 1. Summary of studies where GnRH treatment was given at first or second AI. If a study included several treatment groups (e.g. several doses), the results for the groups are expressed in separate rows but the result for a control group is expressed only in the first row.

Study	Order of AI	Treatment		No. herds	No. AI	Pregnancy rate				P value
		GnRH	Dose, µg			Controls	%	Treated	%	
Günzler et al. (1974)	I	native	1000	Many	200	55/100	55.0	56/100	56.0	n.s.
Goldbeck (1976)	I	native	1000	Many	214	73/107	68.2	87/107	81.3	<0.05[14]
Günzler et al. (1976)	I	native	400	Many	200	59/100	59.0	70/100	70.0	n.s.
Günzler et al. (1976)	II	native	400	Many	200	60/100	60.0	71/100	71.0	n.s.
Grünert et al. (1978)	I [13]	native	1000	?	278	95/140	67.9	112/138	81.2	<0.01/<0.05[15]
Mori and Takahashi (1978)	I ?	fertirelin	100	1	59	19/31	61.3	21/28	75.0	n.s.
Schels and Mostafawi (1978)	I	gonadorelin	125	1	218	54/109	49.5	64/109	58.7	n.s.
Leidl et al. (1979)	I	buserelin	10	Many	397	124/220	56.4	118/177	66.7	<0.05
Leidl et al. (1979)	II	buserelin	10	Many	180	50/85	58.8	56/95	58.9	n.s.
Vahdat and Whitmore (1980) [1]	I	native	100	?	96	27/48	56.3	26/48	54.2	n.s.
Westhuysen (1980) [2]	I	buserelin	20	?	76	17/32	53.1	29/44	65.9	<0.05/n.s.[15]
Holtemöller (1981)	I	buserelin	10	4	119	23/54	42.6	34/65	52.3	n.s.[13]
Moller and Fielden (1981) [3]	I	buserelin	10	3	576	139/284	48.9	170/292	58.2	<0.05
Nakao et al. (1983)	I	fertirelin	100	Many	1194	293/589	49.7	346/605	57.2	<0.05/<0.01[15]
Lee et al. (1983)	I	native	100	3	300	58/146	39.7	76/154	49.4	n.s.[14]
Lee et al. (1983) [2]	I	native	100	3	185	64/93	68.8	59/92	64.1	n.s.
Fielden and Moller (1983) [3,4]	I	buserelin	10	7	1302	371/647	57.3	414/655	63.2	<0.05
Stevenson et al. (1984)	I	native	100	1	328	83/182	45.6	69/146	47.3	n.s.
Stevenson et al. (1984)	II	native	100	1	204	48/104	46.2	55/100	55.0	n.s.
Lee et al. (1985)	I	native	100	1	24	6/12	50.0	6/12	50.0	n.s.
Anderson and Malmo (1985) [3]	I	gonadorelin	250	25	3502	1529/2828	54.1	396/674	58.8	n.s.[16]
Anderson and Malmo (1985) [3]	II	gonadorelin	250	25	1242	654/1156	56.6	50/86	58.1	n.s.
Pennington et al. (1985) [5]	I	native	100	2	313	79/152	52.0	82/161	50.9	n.s.
Pennington et al. (1985) [5]	II	native	100	2	141	37/69	53.6	37/72	51.4	n.s.
Lucy and Stevenson (1986) [6]	I	native	100	1	109	14/55	25.5	23/54	42.6	n.s.
Alacam et al. (1986)	I ?	analogue	25	?	69	23/36	63.9	27/33	81.8	<0.01/n.s.[15]
Dmitriev et al. (1986)	I ?	sulfagon	10	?	100	26/54	48.1	26/46	56.5	n.s.[14]

Coleman et al. (1988) [7]	I	fertirelin	50	?	86	20/28	71.4	23/30	76.7	n.s.
		fertirelin	100					23/28	82.1	n.s.
Lewis et al. (1990) [8]	I	native	100	2	258	60/127	47.2	58/131	44.3	n.s.
Lewis et al. (1990) [8]	II	native	100	2	126	27/66	40.9	27/60	45.0	n.s.
Mee et al. (1990)	I	native	100	1	300	44/117	37.6	34/105	32.4	n.s.[14]
Mee et al. (1990) [9]	I	native	100	1	169	29/67	43.3	23/50	46.0	n.s.
Chenault (1990) [10]	I + II	fertirelin	25	10	1447	117/243	48.1	105/240	43.8	n.s.
		fertirelin	50					100/242	41.3	n.s.
		fertirelin	75					100/240	41.7	n.s.
		fertirelin	100					95/242	39.3	n.s./<0.05[15]
		buserelin	10					95/240	39.6	n.s.
Valks (1996) [11]	I	gonadorelin	250	292	2704	922/1355	68.0	958/1349	71.0	n.s.
Valks (1996) [11]	II	gonadorelin	250	292	989	310/480	64.6	343/509	67.4	n.s.
Ullah et al. (1996) [12]	I	native	100	3	94	8/45	17.8	14/49	28.6	n.s.
Vamerzani et al. (1999) [1]	I+II	buserelin	10	?	35	9/20	45.0	12/15	80.0	n.s.

? Information not available in the reference.
[1] Cows and heifers.
[2] Heifers.
[3] Treatment given 0-6 h before AI.
[4] Includes the material of Moller and Fielden (1981).
[5] In the groups, 23% and 25% were heifers, respectively. Treatment was given on average 6-7 h before AI.
[6] Treatment given 8 h before AI.
[7] Beef cattle.
[8] Approx. 1/3 were heifers.
[9] Treatment given 11-15 h before AI.
[10] First and second inseminations.
[11] Instead of pregnancy rate, non-return rate 56 days was calculated.
[12] Treatment given 10-12 h before AI.
[13] First AI 80%.
[14] Information not available. Calculated here with Chi-square test.
[15] Information in the reference / Calculated here with Chi-square test.
[16] After adjusting for potential confounding variables; uncorrected $P<0.05$.

demonstrated an overall advantage in pregnancy rate of seven percentage points, corresponding to an 18% increase, for repeat breeders treated with GnRH compared with untreated repeat breeders. According to their own findings, they consistently observed an increase in pregnancy rates after GnRH treatment of repeat breeders in three experiments involving cows with either normal or abnormal periparturient conditions in seven different herds (Stevenson et al., 1984; Phatak et al., 1986; Stevenson et al., 1988). They concluded that the decision to utilise GnRH for repeat breeders in any herd should be weighed against the possibility that its efficacy has not been validated consistently in all herds. However, the preponderance of studies with repeat observations in many locations verifies the benefit of GnRH as an effective treatment for repeat breeders.

Tables 1 and 2 show that the results of the studies of the effect of GnRH given at AI on pregnancy rates are inconsistent. This has led to many trials being performed to clarify responses. While most experiments demonstrated an increased pregnancy rate, few trials reached statistical significance. For many trials, lack of significance can be attributed to insufficient sample size (Morgan and Lean, 1993). Meta-analytical techniques have been used to combine data from many studies into a single analysis (L'Abbe et al., 1987). This is a potentially powerful technique for evaluating conflicting clinical trial results and is well suited to resolving concerns about the efficacy of treating cows with GnRH at insemination to improve fertility (Morgan and Lean, 1993). In the study by Morgan and Lean (1993), data from 40 trials described in 27 published papers were subjected to meta-analysis to evaluate the effects of such treatments on a total of 19,019 cows involved in these studies. Morgan and Lean stratified these data by trial and by the effects of dose of GnRH, type of GnRH and number of times inseminated (first, second, or third and greater that were classified as repeat breeders) into five groups as follows: eight trials used GnRH agonistic analogues (buserelin, fertirelin acetate, sulfagon) at first AI, 10 trials used doses of native GnRH of less than 125 µg at first AI, 3 trials used doses of native GnRH greater than, or equal to, 250 µg at first AI, 8 trials investigated the effect of native GnRH or its agonistic analogues at second AI, and 11 trials evaluated the effect of native GnRH or its agonistic analogue on repeat breeders. The collected information was used to calculate relative risk and 95% confidence intervals for relative risk of pregnancy in treated cows using Mantel-Haenszel test. Mantel-Haenszel relative risk estimation is a means of evaluating pooled data that allows one to control for the effect of stratification and is an appropriate method for meta-analysis (L'Abbe et al., 1987).

Table 2. Summary of studies where GnRH treatment was given at the time of the third or subsequent insemination.

Study	Treatment		No. herds	No. AI	Pregnancy rate				P value
	GnRH	Dose, µg			Controls		Treated		
						%		%	
Leidl et al. (1979)	buserelin	10	Many	119	24/49	49.0	38/70	54.3	n.s.[8]
Lee et al. (1983)	native	100	26	346	77/161	47.8	135/185	73.0	<0.001
Stevenson et al. (1984)	native	100	1	97	27/53	50.9	29/44	65.9	n.s.
Anderson and Malmo (1985) [1]	gonadorelin	250	25	361	160/302	53.0	26/59	44.1	n.s.
Pennington et al. (1985) [2]	native	100	2	92	20/43	46.5	27/49	55.1	n.s.
Phatak et al. (1986)	native	100	5	961	177/469	37.7	231/492	47.0	<0.01
Roussel et al. (1988)	native	100	9	379	38/96	39.6	158/283	55.8	<0.01
Stevenson et al. (1988)	native	100	1	140	40/103	38.8	20/37	54.1	n.s.
Stevenson et al. (1988)	native	100	1	513	108/344	31.4	74/169	43.8	<0.05
Lewis et al. (1990) [3]	native	100	2	61	13/29	44.8	11/32	34.4	n.s.
Swanson and Young (1990)	native	100	3	127	36/65	55.4	25/62	40.3	n.s.
Stevenson et al. (1990)	native	100	6	759	112/353	32.1	165/406	41.6	<0.05[8]
BonDurant et al. (1991)	native	100	14	963	184/468	39.3	214/495	43.2	n.s.
Mee et al. (1993)	native	[7]	1	44	2/14	14.3	13/30	30.2	n.s.
Archbald et al. (1993)	native	100	1	281	45/139	32.4	47/142	33.1	n.s.
Archbald et al. (1993) [4]	native	100	1	236	40/129	31.0	43/107	40.2	n.s.
Valks (1996) [5]	gonadorelin	250	292	411	132/204	64.7	139/207	67.1	n.s.
Vamerzani et al. (1999) [6]	buserelin	10	?	35	8/20	40.0	10/15	66.7	n.s.

[1] Treatment given 0-6 h before AI.
[2] In the groups, 23% and 25% were heifers, respectively. Treatment was given on average 6-7 h before AI.
[3] Approximately 1/3 were heifers.
[4] Treatment given about 9 h before AI.
[5] Instead of pregnancy rate, non-return rate 56 days was calculated.
[6] Cows and heifers.
[7] 50 µg, n=8; 100 µg, n=14; 250 µg, n=8
[8] Information not available. Calculated here with Chi-square test.

Although Morgan and Lean (1993) used strictly defined criteria in making their meta-analysis, they made numerous errors in selecting the trials and in reporting the number of cases in each of the five groups. The experimental design used in one of the papers included in the group dealing with GnRH agonistic analogues at first AI does not match the topic of the meta-analysis. In addition, data presented in a study was reported again, along with additional data, in a later study; however Morgan and Lean (1993) treated these two data sets as entirely separate. In their study of low dose of GnRH at first AI, Morgan and Lean (1993) reported only some of the data of two trials, and another paper is so unclear that it may have been impossible for them to refer to it accurately. In their study of native GnRH or its agonistic analogue on repeat breeders, Morgan and Lean (1993) included only some of the data from one paper, and in one instance they included data from a second insemination by referring to the wrong table. The worst situation is in that portion of the meta-analysis which deals with the use of native GnRH or its agonistic analogues at second AI. Four of the eight trials reported here do not study the effect of GnRH given at second AI, but actually the carry-over effects of GnRH treatments given at first AI.

Despite these many errors, it is obvious that they do not bias the results because the number of studies and animals referred is so large and the errors seem to be distributed quite evenly. However, the results of the meta-analysis involving native GnRH or its agonistic analogues at the second AI should be interpreted with caution. The analysis of pooled data of all studies revealed that the use of GnRH at insemination significantly ($P<0.05$) increased the overall risk of pregnancy by 12.5% in treated cows when compared to the controls. There was a significant ($P<0.05$) increase in risk of pregnancy in cows treated with GnRH agonistic analogues at the first AI (increase in risk of 8.0%) or high doses (≥250 µg) of native GnRH at first AI (11.0%), at second AI (9.9%) and in repeat breeder cows (22.5%). Risk of pregnancy from treatment with low doses (<125 µg) of native GnRH at the first AI (5.2%) was not significantly different from that of the control group. However, the relative risk of pregnancy after low doses was similar to that of GnRH agonistic analogues, second AI and high doses of native GnRH categories. The relative risks with 95% confidence intervals for pregnancy after GnRH treatments at AI for these five studies and for overall combined studies are presented in Figure 3. The authors concluded that GnRH treatment at insemination does increase the risk of pregnancy. Results obtained from this analysis were relatively homogenous, except that repeat breeder cows responded better to treatment than the other groups (Figure 3). Individual trials that had extreme results tended to be small and therefore had wide confidence intervals.

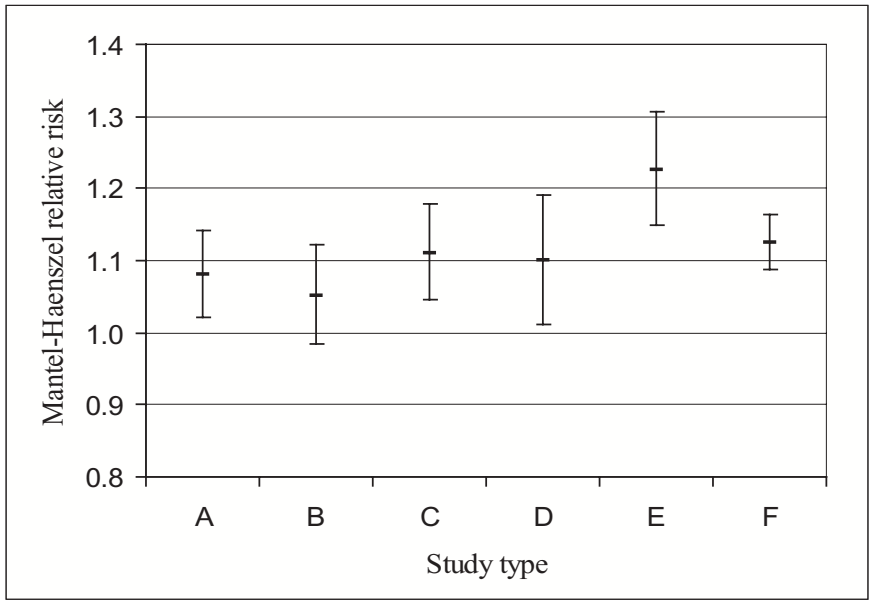

Figure 3. Mantel-Haenszel relative risk and 95% confidence interval for pregnancy after GnRH treatment at AI for five studies (A to E) and for overall combined studies (F). Study A: GnRH agonistic analogues given at first AI, B: low doses (<125 µg) of native GnRH given at first AI, C: high doses (≥250 µg) of native GnRH given at first AI, D: native GnRH or its agonistic analogues given at second AI, E: native GnRH or its agonistic analogues given to repeat breeders at AI, F: all studies combined. A relative risk of >1.0 indicates that pregnancy rates were higher for GnRH-treated cows (redrawn from Morgan and Lean, 1993).

Repeat breeding may be viewed as an effect modifier for the relationship between GnRH treatment and fertility. This observation supports the conclusion that some cattle populations benefit more from treatment than others.

According to Thatcher et al. (1993) the concept of treating repeat breeder cows with GnRH has become clouded because of difficulties in defining a true class of infertile cows and uncertainty as to whether they respond specifically to GnRH. A component of the traditional definition of a repeat breeder, as defined by Casida (1961), is that semen quality and insemination circumstances should be optimal in identifying this class of cows, which have greater rates of defective ova, ova loss, fertilization failure and embryonic loss. If cows with at least three AIs are defined as repeat breeders, it is critical that such management factors as accurate oestrous detection, timing of insemination, handling of semen and inseminator skills are

optimal, and that cows with palpable abnormalities of the reproductive tract are excluded. This array of management factors undoubtedly contributes to the herd-to-herd variability in the success of GnRH treatments.

In an economic assessment under American conditions, Weaver et al. (1988) concluded that herds with conception rates of 45% or less benefited from GnRH treatment at first or second AIs; herds with conception rates of 60% or over benefited from GnRH treatment only at second or later AIs. According to Morgan and Lean (1993), GnRH treatments at the time of AI reduced the number of days open in the following way: GnRH agonistic analogues used at the time of first AI, 1.7 days; low doses (<125 μg) of native GnRH given at first AI, 1.1 days; high doses (≥250 μg) of native GnRH given at first AI, 2.3 days; and native GnRH or its agonistic analogues given to repeat breeders at AI, 4.7 days. It has been estimated that in Finnish conditions the lengthening of one day in calving interval from 11 months causes an economic loss of about 1.0 to 1.3 • (J. Taponen, unpublished). Because of veterinary and drug expenses, it is questionable whether this treatment is cost-effective in Finland, even in repeat breeders.

In conclusion, it seems obvious that administration of GnRH and its agonistic analogues at the time of AI does increase pregnancy rates. The magnitude of the increase is a function of the serial number of AI, being greatest in repeat breeder cows. However, it is important to understand the differences among locations, herds, management and physiological states that contribute to the differential fertility responses to GnRH treatments (Thatcher et al., 1993).

4.2. Mechanisms and factors through which GnRH may modify fertility

GnRH treatment at the time of AI may enhance fertility in a number of ways. However, the mechanisms through which GnRH may modify fertility have not been elucidated (Morgan and Lean, 1993). Treatment with GnRH during oestrus may influence time of ovulation, fertilisation rates, CL development, P_4 secretion, and embryonic survival (Stevenson et al., 1984). GnRH likely acts by effecting the release of FSH and LH, but its direct effect on the reproductive tract cannot be ignored, in view of the fact that GnRH-like molecules have been isolated from ovarian follicles (Ying et al., 1981). Furthermore, ovulation can be induced in hypophysectomised rats following GnRH administration (Corbin and Bex, 1981).

The finding that ovulation can be induced by GnRH (Baker et al., 1973; Seeger and Humke, 1975) led to numerous studies of its effects on the fertility of artificially inseminated cows, since one of the major factors determining the

success of artificial breeding is the proper timing of insemination in relation to the rupture of the ovulatory follicle (Schels and Mostafawi, 1978). To achieve satisfactory pregnancy rates, optimal timing of the deposition of semen and occurrence of ovulation is of utmost importance because of the relatively brief fertile life of both sperm and ovum. Moreover, timing of insemination is under the direct control of man and does not depend on the sexual receptivity of the animal. Therefore the possibility of delays in fertilisation is increased. High conception rates can be expected only if insemination takes place 7 to 18 hours before ovulation (Trimberger and Davis, 1943). This may be impeded if oestrus onset is not observed or if there are physiological or pathological variations of follicle rupture and ovum release (Schels and Mostafawi, 1978). It has been suggested that GnRH-induced ovulation may provide greater synchrony between the time of insemination and the time of ovulation (Moller and Fielden, 1981; Stevenson et al., 1988).

Since the GnRH peak normally occurs near the onset of oestrus (Mori et al., 1974), an injection of GnRH before AI would be more likely to correct an asynchrony of breeding and ovulation than an injection of GnRH at AI (Pennigton et al., 1985). Stevenson et al. (1984) suggested that conception rates increased when cows were treated in the first 5 to 8 hours of oestrus. Therefore, accuracy of heat detection and management could influence the magnitude of response to GnRH treatment. The findings of Rosenberg et al. (1991) and Mee et al. (1990) indicated that timing GnRH injections close to detection of oestrus contributes to the success of GnRH treatment at first postpartum inseminations in cows with low conception rates. In cows inseminated two to four times, GnRH injections close to detection of oestrus needed to be coupled with inseminations 4 to 30 hours after detection of oestrus to obtain an increase in fertility as opposed to early inseminations, less than 3 hours from the detection of oestrus (Rosenberg et al., 1991). GnRH administration early in oestrus and AI 12 to 15 hours later gave better results than the treatment at the time of AI, although not better than in control (Mee et al., 1990). The role of GnRH in indirectly altering the rate or efficiency of oocyte maturation in low fertility cows warrants study (Thatcher et al., 1993).

The effect of GnRH given at oestrus on the subsequent P_4 concentration and its possible influence on fertility through this mechanism has been studied and discussed widely. BonDurant et al. (1991) proposed that GnRH may reduce early embryonic mortality by enhancing the luteinisation of thecal and granulosa cells through the increased LH surge. The subsequent increase in P_4 secretions may improve maternal recognition of the conceptus. Maurer and Echternkamp (1982) found that cows with higher preovulatory LH surges and more rapid rise of P_4

after ovulation showed higher embryonic survival. Also Lee et al. (1985) and Ullah et al. (1996) proposed that higher pregnancy rates in cows injected with GnRH are probably the result of detected increased P_4 production and enhanced embryo survival. In addition, Mee et al. (1993) found that higher P_4 levels were due to an increased proportion of large luteal cells in the CL and possibly due to increased concentration and pulse frequency of FSH secretion. However, Lewis et al. (1990) did not detect any significant increase in P_4 concentrations after GnRH treatment, and Lucy and Stevenson (1986) found that injection of GnRH during perioestrus, either shortly before or concurrent with the preovulatory surge of LH, attenuated subsequent progesterone concentrations in serum during the first 7 days of the oestrous cycle in repeat breeders. Based on this finding they concluded that improved fertility detected in their study may be associated with delayed or slowly rising P_4 concentrations after ovulation. Despite the variable nature of results, it is obvious that GnRH increases P_4 production, in view of few results to the contrary.

Based on their meta-analytical study, Morgan and Lean (1993) concluded that the type of GnRH (native or agonistic analogue) used at the time of AI did not greatly influence the risk of pregnancy for cows at first service. Both groups had very similar relative risks and confidence intervals (Figure 3). GnRH dosage may influence results slightly, as all higher dose studies showed a positive fertility effect from GnRH treatment, but relative risk of pregnancy did not differ markedly from that achieved with lower doses of GnRH.

There are many other sources for considerable variations in the results of experiments studying the effect of GnRH administered at AI on pregnancy rate, and not nearly all of them have been elucidated. Geographic location, physiological status of cows, breed of cattle, nutrition, herd management and overall herd fertility have also been proposed as factors contributing to the variation of results (Morgan and Lean, 1993). BonDurant et al. (1991) ascribed part of the positive result in their study to an improvement in management practices at the farms involved in the experiment. The most positive responses to treatment were recorded in cattle that had high daily milk production at the time of AI (>25 kg, Grünert et al., 1978; >30 kg, Goldbeck, 1976), but contrary to this, Holtemöller (1981) and Nakao et al. (1983) detected the best response in the middle level (20 to 25 kg and 25 to 30 kg, respectively) and no response in the highest level (>25 kg and >30 kg, respectively). Lee et al. (1983) found no effect of milk production. In addition, the effect of the length of time from calving to first AI has been inconsistent. The best treatment effect has been found in cattle mated relatively early, 4 to 6 weeks, after calving (Grünert et al., 1978; Goldbeck, 1976). However, Nakao et al. (1983) observed a greater response to treatment

when cows were mated after 101 days postpartum, and Moller and Fielden (1981), Lee et al. (1983) and Chenault (1990) did not detect any influence. Young cows (parity 1 to 2 or 3) seem to benefit more from the GnRH treatment than older cows (Grünert et al., 1978; Holtemöller, 1981; Nakao et al.; 1983). However, no effect has been detected on heifers, probably due to the fact that they are not exposed to the type of physiological demands and changes placed on lactating cows (Lee et al., 1983). Overall herd fertility has been proposed to have a great impact on the effect of GnRH treatment. Coleman et al. (1988) have summarised that treatment with GnRH at AI might be more beneficial in herds with below-average conception rates or in identified low-fertility cows compared with herds or cows of average or above-average fertility. The better response to GnRH treatments seen in repeat breeder cows may be explained by this fact, since the fertility of these cows is generally low. In the studies summarised in Table 2, the initial pregnancy rates of the study populations, repeat breeders, were below 50% in 14 of 18 trials and even below 40% in 7 trials.

5. Possible negative effects of GnRH administered during perioestrus

Although the effect of GnRH treatment given at the time of AI on pregnancy rate has been mainly positive at least numerically, ten of 55 trials (or subgroups of trials) showed a lower pregnancy rate in treated animals than in control animals (Tables 1 and 2). Although the decrease was generally small, two studies showed a significant negative effect of GnRH on pregnancy rate (Mee et al., 1990; Chenault, 1990). In Mee et al. (1990), where both the timing of GnRH treatment and the timing of AI (early / late after first detected oestrus) were studied, only treatments of GnRH early in oestrus and AI late in oestrus were found to restore pregnancy rates to control values. In the other groups, pregnancy rates were lower than in control group. The authors concluded that this treatment contrast illustrates an inhibitory effect of GnRH on pregnancy rates, based on its timing relative to the onset of oestrus or the onset of the preovulatory surge of gonadotropins.

When GnRH treatment was used in combination with oestrus induction using PG treatment, some evidence was detected of possible negative effects of GnRH on subsequent luteal function or fertility. Stevens et al. (1993) administered GnRH simultaneously with PG on day 8 or 10 of the oestrous cycle. Cows that ovulated prematurely (day 8: 4/8, day 10: 1/8) failed to develop and maintain a fully functional CL, and all returned to oestrus 7 to 13 days after the induced ovulation. In addition, Schmitt et al. (1996) reported a significantly lower pregnancy rate

after timed AI following GnRH, when GnRH was injected 24 h after PG, compared to AI at detected oestrus after PG. No difference was found when GnRH was given 48 h after PG.

Despite intensive research on the effects of GnRH, very few studies have been published on GnRH treatments given during the first days after ovulation in cattle. Macmillan et al. (1986) reported a reduction in pregnancy rate by 10.9% units when buserelin (5 µg) was administered 1 to 3 days after insemination. Ford and Stormshak (1978) found significantly reduced P_4 concentrations when native GnRH (100 µg) was given 55 h after detected oestrus. Later, Rodger and Stormshak (1986) and Martin et al. (1990) studied the influence of native GnRH (100 µg) given on day 2 post-oestrus (day of oestrus = day 0) on subsequent luteal function. In both studies, a tendency toward reduced P_4 concentrations was detected. It has been shown that early embryonic loss is probably associated with a complex endocrine syndrome, which could be manifested by a lower capacity to synthesise and/or release CL-progesterone (Lamming et al., 1989). It is, however, questionable that the reduced fertility reported by Macmillan et al. (1986) can be explained by an impaired luteal function, because the reasons behind the decline of P_4 concentration caused by the GnRH given during metoestrus are not yet known.

AIMS

The overall aim of the present thesis was to find and characterise possible negative effects of GnRH treatments given during perioestrus on reproductive functions, with special focus on the prostaglandin-induced oestrus period, and their effects on the ovulatory follicle, ovulation, subsequent follicular growth, and luteal function. The specific aims of the studies were as follows:

- to investigate, under controlled study conditions, the influence of exogenous GnRH on oestradiol secretion of the ovulatory follicle, on occurrence of ovulation, development and function of the corpus luteum and growth of a dominant follicle after ovulation, when GnRH treatment was given before the expected physiological LH surge;

- to investigate, under controlled study conditions, the influence of exogenous GnRH given shortly after ovulation on the development and function of the corpus luteum and the length of the subsequent oestrous cycle;

- to confirm, under field conditions, our earlier findings that PG treatment given 8 days after oestrus followed by administration of GnRH 24 h later can cause remission of luteal function and, thus, short oestrous cycles;

- to study the effects of PG treatment alone before mid-diestrus, and PG+GnRH treatment on oestrous signs under farm conditions;

- to confirm, under controlled study conditions, that CL regression in normally cycling dairy heifers seen during short oestrous cycles induced with cloprostenol and GnRH 24 h apart is caused by a premature release of $PGF_{2\alpha}$ (monitored by its main metabolite, 15-ketodihydro-$PGF_{2\alpha}$), and further, to study the $PGF_{2\alpha}$ release pattern more closely to determine whether it resembles the spontaneous release occurring during normal luteolysis or whether $PGF_{2\alpha}$ is continuously secreted after the induced ovulations leading to short oestrous cycles.

MATERIALS AND METHODS

Two types of studies were included in the present work. Firstly, three experiments were carried out in controlled environments at research farms at the College of Veterinary Medicine, Department of Obstetrics and Gynaecology, Hautjärvi, Finland (Studies I and II) and at the University of Helsinki, Viikki Research Farm (Study IV). Secondly, in the field study, data were collected on 19 commercial dairy herds on typical Finnish family-managed dairy farms (Study III).

1. Animals

A total of 102 animals, heifers and cows, were used in these experiments. Number of animals in each study, breed, parity, age of the heifers at the beginning of the experiment and interval from calving to the beginning of the experiment in the cows are presented in Table 3. All animals had shown normal oestrous cycles and were clinically healthy. They were stanchioned and fed grass silage, dry hay, and corn according to Finnish standards (Studies I, II, IV). In the field study (Study III), the housing and management practices were not strictly monitored, but were according to Finnish standards.

2. Experimental design

The effects of GnRH - when given during prooestrus (I) or during metoestrus (II) - on ovulation, subsequent follicular growth, luteal function and cycle length were investigated in the two first experiments. In addition, the purpose was to undertake a closer examination of the short oestrous cycles observed in Study I: their appearance (III) and mechanisms behind these cycles (IV). In Studies I, III and IV, where the effects of GnRH given during prooestrus (0 to 2 days before LH surge) were studied, PG was administered on days 6 to 9 after ovulation in order to induce prooestrus. In Studies I, II and IV, PG treatment was used also to induce oestrous cycle synchronisation. In all of these PG treatments, a single i.m. injection of 0.5 mg of cloprostenol [Estrumat^{R} 0.25 mg/ml, Mallinckrodt Veterinary Ltd., Harefield, Uxbridge, England (I, II, III); Estrumat^{R} 0.25 mg/ml, Schering-Plough A/S, Denmark (IV)] was administered.

In all studies, gonadorelin (Fertagyl^{R} 0.1 mg/ml, Intervet International B. V., Boxmeer, Holland) was used as a GnRH treatment. The dose was 100 µg i.m. in Studies I, III and IV, while in Study II, 250 µg i.m. was used. GnRH is used in the text to indicate gonadorelin treatment.

Table 3. Number of animals in each study, breed, parity, age of the heifers at the beginning of the experiment and interval from calving to the beginning of the experiment in cows.

Study	Number of animals	Breed	Parity	Age at beginning of experiment, months	Interval from calving to experiment, months
I	6 heifers 3 cows	Ay	1-2	18-21	3-6
II	6 heifers 3 cows	Ay	1-2	12-15	9-13
III	60 cows	Ay (50) HFr (10)	1-5, average 2.6		2-15, average 6.1
IV	24 heifers	Ay		11-20	

In Studies I, II and IV, transrectal ultrasonographic examinations of the ovaries were performed once daily after PG treatment to monitor ovulation. After this, ovarian structures were followed during the first half of the subsequent oestrous cycle (I, IV) or throughout the subsequent oestrous cycle (II). Blood (I, II, IV) or whole milk (III) samples were collected for hormone determinations. An overall summary of the treatments, ultrasonographic examinations and samplings in all studies are presented in Table 4.

2.1. GnRH during induced prooestrus (I)

The experiment included GnRH treatments at three different time points (T24, T48, T72) and one control manipulation (C). Every animal was assigned once to each of these four manipulations. From day 5 or 6 after ovulation, daily transrectal ultrasound examinations were carried out to determine the day when the dominant follicle ceased to grow. On day 8 - or if the dominant follicle had continued to grow between days 7 and 8 - on day 9 after ovulation, PG was administered to induce luteolysis. After this, the animals were given GnRH as follows: treatment 1) 24 h after PG (T24; in article I called T1), 2) 48 h after PG (T48; T2), 3) 72 h after PG (T72; T3), or 4) no gonadorelin (control manipulation, C).

Ultrasonographic examinations were performed daily from the day of PG treatment until ovulation and thereafter on days 1, 2, 3, 5, (6), 7, 8, and (9) (On days 6 and 9 if necessary or possible; day 0 = day of ovulation).

Table 4. Summary of the treatments, ultrasonographic examinations and samplings in Studies I, II, III and IV. PG treatments include only treatments given to study manipulations, not for synchronisation purposes.

Study	PG treatment	Timing of GnRH treatment after PG	Ultrasonographic examinations	Sampling
I Prooestrus	Day 8 or 9 after ovulation	24 h (Group T24) 48 h (Group T48) 72 h (Group T72) None (Group C)	1) plateau phase of dominant follicle 2) ovulation control 3) days 1 to 9 post-ovulation	Blood (serum) from PG to day 9 post-ovulation (almost daily)
II Metoestrus		After ovulation: 0-24 h (Group T1) 24-48 h (GroupT2) None (Group C)	1) ovulation control 2) throughout the subsequent oestrous cycle until next ovulation	Blood (serum) from day 1 post-ovulation to next oestrus (daily)
III Incidence of short cycles	Day 8 after oestrus	24 h (Group PG+GnRH) None (Group PG)		Whole milk from PG to next oestrus (daily)
IV Short cycles & $PGF_{2\alpha}$	Day 6 or 7 after ovulation	24 h (Groups T) None (Groups C)	1) ovulation control 2) day 1 to 9 post-ovulation	Blood (plasma) from PG to day 9 post-ovulation (every 3 h)

Blood samples for progesterone and oestradiol-17β determinations were collected by vacuum puncture of a tail blood vessel into silicone plain tubes (Venoject, Terumo Europe N.V., Leuven, Belgium). Samples were taken daily from the day of PG treatment until ovulation and thereafter on days 1, 2, 3, 5, 7, and 8 or 9. Serum was harvested, frozen and stored in plastic tubes at -20°C until analysed. All procedures were performed in the morning from 8:00 to 10:00.

2.2. GnRH during metoestrus (II)

The experiment consisted of two different GnRH treatments (T1, T2) and one control manipulation (C). Every animal was assigned once to each of these three manipulations. Oestrus was induced with PG in 18 cases. In 9 cases, oestrus was induced with an intravaginal device containing 1.9 mg of progesterone and 10 mg of encapsulated oestradiol benzoate (EAZI-BREED™ CIDR® device, InterAg, Hamilton, New Zealand; Cidirol® capsules, InterAg, Hamilton, New Zealand). The device was inserted for 12 days. From the second day after PG or device removal, the animals were examined daily by transrectal ultrasound to determine the occurrence of ovulation. After detection of ovulation, the animals were given 250 µg of gonadorelin as follows: 0-24 h after ovulation (T1), 24-48 h after ovulation (T2), or no gonadorelin (control manipulation, C).

Ultrasonographic examinations were performed daily from the second day after PG treatment or device removal until ovulation. After this, examinations were carried out on days 1, 4 or 5, 7 or 8, 11 or 12, 14 or 15 and daily from the beginning of the next oestrous signs until ovulation (day 0 = day of ovulation).

Blood samples for progesterone determinations were collected by vacuum puncture of a tail blood vessel into silicone plain tubes (Venoject, Terumo Europe N.V., Leuven, Belgium). Samples were taken daily from day 1 after ovulation until the next oestrus. Serum was harvested, frozen and stored in plastic tubes at -20°C until analysed. All procedures were performed in the morning, from 08:00 to 10:00.

2.3. Incidence of short oestrous cycles and oestrous signs after GnRH (III)

Eight days after spontaneous oestrus, the cows were palpated per rectum to determine the existence of a CL, and a new oestrus was induced with PG in the morning. The animals were divided into two groups by their identification number. Animals having even numbers were given 100 µg of gonadorelin 24 h after the PG treatment (PG+GnRH group), while animals having odd numbers served as controls without any further treatment (PG group). Oestrous signs and possible metoestrous bleeding were recorded by the herdsmen according to our instructions. The signs were classified into three categories as follows: clear signs, weak signs or absence of signs. To minimise subjectivity, the herdsmen were asked to compare these signs to the signs of the preceding spontaneous oestrus as follows: weaker, similar or clearer.

Samples of whole milk for progesterone assays were collected into plastic tubes containing a tablet of sodium azide or bronopol immediately before PG treatment. Thereafter, daily samples were taken immediately after the morning milking until the next oestrous signs had been detected for two days. If an animal showed a short oestrous cycle (less than 16 days), samples were collected daily until the second oestrus. Milk samples were frozen and stored in original tubes at -20°C until analysed.

2.4. Short oestrous cycles and 15-ketodihydro-$PGF_{2\alpha}$ (IV)

The animals were allotted to four groups of 6 heifers. In all groups, oestrus was induced with PG. From the second day after PG treatment, the heifers were examined daily by transrectal ultrasonography to determine the occurrence of ovulation. Luteolysis was thereafter induced with the second PG. This was done

on day 6, 120 to 144 h after ovulation, in 2 groups (groups d6), and on day 7, 144 to 168 h after ovulation, in the other 2 groups (groups d7). Twenty-four hours after the second PG administration, the heifers of one d6 and one d7 group were administered 100 µg of gonadorelin to induce premature ovulation (hereafter named treatment groups T-d6 and T-d7), while the remaining two groups served as controls without any further treatment (groups C-d6 and C-d7). All injections were administered at 9:00 a.m.

Transrectal ultrasonographic examinations of the ovaries were performed once daily (morning) from the day of the second PG treatment and for at least 9 days after ovulation. On the day of expected ovulation, ultrasonography was repeatedly performed every 6 h in group T-d6, every 3 h in group T-d7 and every 6 to 12 h in groups C-d6 and C-d7.

Blood sampling for progesterone and 15-ketodihydro-$PGF_{2\alpha}$ determinations began from the day of the second PG treatment. The first sample was taken immediately before PG treatment by vacuum puncture of a tail blood vessel into heparinized tubes (Vacutainer, Becton Dickinson Vacutainer Systems, Plymouth, UK). After this, the jugular vein was catheterised with an indwelling catheter (Cook^{R} Veterinary Products V-PPC-7.0U-25, Cook Australia, Queensland, Australia). The second sample was taken 24 h after the first one, in groups T immediately before the GnRH treatment. Beginning from the second sample, the heifers were bled every 3 h, for 9 to 10 days in the T groups and for 11 to 13 days in the C groups. This scheme allowed blood sampling in all groups for about 9 days postovulation. After immediate centrifugation (2,500 g, 15 minutes), blood plasma was harvested, frozen and stored in plastic tubes at -20°C until analysed. During each sampling, possible oestrous signs and metoestrous bleeding were recorded.

3. Ovarian examinations

In Study III, the ovaries were palpated per rectum to determine the existence of a CL 8 days after natural oestrus, immediately before the administration of PG. In Studies I, II and IV, ovarian structures, the growth of the CL and follicles and the occurrence of ovulation were examined using transrectal ultrasonography (a real-time B-mode ultrasound scanner, Aloka SSD-210DXII, Aloka, Japan, equipped with a 7.5 MHz rectal linear array transducer). All examinations were carried out by the same operator.

The ovaries were scanned several times to determine the largest gross-section of follicles and/or CL. After freezing the image, the largest and the smallest diameters were measured and recorded, the average diameter was calculated later. The central cavities of CL were measured and recorded in the same way. All follicles equal to or larger than 5 mm (Studies I, IV) or 8 mm (Study II) were measured, whereas those follicles smaller than 5 mm (Study I) or 8 mm (Study II) were only counted. Locations of follicles larger than 6 mm (equal to or larger than 8 mm, Study II) were coded in order to follow their growth. Occurrence of ovulation was defined as a sudden disappearance of a large follicle between two consecutive ultrasound examinations. Day of ovulation (day 0) was determined to be the last day when the follicle was intact before the examination next day when the follicle had disappeared.

4. Hormone analyses

4.1. Progesterone

The measurements of blood progesterone concentration were performed by radioimmunoassay (RIA) by use of a commercial kit (Coat-A-Count^{R} Progesterone, Diagnostic Products Corporation, Los Angeles, USA). The intra-assay coefficient of variation for progesterone was 5.8 (Study II) and 6.3% (Study IV), when calculated from duplicates of measurements between 1.0 and 20.0 nmol/l, and 11.5% when calculated from duplicates of the whole material (Study I). The inter-assay coefficient of variation varied between 3.6 and 6.7% (I, II, IV). The detection limit of the assay was 0.3 nmol/l.

In Study III, progesterone concentrations of samples of whole milk were determined by a microplate ELISA method based on the use of an anti-progesterone monoclonal antibody (5D4) (Siklodi et al., 1995). The method was developed and described originally for P_4 determination in equine plasma (Nagy et al., 1998), and was later modified to assay also skimmed (Huszenicza et al., 1998) and whole bovine milk (Kulcsár M. et al., unpublished data). Shortly before analysis, the samples were allowed to thaw at room temperature. After thawing, they were put into a +45°C water bath for 15 minutes and then carefully shaken for 30 seconds using a single tube vortex in order to redisperse their fat content. The samples were pipetted into the wells of the microplate immediately after shaking. All samples were assayed in triplicates in 14 runs, using in total 89 evaluated microplates. Triplicates of standards, as well as of control samples (stored frozen) with known low (1.71 nmol/l), medium (4.39 and 5.29 nmol/l) and high (9.42 and 9.93 nmol/l) P_4 concentrations, were placed in each microplate.

The mean (±SD) sensitivity of the assay runs was 0.18±0.05 nmol/l. Depending on the actual concentrations, the inter- and intra-assay coefficients varied between 10.3 and 12.3%, and between 5.1 and 11.6%, respectively.

4.2. Oestradiol-17β (I)

Oestradiol-17β (E-17β) concentrations were determined by the radioimmuno-assay procedure previously reported by Sirois and Fortune (1990). The intra-assay coefficient of variation for E-17β was 4.0% when calculated from duplicates of the entire material. The inter-assay coefficient of variation was 10.9% (116.3 pmol/l, n=6). The detection limit of the assay was 2.9 pmol/l.

4.3. 15-Ketodihydro-PGF$_{2\alpha}$ (IV)

Prostaglandin F$_{2\alpha}$ secretion was studied as a concentration of its main metabolite, 15-ketodihydro-PGF$_{2\alpha}$, which was analysed from all plasma samples beginning 24 h after the second PG treatment, according to Granström and Kindahl (1982). The intra-assay coefficients of variation ranged between 6.6 and 11.7% for the different ranges of the standard curve and the inter-assay coefficient of variation was 14%. The detection limit was 75 pmol/l when analysing 0.2 ml plasma.

The basal levels of 15-ketodihydro-PGF$_{2\alpha}$, the number of pulses, time of their occurrence and intervals between pulses were calculated during the entire follow-up period. Peaks of the 15-ketodihydro-PGF$_{2\alpha}$ were identified following the method described by Zarco et al. (1984), referred to below as a skewness method. The mean and standard deviation (SD) of all samples were calculated for each heifer, and values greater than 2 SDs above the mean were arbitrarily considered to represent a significant elevation, a peak. These values were then eliminated, and the calculation was repeated until no new peaks were detected. The mean of the remaining values was considered to represent a mean basal production of 15-ketodihydro-PGF$_{2\alpha}$ in each heifer, and all values greater than mean basal value plus 2 SD, peaks, were considered to represent a significant synthesis and release of PGF$_{2\alpha}$.

5. Statistical analysis

The effects of manipulations on progesterone concentrations were analysed by repeated measures analysis of variance with manipulation and day as within factors (I, II) and with manipulation as a between factor and day as a within factor (III, IV). Oestradiol concentrations and diameter, growth and growth rate of the ovulatory follicles (I), interoestrous intervals and diameter of CL (II) were analysed by repeated measures analysis of variance with manipulation and/or day as the within factors. In addition to these, repeated measures analysis of variance, with group as a between factor and day as a within factor, was used in analysing the differences in the growths of CL and dominant follicles of the first follicular waves between the treatment groups as well as between the groups re-allotted according to cycle lengths (IV). Significance of within effects was evaluated by use of Greenhouse-Geisser-adjusted P-values.

Analysis of variance was used in testing the differences in the diameters of ovulatory follicles between treatment and day groups (two-way analysis of variance, IV) and in the basal levels of 15-ketodihydro-$PGF_{2\alpha}$ between the groups re-allotted according to cycle lengths (one-way analysis of variance, IV). The effects of treatment and day on the growth of the dominant follicle and the CL were studied by covariance analysis (I). The lengths of the two subsequent luteal phases in cows that showed short cycles (short vs. normal) were analysed by paired samples t-test (III). The differences in lengths of normal luteal phases between manipulations were analysed by independent samples t-test (III), as were the differences in diameter and growth of ovulatory follicle (combined data, I, II, IV).

The differences in the incidence of short cycles (III, IV), in the intensity of oestrous signs and in the incidence of metoestrous bleeding (III) between the manipulations were evaluated by use of a chi-square test. If any of the expected values in two by two tables was below 5, Fisher's exact test was used. All differences were considered significant at $P<0.05$.

RESULTS

1. Effect of PG (I, III, IV)

1.1. Luteolytic effect

In this thesis, PG was used in order to induce oestrous periods, during which prematurely induced ovulations caused by GnRH were studied (I, III, IV). These PG treatments were always given during early dioestrus or early mid-dioestrus, 6 to 9 days after ovulation.

When PG treatment was administered either on day 8 or day 9 after ovulation (I), the regression of the CL was rapid and complete in all 36 induced oestrous periods in 3 cows and 6 heifers, four inductions per each animal. Progesterone concentration always declined below 2.0 nmol/l within 24 h after the PG treatment.

In Study IV (IV unpublished data), all animals were heifers, and oestrus was induced either on day 6 or on day 7 after ovulation. When PG was administered on day 7, all 12 heifers showed complete luteolysis. In contrast, 3 of 12 heifers had incomplete luteolysis after the PG treatment administered one day earlier. In 2 of these 3 heifers, P_4 concentrations declined in 24 h to 6.9 and 3.8 nmol/l, but began to rise again, reaching 7.9 and 6.4 nmol/l at 48 h. In the third heifer, signs of luteolysis were detected in ultrasonography, but P_4 concentration remained at suprabasal level (between 2.6 and 4.8 nmol/l). In the heifers having complete luteolysis, P_4 concentration declined rapidly in most cases: 24 h after the treatment it was below 3 nmol/l and after 48 h below 1 nmol/l. In one heifer treated on day 7 (control group), the concentration was 5.6 nmol/l 24 h after treatment, not falling below 1 nmol/l until 96 h post-treatment. In one heifer treated on day 6 (treatment group), P_4 concentration was 5.1 nmol/l 24 h after treatment, but the decline continued linearly and fell below 1 nmol/l 48 h after treatment. Beside these, in one heifer treated on day 6 (treatment group), P_4 concentration declined to 1.9 nmol/l within 24 h, but immediately after this, the concentration began to rise, even before the occurrence of GnRH-induced ovulation.

In the field study (III), 7 of 60 cows did not respond with luteolysis at all to PG treatment given on day 8 after oestrus. In 4 cows, the decrease of P_4 concentration was slow, occurring 2 to 5 days after treatment. In addition, 9 cows, 7 in the PG group and 2 in the PG+GnRH group, developed ovarian dysfunction after treatment.

1.2. Effect on ovulation

In all control group animals of Studies I and IV, a large dominant follicle was present at the time of PG treatment. When treatment was given on day 8 or 9 after ovulation (I), all animals showed an ovulatory oestrus. Spontaneous ovulations occurred in 8 animals (2 cows, 6 heifers) between 72 and 96 h, and in one cow between 120 and 144 h.

When cloprostenol was given on day 7 (IV, IV unpublished data), all 6 heifers exhibited an ovulatory oestrus after treatment. One heifer ovulated 60 to 72 h, three heifers 78 to 84 h, one heifer 84 to 96 h and one heifer 108 to 120 h after treatment. Of the four heifers that showed luteolysis after cloprostenol treatment given on day 6, only two had an ovulatory oestrus. These heifers ovulated 72 to 96 and 78 to 84 h after treatment.

2. Effect of GnRH on size and growth of ovulatory follicle (I, II, IV)

The diameters of the ovulatory follicles at PG treatment and at ovulation as well as total growth and growth rate in Studies I, II and IV are presented in Table 5. When analysing the diameter of the ovulatory follicle during the last 24 h before spontaneous ovulation, the data of Studies I and II were combined and re-divided into cows and heifers. Oestruses during which the ovulatory follicles were followed were induced with PG treatment or a progesterone-releasing intravaginal device was inserted for 12 days. In Study I, groups T72 and C were combined, because it is obvious that natural LH release had occurred in most cases before exogenous GnRH was given 72 h after PG, and thus, GnRH did not have any effect on the size and growth of ovulatory follicle. The average diameters of spontaneously ovulated follicles were from 16.9 to 18.5 mm and from 14.5 to 16.6 mm in cows and heifers, respectively. The ovulatory follicles were significantly larger in cows ($P<0.001$). The follicles seemed to be somewhat smaller in Study II than in I and IV.

During the last 24 h before ovulation, the ovulatory follicles were significantly ($P<0.01$, IV) or almost significantly ($P=0.08$, I) smaller when ovulation was induced with GnRH 24 h after PG as compared to spontaneous ovulations. After GnRH induction the ovulatory follicle grew significantly less ($P<0.05$, I; $P<0.01$, IV unpublished data) than in control groups due to earlier ovulations. In addition, there was a significant difference ($P<0.05$) in the growth rates between groups T and C (IV unpublished data). Also in Study I, a similar difference was noticed, although significance was not reached.

Table 5. Size and growth of ovulatory follicles in Studies I, II and IV.

Study	n	Diameter at PG (mm)	Diameter at ovulation (mm)	Total growth (mm)	Growth rate (mm/day)
Study I					
Group T24					
all animals	8	14.7±1.3	15.7±1.4	1.0±1.1	0.5±0.6
heifers	6	14.7±1.6	15.2±1.2	0.5± 0.6	0.3±0.3
Group T48					
all animals	8	14.7±0.9	16.1±1.7	1.4±1.1	0.5±0.4
heifers	5	14.3±0.6	15.9±1.3	1.6±0.9	0.5±0.3
Groups T72+C					
all animals	17	14.1±1.4	16.6±1.6	2.6±1.0	0.9±0.3
heifers	12	13.7±1.1	15.9±0.9	2.2±0.7	0.7±0.2
cows	5	15.1±1.5	18.5±1.5	3.4±1.0	1.1±0.3
all animals	33	14.4±1.3			
heifers	23	14.1±1.2			
cows	10	15.1±1.2			
Study II					
all animals	25		15.4±2.2		
heifers	16		14.5±2.0		
cows	9		16.9±1.9		
Study IV					
Groups d6	8	11.8±1.1	14.0±2.5	2.2±1.9	0.9±0.7
Groups d7	12	13.5±1.7	15.8±1.2	2.3±1.7	0.9±0.6
Groups T	12	12.7±1.6	14.0±1.7	1.3±1.2	0.7±0.6
Groups C	8	12.9±1.9	16.6±1.3	3.7±1.5	1.2±0.4

When the dominant follicle of the first follicular wave reached its plateau phase, its average diameter was 1.0 mm larger in cows than in heifers ($P<0.05$) (I). This plateau phase was reached on day 7 or day 8 after ovulation (I). The average diameter of the dominant follicle in heifers was significantly larger on day 7 than on day 6 ($P<0.05$), indicating that growth continued at least until day 7 after ovulation (IV). On day 7 the dominant follicle probably reached the plateau phase in Study IV, because the average diameter (13.5±1.7 mm) was about the same than that in heifers, whose dominant follicle had reached the plateau phase in Study I (14.1±1.2 mm).

3. Effect of GnRH on oestradiol-17β during PG-induced oestrus (I)

On the day of PG treatment, 8 or 9 days after ovulation, during the non-growing phase of the dominant follicle, the concentration of E-17β was low, less than 9.5 pmol/l, in all animals during every manipulation period. This was followed by a rapid rise within 24 h after PG administration. This rise continued at a slower rate until 48 h in groups T48, T72 and C. In group T24, where GnRH was given 24 h after PG treatment, the concentration had dropped back to basal level, causing a significant difference in E-17β concentration between T24 and C at 48 h ($P<0.001$). The other treatments did not differ from control. In all treatment groups, E-17β concentration was approximately at basal level 24 h after GnRH treatment.

4. Ovulations induced with GnRH (I, III, IV)

Premature ovulations were induced by GnRH treatment in Studies I, III and IV. When GnRH was administered either 24 h or 48 h after PG treatment (I), induced ovulation occurred in 16 of 18 cases 24 h to 48 h after GnRH administration. In one case, the dominant follicle of a cow continued to grow for 6 days despite GnRH administration and lost its dominance after that. In another case, the emergence of a subsequent follicular wave was detected at the time of GnRH treatment.

With more frequent (3 to 6 h) ultrasound scanning (IV), time intervals between GnRH treatment and ovulation were as follows: 24 to 27 h in 6 heifers, 24 to 30 h in 5 heifers and 30 to 36 h in one heifer. All animals ovulated in response to GnRH treatment.

In the field study (III), day of ovulation (day 0) was estimated based on the rise of P_4, because no ultrasound examination was used. The day when P_4 concentration exceeded 1 nmol/l for the first time was found to be day 3. In most cases this was also in close accordance with the behavioural signs of oestrus. Thus, ovulations occurred approximately 1.8 days after GnRH treatment. However, in some cases ovulation seemed to take place as late as 4 to 5 days after GnRH treatment.

5. Effect of GnRH administered during PG-induced prooestrus

5.1. Effect on oestrous cycle length (I, III, IV)

Length of oestrous cycle following GnRH-induced ovulation was estimated using detection of oestrous signs, progesterone profiles (I, III, IV) and ultrasound scanning (I, IV). When ovulation was induced with GnRH treatment during early prooestrus 24 h after PG administration, the subsequent oestrous cycle was clearly shortened in some animals. In Study I, of eight animals (2 cows, 6 heifers), one heifer and one cow ovulated 9 (cow) and 10 (heifer) days after induced ovulation. The animals also showed behavioural signs of oestrus and an increased uterine tone at rectal palpation in connection with these ovulations. No premature luteolyses were detected in control animals as well as in those animals treated with GnRH 48 h or 72 h after PG administration.

In Study IV, some heifers had a premature decline in P_4 concentration. All of these heifers showed behavioural signs of oestrus, or at least typical vaginal discharge, for some days after the decline. The duration of the oestrous cycles was calculated from the day of GnRH treatment (one day before ovulation) to the subsequent spontaneous behavioural signs of oestrus. Ovulation during this oestrus was followed by ultrasonography, and the duration of these cycles was also calculated from the GnRH-induced ovulation to the subsequent ovulation. Durations of oestrous cycles after the PG+GnRH treatments from GnRH to the subsequent oestrus, from an ovulation to the subsequent ovulation, and in the case of a short oestrous cycle, duration of the subsequent cycle in groups T-d6 and T-d7 are presented in Table 6. Seven short oestrous cycles, 8 to 12 days in duration, were detected among 12 treated cycles, while no short cycles were found in 8 control cycles. The difference in the incidence was statistically significant ($P<0.05$). The incidence of short cycles after PG and GnRH administrations 24 h apart was 58% (95% confidence interval 30% to 86%).

In the field study (III), ovulation was not monitored, but lengths of oestrous cycles were assessed based on P_4 profiles. Luteal phase was defined to be the interval during which P_4 concentration of whole milk exceeded 1 nmol/l as determined by the ELISA method described in Section 4.1. When the luteal phase was shorter than 10 days, the oestrous cycle was called a short cycle. No short cycles were observed in the control group, whereas four of twelve cows treated with PG+GnRH showed a short oestrous cycle ($P<0.05$). The incidence of short cycles was 33% (95% confidence interval 6 to 60%). The length of the short luteal phases varied from 3 to 6 days with an average of 4.0±1.4 days. The approximated length of these short cycles was 8 to 10 days. The next luteal

Table 6. Durations of oestrous cycles after cloprostenol (0.5 mg, i.m.) and GnRH (100 µg, i.m.) treatments 24 h apart from GnRH treatment to subsequent oestrus, and in case of short oestrous cycle, from ovulation to subsequent ovulation, and duration of subsequent cycle in groups T-d6 (cloprostenol given on day 6 after ovulation) and T-d7 (cloprostenol given on day 7 after ovulation).

Heifer	Duration of the cycle, in days		Duration of the next cycle, in days
	GnRH to next oestrus	Ovulation to next ovulation	
Group T-d6			
221	22		
223	21		
225	12	12	18
226	9	anovulation	-
228	10	9	20
941	10	anovulation	-
Group T-d7			
229	9	9	19
231	23		
232	18		
234	18		
235	9	9	18
240	9	8	19

phases during the subsequent untreated oestrous cycles were significantly longer ($P<0.001$) than the short ones, with an average of 18.0±2.0 days. The lengths of the luteal phases after treatments in the control group and in those cows treated with PG+GnRH but having normal cycles were 15.9±2.3 and 17.1±4.6 days, respectively. There were no differences between the means, but the variance in the latter group was significantly larger ($P<0.05$). After GnRH-induced ovulation, in addition to the short cycles, some "normal" cycles seemed to be lengthened.

5.2. Effect on progesterone concentration (I, III, IV)

Significant differences in P_4 concentrations were detected between treatment and control groups (III, IV). After GnRH-induced ovulation, the concentration remained at a lower level from day 5 onwards (Study IV, group T-d6, $P<0.01$), from day 7 (Study IV, group T-d7, $P<0.05$) or from day 8 (Study III, $P<0.05$) than in control groups. In Study I, however, no significant differences were detected, although the daily rise in P_4 concentration seemed to be slower in group T24.

Evaluating the individual progesterone profiles, it becomes obvious that the arrested rise in group averages of P_4 concentration in the treatment groups is caused by a bipartite reaction of the individuals (I, III, IV). As explained in Section 5.1., some animals showed a short oestrous cycle after prematurely

induced ovulation. For further analysis, the animals in the treatment groups were re-divided into those with a short cycle (group TS) and those with a normal cycle (group TN) (In Article III, group TS is called SGS and group TN is called SGN.) (III, IV). No significant differences in P_4 levels and profiles of P_4 curves were found between group TN and the control group (III, IV), while the P_4 curve of group TS was significantly different ($P<0.001$) from that of the control group. No differences in P_4 levels could be detected during the first 5 days after ovulation, but from day 6 onwards the concentration declined rapidly in group TS (III, IV). In individuals (IV), the highest P_4 concentration was attained on day 5 after ovulation in four of seven heifers. In two heifers, the highest concentration occurred on day 4; however, in these heifers, the P_4 concentration did not decline below 1 nmol/l, but remained at suprabasal levels, around 2 and 3 nmol/l, during the entire sampling period after the decline (i.e. 6 to 7 days). In one heifer, the concentration remained more or less unchanged for 4 days, between days 5 and 8, and was then followed by a sudden decline.

5.3. Effect on corpus luteum (I, IV)

Diameters of corpora lutea among the treatment and control groups were analysed from day 3 (I) or from day 4 (IV) to day 8 after ovulation. Significant differences in the growth profiles of corpora lutea were found in Study IV (IV unpublished data) between groups T-d6 and C ($P<0.05$) as well as between T-d7 and C ($P<0.05$). In Study I, however, no differences were found. For further analysis in Study IV, the heifers of the treatment groups were re-allotted into groups TS and TN, as was done in the analysis of P_4 concentrations. The mean estimated growth rates of CL from day 4 to day 8 were –0.2±0.8, 1.5±0.5, and 1.8±0.6 mm/day in groups TS, TN, and C, respectively. No significant differences in diameter or growth of CL were found between groups TN and C. However, the development of CL in group TS was significantly different from that in group C ($P<0.001$). In group TS, the growth and diameter of CL were similar to those in groups TN and C until day 5, but thereafter, the growth ceased and the diameter began to decrease.

5.4. Effect on follicular growth after induced ovulation (I, IV)

The growth pattern of the dominant follicle of the first follicular wave following GnRH-induced ovulation was not significantly different from that following spontaneous ovulation (I, IV). The mean estimated growth rates after spontaneous ovulations were 1.5±0.4 (from day 2 to day 7, I) and 1.5±0.4 mm/day (from day 2 to day 6, IV). After GnRH-induced ovulations they were 1.3±0.2 mm/day

(PG+GnRH 24 h apart, from day 2 to day 7, I), 1.6±0.3 mm/day (PG+GnRH 48 h apart, from day 2 to day 7, I), and 1.3±0.2 (PG on day 6+GnRH 24 h apart, from day 2 to day 6, IV) and 1.2±0.3 (PG on day 7+GnRH 24 h apart, from day 2 to day 7, IV).

However, a significant difference was observed in the levels of the growth curves between groups T24 and C ($P<0.01$, I) as well as between groups T-d7 and C ($P<0.05$, IV). The follicles in groups T24 and T-d7 were on average 1 to 2 mm larger than in the control groups throughout the growth period. No differences were found, however, between groups T48 and C (I) and between T-d6 and C (IV unpublished data). The appearance of a short oestrous cycle did not seem to have any influence on the size of the dominant follicle, or vice versa, since no differences were found between groups TS, TN and C (IV).

6. Effect of GnRH given during metoestrus (II)

6.1. Effect on follicles and corpus luteum

On day 1 (0-24 h after ovulation), follicles larger than 8 mm were found in four instances, twice in group T1, once in group T2 and once in group C. As GnRH treatment did not induce ovulation of larger or smaller follicles, no accessory corpora lutea were found later.

Corpus luteum size was analysed on day 11 or 12 and day 14 or 15 after ovulation, and the results are presented in Table 7. In group T1, on day 14 or 15, CL was on average 1.3 mm smaller than in group C ($P<0.01$), but no significant differences were found on day 11 or 12, nor between groups T2 and C.

6.2. Effect on progesterone patterns

A significant effect of day was observed on P_4 concentration in all manipulations ($P<0.001$). On day 3, P_4 concentration began to rise steadily until day 11, with P_4 levels remaining unchanged thereafter. The mean estimated daily rise in P_4 concentration from day 2 to day 11 was 1.8±0.5 nmol/l. The group averages are presented in Table 7. No significant differences were found in levels or profiles of P_4 curves between GnRH treatments and control manipulation.

In group C, P_4 concentration rose continuously from day 2 to 11, but a slight decline in P_4 concentration was noticed in GnRH treatments between days 7 and 8 after ovulation in group T1, and between days 8 and 9 in group T2. In both

Table 7. Size of CL on days 11 or 12 and 14 or 15, and daily rise in P_4 concentration from day 2 to day 11 after ovulation, when GnRH (250 μg, i.m.) was administered 0 to 24 h (T1) and 24 to 48 h (T2) after ovulation, or when no GnRH was given (C).

	T1	T2	C
Diameter of CL, mm			
Day 11/12	22.2±2.0	22.7±3.2	22.7±3.2
Day 14/15	21.2±2.0[a]	22.1±2.9	22.6±2.0
P_4 concentration, nmol/l			
Daily rise, D2 – D11	1.9±0.6	1.8±0.4	1.7±0.5

[a] Significantly different from control (P<0.01)

treatment groups (T1 and T2), the decline occurred between days 6 and 7 after GnRH administration. In individual animals, this decline occurred in 8 of 9 animals in groups T1 and T2, and in 2 of 9 in group C between days 5 and 9 after ovulation. The decline occurred in six cases between days 7 and 8 in group T1; in group T2 the decline was more inconsistent.

During the last 8 days before the next ovulation, a significant decline in P_4 concentration was detected between days -4 and -3 (P<0.01) in groups T1, T2 and C. There were no significant differences in the levels or profiles of P_4 curves during these days between GnRH treatments and control manipulation.

6.3. Effect on oestrous cycle length

The length of the oestrous cycle in groups T1, T2 and C was 21.9±1.8, 22.3±2.1 and 21.6±1.8 days, respectively. GnRH treatment given on day 1 or 2 after ovulation did not have any significant effects on inter-oestrous intervals. Instead, the animal effect was significant on the mean length of the oestrous cycle (P<0.01).

7. Short oestrous cycles and $PGF_{2\alpha}$ secretion (IV)

The P_4 and 15-ketodihydro-$PGF_{2\alpha}$ profiles of heifers 225, 226 and 228, of heifers 229, 235 and 240, as well as of heifers 231, 234 and 239 are illustrated in Figures 4, 5 and 6, respectively. The basal levels of 15-ketodihydro-$PGF_{2\alpha}$ during the entire follow-up period were 188±61 pmol/l (min 106, max 297), 147±33 pmol/l (min 111, max 200) and 141±36 pmol/l (min 100, max 214) in the treatment group heifers with a short and a normal oestrous cycle and in the control group heifers, respectively. Heifer 228 in group TS had a divergently high basal level, 297 pmol/l. If it was removed from the results, the basal level in group TS was

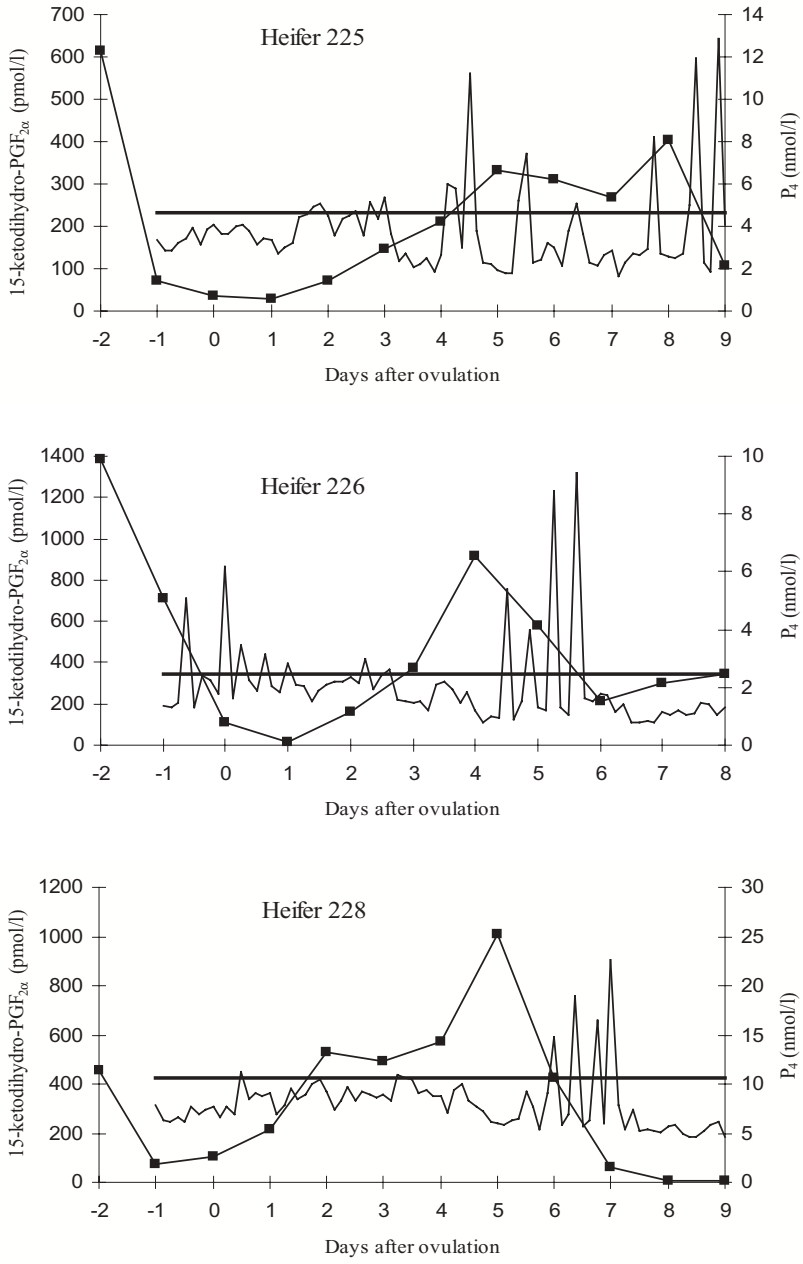

Figure 4. Peripheral blood plasma progesterone (—■—) and 15-ketodihydro-$PGF_{2\alpha}$ (——) concentrations in heifer nos. 225, 226 and 228, which showed a short oestrous cycle after cloprostenol (0.5 mg, on day –2) and GnRH (100 µg) treatment 24 h apart. The horizontal line denotes the line of significance (mean basal value + 2SD) for 15-ketodihydro-$PGF_{2\alpha}$ concentrations.

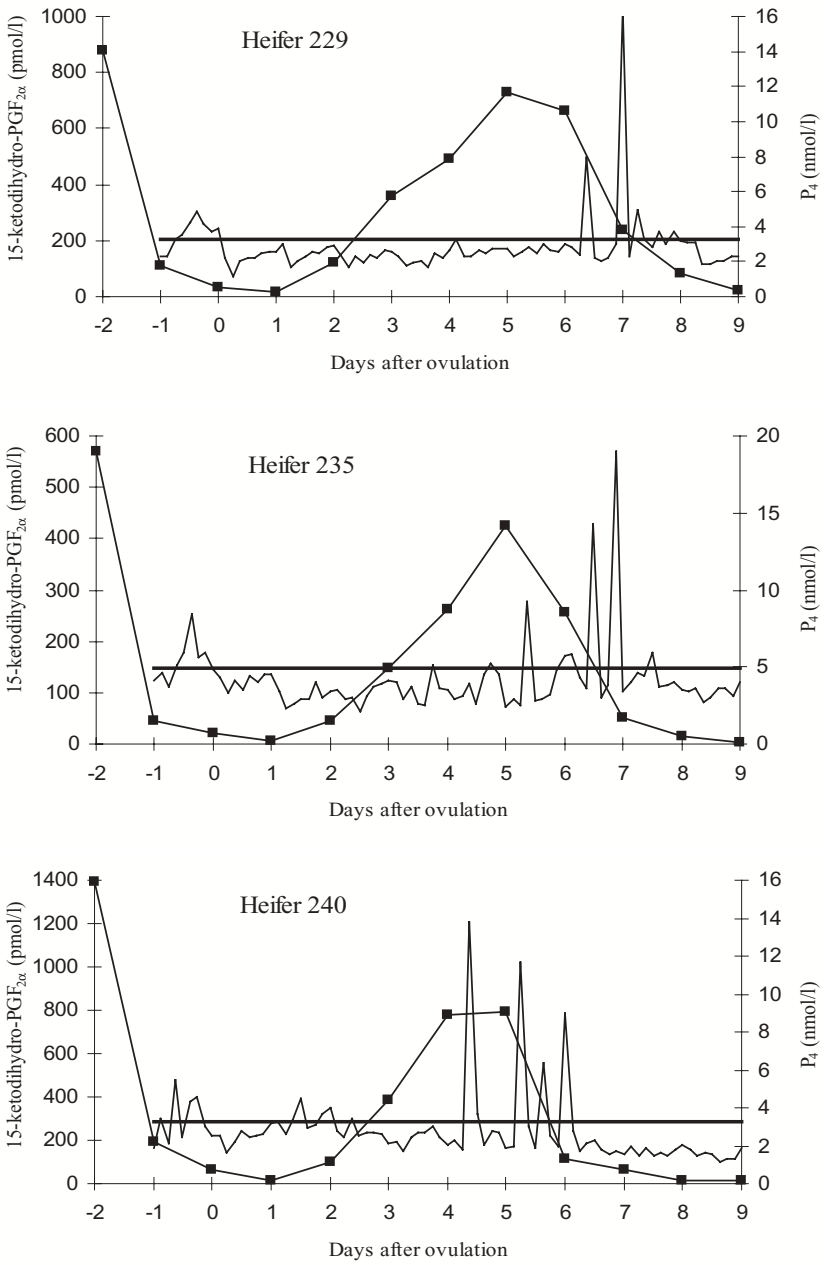

Figure 5. Peripheral blood plasma progesterone (—■—) and 15-ketodihydro-$PGF_{2\alpha}$ (——) concentrations in heifer nos. 229, 235 and 240, which showed a short oestrous cycle after cloprostenol (0.5 mg, on day –2) and GnRH (100 µg) treatment 24 h apart. The horizontal line denotes the line of significance (mean basal value + 2SD) for 15-ketodihydro-$PGF_{2\alpha}$ concentrations.

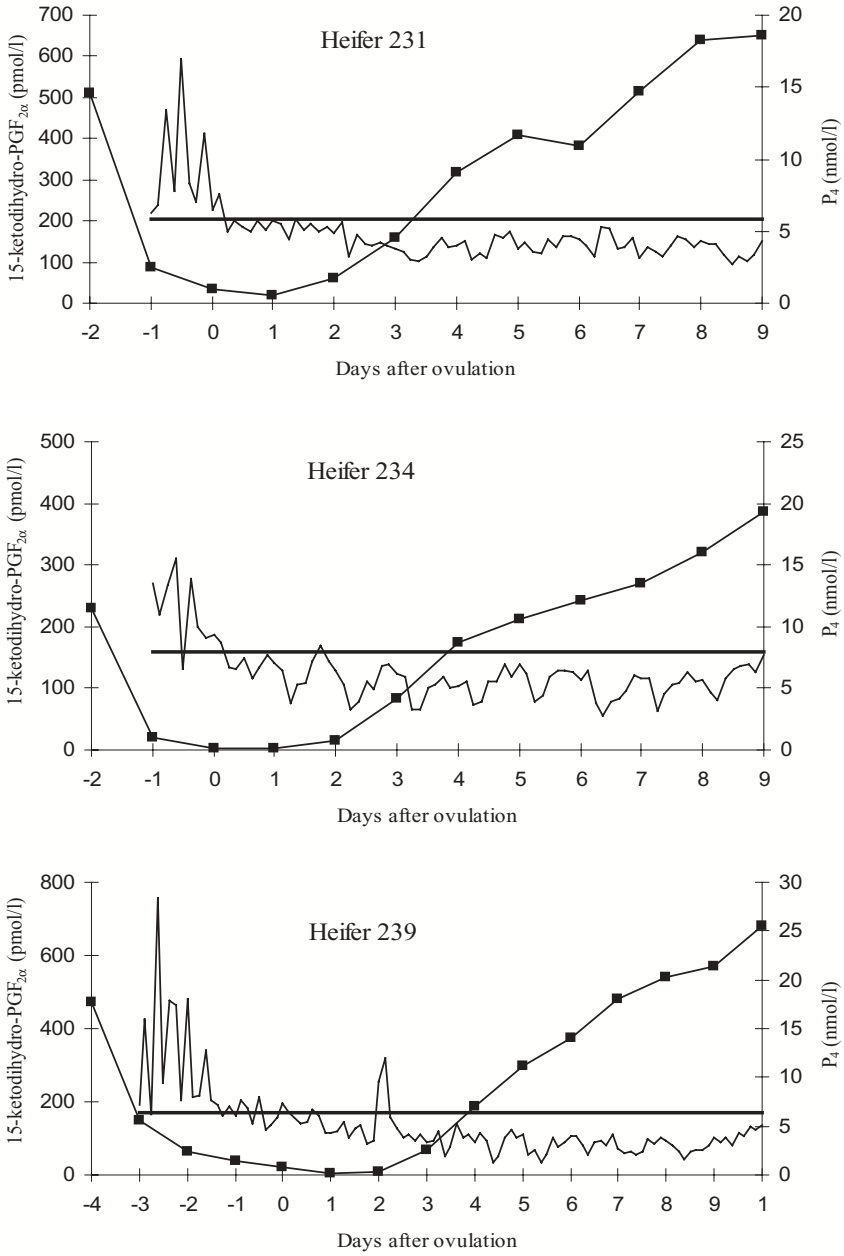

Figure 6. Peripheral blood plasma progesterone (—■—) and 15-ketodihydro-$PGF_{2\alpha}$ (——) concentrations in heifers nos. 231, 234 and 239. Heifer nos. 231 and 234 were treated with cloprostenol (0.5 mg, on day –2) and GnRH (100 µg) 24 h apart, while heifer no. 239 was treated with cloprostenol (0.5 mg, on day –4), but no GnRH was given. All heifers had oestrous cycles of normal length. The horizontal line denotes the line of significance (mean basal value + 2SD) for 15-ketodihydro-$PGF_{2\alpha}$ concentrations.

170±41 pmol/l (min 106, max 216). No significant differences in the basal levels of 15-ketodihydro-$PGF_{2\alpha}$ were found among the groups, with or without heifer 228.

After P_4 exceeded 3 nmol/l (day 2 or 3, heifer 228 day 0, heifer 224 day 1), significant elevations in 15-ketodihydro-$PGF_{2\alpha}$ concentrations were detected in all heifers that showed short oestrous cycles (group TS), but not in any heifers with normal oestrous cycles (group TN) in groups T. The pattern of significant elevations of 15-ketodihydro-$PGF_{2\alpha}$ concentrations in heifers in group TS, number of peaks, intervals from ovulation to the first peak and intervals between the peaks are presented in Table 8. Some of these peaks, the first one in heifer 228 and the first two in heifer 235, were fairly low, exceeding the level of significance by not more than 12 pmol/l. In group C, significant elevations in 15-ketodihydro-$PGF_{2\alpha}$ concentrations were detected in 4 heifers (222, 224, 244 and 246). In heifers 222, 224 and 244, a low single peak was seen around day 3 or 4. In heifers 222 and 224, two peaks and in heifer 246 one peak was detected during day 9. None of these peaks exceeded the level of significance by more than 35 pmol/l, except in heifer 222 on day 9, 141 pmol/l. However, neither this nor any of the other peaks seemed to have any influence on P_4 concentration.

8. Oestrous signs during PG-induced oestrus with or without GnRH-induced ovulation (III)

The intensities of oestrous signs in both PG and PG+GnRH groups are shown in Table 9. After PG-only treatment, 30 of 35 cows showed oestrous signs of varying intensity. Of those animals that really were in ovulatory oestrus (P_4<1 nmol/l), all except one showed oestrous signs. Oestrous signs after the PG+GnRH treatment were significantly weaker than those observed after the PG treatment (P<0.001). In the PG+GnRH group, none of the cows in ovulatory oestrus showed clear oestrous signs, and 10 of 13 did not exhibit any signs at all.

The intensity of oestrous signs after the treatments was also compared to those of the signs observed during the previous spontaneous oestrus. After the PG-only treatment, no differences in intensity were found, either among all cows, or among those that really were in ovulatory oestrus. Thus, PG had no effect on the intensity of oestrous signs. On the other hand, GnRH treatment 24 h after PG treatment weakened the oestrous signs significantly (P<0.01).

Table 8. Significant elevations in 15-ketodihydro-$PGF_{2\alpha}$ as indicated by number of peaks, interval from ovulation to first peak and intervals between peaks, during a period of high P_4 in the heifers that showed short oestrus cycle (group TS). Values of 15-ketodihydro-$PGF_{2\alpha}$ were considered significantly elevated when they exceeded mean basal value +2SD as calculated by skewness method.

Heifer	Number of peaks	Interval from ovulation to 1st peak, h	Intervals between peaks, h						Complete luteolysis
			1.-2.	2.-3.	3.-4.	4.-5.	5.-6.	6.-7.	
225	7	96	9	24	21	33	18	9	yes
226	4	105	9	9	9				no
228	5	75	66	9	9	6			yes
229	5	151	15	6	9	6			yes
235	7	88	24	15	18	9	9	15	yes
240	4	103	21	9	9				yes
941	5	99	15	15	15	15			no

Table 9. Intensity of oestrous signs of all cows and cows in ovulatory oestrus in PG and PG+GnRH groups. In both groups, cloprostenol (0.5 mg, i.m.) was given 8 days after oestrus. In the PG+GnRH group, gonadorelin (100 µg, i.m.) was administered 24 h after cloprostenol treatment, while no further treatment was administered to the PG group.

	PG group		PG+GnRH group	
	all cows (n)	cows in oestrus (n)	all cows (n)	cows in oestrus (n)
Total cows (n)	35	21	25	13
Oestrous signs				
Clear	24	17	2	0
Weak	6	3	10	3
None	5	1	13	10
Comparison to previous signs of spontaneous oestrus				
Clearer	8	7	2	1
Similar	13	8	4	1
Weaker	9	5	6	1
None	5	1	13	10

After spontaneous oestrus, the incidence (±SEM) of metoestrous bleeding was 57±6%. It can be expected that the accuracy in detecting vaginal discharge decreases when cows are grazing. When the period on pasture is removed from the analysis, the incidence (±SEM) of metoestrous bleeding was 64±7%. After PG-only treatment, 8 of the 21 cows that were really in oestrus showed bloody discharge, whereas 11 of these animals had displayed metoestrous bleeding after the previous spontaneous oestrus. Thus, PG did not have any significant influence on metoestrous bleeding. After PG+GnRH treatment, none of the animals showed bloody discharge.

DISCUSSION

1. Effect of PG

Prostaglandin $F_{2\alpha}$ and its agonists cause luteolysis very effectively, resulting in a premature new oestrus and ovulation. When PG treatment was administered either on day 8 or day 9 after ovulation to cows and heifers (I), or on day 7, 144 to 168 h after ovulation – that is, 7 to 8 days after oestrus – to heifers (IV), all animals were in oestrus within 6 days after the treatment, as expected. By contrast, this was not the case in Study III, where PG was administered to cows on day 8 after oestrus. As P_4 did not decline in 7 of 60 cows (III), CL did not regress after PG treatment, and the animals returned to oestrus 18 to 25 days after the preceding oestrus. The luteolytic potency of PG is dependent on the stage of the luteal phase. During early dioestrus, PG is less effective in inducing regression of the CL than later (King et al., 1982, Wiltbank et al., 1995). In lactating dairy cows, no luteolytic response to PG was observed during the first 5 days after oestrus, but on days 6 and 7 after oestrus, a 25% and 66% response, respectively, and greater than 90% response thereafter has been shown (Seguin, 1997; Wiltbank, 1997). In heifers, responsiveness to PG is regained somewhat earlier than in cows (Seguin, 1997; Wiltbank, 1997). On day 5 after oestrus, 41% of heifers responded to PG treatment with luteolysis (Wiltbank, 1997). Because the day of oestrus was determined by detection of external oestrous signs in Study III, there may be a variation of about ± 1 day in the day of cycle. This variation could explain the high incidence of unresponsiveness to PG, and other ovarian dysfunctions seen in this study. Also in Study IV, when PG was administered to six heifers on day 6, 120 to 144 h after ovulation, three animals had incomplete luteolysis and one animal did not ovulate. Similar failures in oestrus synchronisation during early dioestrus, after day 4, were shown by King et al. (1982). These findings indicate that the oestrus synchronisation effects of PG on day 7, and maybe also on day 8 after oestrus in cows, and one day earlier in heifers, could be poor. Thus oestrus synchronisation should be avoided on these days, whenever possible.

The slow decrease in P_4 concentrations after PG administration seen in some animals in Studies III and IV is not in accordance with many earlier findings. After PG treatment, P_4 concentration has been reported to start to decrease in 10 min (Hafs et al., 1974; Oxender et al., 1974). A reduction in P_4 concentration by about 60% within 12 h (Louis et al., 1973), less than 3.2 nmol/l by 24 h (King et al., 1982), and a plateau 48 to 72 h (Stellflug et al., 1975) after PG treatment has

been documented. The slow decrease, like some other disturbances, may be related to the early administration of PG during a time when CL was still completely or partially refractory to $PGF_{2\alpha}$.

The interval from PG treatment to spontaneous ovulation can be studied in the control groups of Studies I and IV. Eight of the 9 animals (2/3 cows, 6/6 heifers) (I) and 6 of the 8 heifers (IV) ovulated 72 to 96 h after PG administration. The mean interval to ovulation has been reported to vary from 79 to 104 h (Hafs et al., 1974; Oxender et al., 1974; Cooper, 1974; Wishart, 1974). However, it seems to depend on the stage of dioestrus when administration occurs. It is about half a day shorter during early than during late dioestrus, and about half a day shorter in heifers than in cows (King et al., 1982). More precisely, the mean response intervals are longer among cows treated around mid-dioestrus and shorter for early and late dioestrus (Macmillan and Henderson, 1984). The time of onset of oestrus is dependent on the stage of the follicular wave when CL regression is induced. The interval is shortest when a mature dominant follicle is present at PG administration, and longest when a follicle wave is emerging or undergoing selection (Roche et al., 1996). When PG was administered on day 8 after ovulation in heifers, the mean time interval between treatment and ovulation was 89 h (Kastelic et al., 1990a). Following two cloprostenol treatments 11 days apart in heifers, ovulation had not begun within 72 h, while 95% had ovulated within 96 h (Roche, 1977). In Roche's study, the second treatment was given approximately 6 to 8 days after ovulation, which is close to the time point in Study IV. In Study I, the treatment was given 8 or 9 days after ovulation, but this probably should not have caused any difference, because the time of treatment was fixed to the early static phase of the dominant follicle. Thus, our findings are in good accordance with their reports. In the heifer that ovulated 108 to 120 h after PG treatment, P_4 concentrations did not fall below 1 nmol/l until 96 h after the PG treatment. Thus, a slow decrease in P_4 concentration seems, at least in some cases, to affect the interval from PG administration to oestrus and ovulation.

2. Effect of GnRH on size and growth of the ovulatory follicle

The mean maximum diameter of the ovulatory follicle during the last 24 h before ovulation in heifers was 14.5 to 16.6 mm. This is in good agreement with the earlier reports: between 15.3 and 20.3 mm in two-wave cycles and between 12.8 and 18.6 mm in three-wave cycles after natural luteolysis (Savio et al., 1988; Sirois and Fortune, 1988; Knopf et al., 1989; Ginther et al., 1989a; Ginther et al., 1989b) and between 13.7 and 19.5 mm after PG-induced luteolysis (Kastelic et

al., 1990a; Kastelic and Ginther, 1991). Much less information is available about follicles in cows. Our results indicate that ovulatory follicles are 2 to 2.5 mm larger in cows than in heifers.

It has been reported that the dominant follicle of the first wave reaches its maximum diameter on day 7 after ovulation (Knopf et al., 1989), or between days 6 and 7 (Ginther et al., 1989a; Ginther et al., 1989b) in heifers. In Studies I and IV, the growth phase seemed to be slightly (0.5 to 1 day) longer. The mean maximum diameter of the dominant follicle in heifers has varied between 15 and 18 mm (Savio et al., 1988; Knopf et al., 1989; Ginther et al., 1989a; Ginther et al., 1989b; Kastelic et al., 1990a; Kastelic and Ginther, 1991; Bodensteiner et al. 1996), which is somewhat larger than our findings (mean 14.1 mm, range 11.5 to 16.5 mm). Only Savio et al. (1988) in two-wave cycles, and Sirois and Fortune (1988), have reported smaller diameters, 14 and 12 to13 mm, respectively. As is the case for ovulatory follicles, little information about dominant follicles in cows is available. As earlier observations suggest (Kastelic et al., 1990a; Kastelic and Ginther, 1991), the static-phase dominant follicle, though it has apparently reached its natural maximum diameter, is capable of further growth after induced luteolysis. Growth of the ovulatory follicle and linear growth rate from PG treatment to ovulation were consistent with findings of Kastelic et al. (1990a) and Kastelic and Ginther (1991).

Early induction of ovulation (24 h after PG treatment) with GnRH led to a smaller total growth of a dominant follicle and a smaller follicle at ovulation. In addition, the follicular growth rate was slower after GnRH treatment, possibly indicating that GnRH retards follicular growth. Although the mean diameter of the ovulatory follicle at ovulation was smaller in the GnRH-treated groups than in the control groups due to earlier ovulations induced with GnRH, the sizes of ovulatory follicles were within the range of normal ovulatory follicles during induced and spontaneous oestrus described above. Hence, it is obvious that the size and growth of the ovulatory follicle provide sufficient capacity for further normal development after ovulation.

3. Effect of GnRH on oestradiol-17β during PG-induced oestrus

On the day of PG treatment, 8 or 9 days after ovulation, the concentration of oestradiol in all animals in every treatment was at the basal level. This is consistent with reports from Glencross and Pope (1981) and Dieleman et al. (1986). Luteolysis caused by PG treatment was followed by a rapid rise in oestradiol concentration. A peak value was reached 48 h after the treatment when

GnRH was given 48 h or later after PG (groups T48, T72, C). The time to peak value was similar to that shown by Dieleman et al. (1986) in natural luteolysis, but one day shorter than that observed by Glencross and Pope (1981) after PG-induced luteolysis. Glengross and Pope administered PG later in the oestrous cycle, when newly recruited follicles were in the growth phase, which may explain the difference.

In group T24, when GnRH was administered 24 h after PG treatment, oestradiol concentrations failed to continue to rise after day 1 as in other treatments, but reached a peak value 24 h after PG administration. This was followed by a precipitous decline to the basal level during the following 24 h. A similar fall was seen in other treatments and control after 48 h post-treatment. It has been shown that the LH surge inhibits oestradiol secretion of the preovulatory follicle (Dieleman et al., 1986), and the amount of GnRH used in this study causes an LH surge (Chenault et al., 1990). The decline in oestradiol to the basal level occurs in 24 h, as reported by Dieleman et al. (1986). Duchens et al. (1994) found a slower decline during 48 h, but this was probably due to less frequent sampling. The fall in oestradiol concentration between 48 and 72 h in Study I can be explained by a natural LH surge in groups T72 and C. In group T48, it may have been caused either by a natural or by a GnRH-induced LH surge. When GnRH was administered 24 h after PG treatment, it seemed to cause an LH surge, which stopped the oestradiol secretion of the preovulatory follicle. This occurred although the follicle had not reached final maturity, as in other treatments.

4. Ovulations induced with GnRH

One hundred µg of gonadorelin given during early dioestrus caused ovulation very effectively in 16 of 18 (I) and in 12 of 12 animals (IV). As detected by frequent ultrasound scanning (IV), 11 of 12 heifers ovulated 24 to 30 h, and one 30 to 36 h, after treatment. This accords well with earlier findings that GnRH causes ovulation between 24 and 32 h after treatment when a dominant follicle is present (Pursley et al., 1995). In Study III, the approximate time of ovulation was 1.8 days (about 43 h) after GnRH treatment. Hence, it is obvious that in some animals GnRH did not cause ovulation, possibly due either to an inadequate dose of GnRH or to the fact that the follicle had lost its dominance. In some animals, however, ovulation seemed to occur as late as 4 to 5 days after GnRH treatment, which suggests that the dose of 100 µg may be inadequate, at least in cows.

5. Effect of GnRH administered during PG-induced prooestrus

When GnRH was given 24 h after PG treatment, some animals exhibited a short oestrous cycle. In all the 35 control cycles, the length of the oestrous cycle was normal, but the incidences of short cycles were 25%, 33% and 58% in Studies I, III and IV, respectively. In Studies III and IV, the difference in the incidence was statistically significant. Similar findings have been reported by Stevens et al. (1993), when GnRH was administered simultaneously with PG. Significantly lowered pregnancy rates after timed AI have been detected following GnRH injection 24 h after PG (Schmitt et al., 1996).

Because ultrasound follow-up was not used in Study III to confirm complete luteal regression following PG treatment, occurrence of ovulation after GnRH treatment, and formation of a new CL, we cannot be absolutely sure that the detected short cycles were real oestrous cycles with ovulation and CL formation. GnRH administration 12, 24 and 48 hours after PG treatment has been shown to result in increased P_4 concentrations, suggesting either rescue or stimulation of the CL (Thatcher and Chenault, 1976). The increase was, however, small and the duration only a few hours. In theory, the administration of GnRH could have rescued luteal tissue, and this original luteal tissue might have regressed at the end of the initial oestrous cycle, resembling a short oestrous cycle. In all cases of short cycles, P_4 concentrations declined to the basal levels and remained there for at least 4 days. In the case of rescued luteal tissue, the decline in P_4 concentrations should have been shorter in duration. Thus, it is obvious that regression of a CL, ovulation and formation of a new CL occurred after the treatments followed by the short cycles described here, as we have seen in Studies I and IV, where ultrasound examinations were used to confirm ovarian function.

Short oestrous cycles have been studied widely. Premature luteolysis shortening the normal length of the bovine oestrous cycle is a common phenomenon following the first ovulation during puberty or following first spontaneous or gonadotropin-induced ovulations postpartum [for reviews see: Garverick and Smith, (1986); Hunter, (1991); Lishman and Inskeep, (1991); Garverick et al., (1992)]. Most studies have been performed with suckled beef cows, because the first postpartum ovulation after early weaning of calves (30 to 35 days of age) is normally followed by a short luteal phase. In dairy cows as well, a higher incidence of short oestrous cycles has been observed after first postpartum ovulations (Kesler et al., 1979; Hinshelwood et al., 1982; Peter et al., 1989). Odde et al. (1980) reported that among short cycles, duration of 7 to 10 days was common, but 8 days duration was the most frequent.

Most studies investigating mechanisms associated with subnormal luteal function have utilised the model of CL formed following the first induced or spontaneous ovulation postpartum. In theory, the mechanisms behind the short oestrous cycles may include: (1) inadequate preovulatory follicular development, (2) decreased luteotropic support, or (3) premature release of a luteolysin [for reviews see: Garverick and Smith, (1986); Hunter, (1991); Lishman and Inskeep, (1991); Garverick et al., (1992)]. While the mechanisms are not yet fully understood, recent findings indicate that early demise of the CL is the result of premature release of $PGF_{2\alpha}$ from the uterus (Copelin et al., 1987; Lishman and Inskeep, 1991; Garverick et al., 1992). When postpartum cows were hysterectomized prior to the first ovulation, CL were maintained, and secretion of P_4 was similar to that observed during a normal oestrous cycle (Copelin et al., 1987). Armstrong and Hansel (1959) have shown that daily administration of oxytocin during the first week after oestrus results in shortened oestrous cycles (8 to 12 days). In cows exhibiting subnormal luteal function, the uterus has a greater potential to release $PGF_{2\alpha}$ in response to oxytocin earlier during the oestrous cycle than in cows exhibiting normal luteal function (Zollers et al., 1989). The luteolytic effect on CL appears by day 6 after the first oestrus (Copelin et al., 1987). During short oestrous cycles, there are more oxytocin receptors and fewer progesterone receptors in the endometrium, which may explain the greater response to oxytocin (Zollers et al., 1989; Zollers et al., 1993). Progestagen pretreatment prevents premature luteolysis, but it is unclear whether its effect on $PGF_{2\alpha}$ secretion is indirect or direct (Ramirez-Godinez et al., 1981; Sheffel et al., 1982; Garverick et al., 1992).

The short oestrous cycles described in this study occurred in normally cycling animals having a luteal phase immediately prior to the short cycle. Thus, there seems to be a difference between the short cycles described here and those occurring after the first ovulation postpartum. We have shown (IV) that the luteal regression seen in these short cycles is caused by a premature release of $PGF_{2\alpha}$. But why $PGF_{2\alpha}$ is released prematurely remains to be elucidated. Quintal-Franco et al. (1999) have shown that episodic release of LH pulses before, during and after the time of a preovulatory LH surge may stimulate CL development and function. Compared with those in control animals, P_4 concentrations were less and diameters of CL smaller in animals in which LH release was inhibited by treatment with an antagonist. However, during the short cycles described here, no differences were found in the size of CL or P_4 concentrations before the onset of premature luteal regression.

6. Effect of GnRH administered during metoestrus

In 4 of 27 cases, a follicle larger than 8 mm was detected during the first 24 hours after ovulation. These follicles were most probably regressing dominant follicles from the preceding anovulatory follicle waves, as can be seen from the studies of Ginther et al. (1989a) and Knopf et al. (1989). None of these follicles ovulated after GnRH treatment, which confirms their suggested atretic nature.

Kastelic et al. (1990b) have shown that the correlation between the size of CL and plasma P_4 concentration is between 0.7 and 0.8. Thus, the size of CL can be used as a method for assessment of luteal function. In our study, the difference of 1.3 mm in the diameter of the CL on day 14 or 15 between groups T1 and C was statistically significant, implying a difference of about 20% in spherical volume. The difference is, however, so small and close to the precision of measurement that its practical significance may be questionable.

Although there were no significant differences in levels or profiles of P_4 curves between GnRH and control treatments in our study, a slight decrease in P_4 concentrations was noticed in group T1 beginning from day 8 and in group T2 from day 9 (7 days after the GnRH administration), but not in group C. This finding is in agreement with the results reported by Rodger and Stromshak (1986) and Martin et al. (1990). Although the differences in concentrations were so small that statistical significance could not be reached, the similarity of the findings shows that GnRH given during metoestrus can cause a reduction in P_4 production after about one week. Martin et al. (1990) suggests that LH secreted in response to GnRH acts on the CL to promote the conversion of small luteal cells to large cells, which could explain alterations in P_4 production. Although follicular growth was not followed in the present study, it is obvious that the normal pattern of follicular growth was likely perturbed by GnRH administration, and thus, the rise in oestradiol concentration was delayed. Also this may have some effects on the development and function of corpora lutea.

Many known biological effects of GnRH are caused by LH release some hours after GnRH release/administration (Chenault et al., 1990). In the present study, the LH surge as a consequence of GnRH treatment was not measured. Lucy and Stevenson (1986) and Rosenberg et al. (1991) have, however, shown that GnRH administered after the spontaneous LH surge caused significantly lower LH release than when it was administered before the spontaneous surge. This has been explained either by the decrease in oestradiol production, which follows the LH surge (Zolman et al., 1974; Kesner et al., 1981), or by the refractoriness of pituitary cells to GnRH after the LH surge (Kesner et al., 1981). However, the

GnRH-induced LH surge in the study of Rosenberg et al. (1991) did not differ from the spontaneous LH surge. The time interval between the spontaneous and GnRH-induced LH surge was not described. It can be estimated from the experimental protocol that the interval was, in most cases, most probably less than 24 h. In our experiment, the time interval from the spontaneous LH surge to GnRH administration was 30 to 54 h in group T1 and 54 to 78 h in group T2. Because of this, it is unlikely that a weak LH response to GnRH administration after the spontaneous LH surge in our study can explain the non-altered P_4 production.

It is recommended for practical use, based on the findings of Macmillan et al. (1986), that GnRH should not be administered during at least the first 3 days after ovulation. In the present study, exogenous GnRH given 0 to 48 hours after ovulation had no influence on P_4 concentrations during the subsequent oestrous cycle or on the length of the cycle. The only significant effect was the slight decrease in the size of CL. These findings are not relevant enough to explain the reduced pregnancy rate after the GnRH treatment given 1 to 3 days after insemination, as reported by Macmillan et al. (1986). However, considering previous and present results, administration of GnRH after ovulation should be avoided.

7. Short oestrous cycles and $PGF_{2\alpha}$ secretion

Peaks of 15-ketodihydro-$PGF_{2\alpha}$ were identified by using the skewness method described by Zarco et al. (1984). In this method, concentrations greater than 2SD (Bekana et al, 1996; Kask, 1999; Königsson, 2001) or 3SD (Zarco et al., 1984; Albihn et al., 1991) above the mean are arbitrarily considered to represent a significant elevation. It is, however, possible that the use of 3SD may eliminate some peaks that represent a significant synthesis and release of $PGF_{2\alpha}$. On the other hand, the use of 2SD may find some "peaks" whose significance is questionable. Considering the relatively short half-life of 15-ketodihydro-$PGF_{2\alpha}$ in the circulation of about 8 min (Kindahl et al., 1976a), the sampling interval of 3 h used in the present study is rather infrequent. Little can therefore be said about the magnitude of the peaks, and the values that just barely reach the level of significance may represent, in reality, much higher peaks. Because of this, 2SD was selected to represent a significant elevation in the present study.

In the heifers showing a short oestrous cycle (group TS), frequent fluctuation with 4 to 7 peaks of 15-ketodihydro-$PGF_{2\alpha}$ was observed during the period of high P_4 (>3 nmol/l). The intervals between the pulses usually varied from 6 to 15 hours,

but the intervals between the first 2 or 3 peaks tended to be somewhat longer, concurring with the findings of Basu and Kindahl (1987) and Albihn et al. (1991) obtained during spontaneous luteolysis. These fluctuations coincided with a decrease in P_4 concentration, indicating premature luteolysis. The manner of fluctuations, duration, number of peaks, and intervals between peaks are consistent with the findings obtained during spontaneous luteolysis in many studies (Kindahl et al., 1976a; Kindahl et al., 1976b; Basu and Kindahl, 1987; Albihn et al., 1991). The onset of this frequent pulsatility occurred 75 to 151 h after ovulation. However, the first peaks in heifers 228 and 235 were so low that it remains questionable whether these represent a significant release of $PGF_{2\alpha}$ (see the former paragraph). If these peaks are excluded, the onset occurred 96 to 151 h after ovulation. In heifers with an oestrous cycle of normal length, either no or only single peaks of 15-ketodihydro-$PGF_{2\alpha}$ were observed during the period of high P_4. No frequent fluctuation similar to that in heifers showing a short oestrous cycle was observed.

In heifer 225 (Table 8, Figure 4), the duration of the pulsatile pattern of 15-ketodihydro-$PGF_{2\alpha}$ concentration was divergently long, for at least 6 days (the pulsatility possibly continued after the end of the sampling period), as compared with the durations of the other heifers with a short oestrous cycle and durations seen during spontaneous luteolysis in other studies, often a period of 2 to 3 days (Kindahl et al., 1981). In this heifer, the rise in P_4 concentration ceased and the level was more or less unchanged, between 5 and 8 nmol/l, for 4 days, followed by a sudden decline. The cessation coincided with the first peaks of 15-ketodihydro-$PGF_{2\alpha}$. The pulses in 15-ketodihydro-$PGF_{2\alpha}$ continued during the period of stable P_4 concentrations. This accords well with findings in which the duration of the prostaglandin release continued for as long as the P_4 concentration was maintained at approximately 3.8 nmol/l by the P_4 implants (Kindahl et al., 1979; Kindahl et al., 1980). In this heifer, total responsiveness of the CL to $PGF_{2\alpha}$ may not have been achieved during the first days of $PGF_{2\alpha}$ secretion, and thus, the secretion continued at least until completion of luteolysis. However, the pulsatility in 15-ketodihydro-$PGF_{2\alpha}$ finished after a normal period of 2 to 3 days in two other heifers (226 and 941), although P_4 concentration remained at a suprabasal level, around 2 and 3 nmol/l, after the luteolysis. Possibly, the P_4 concentration of 3 nmol/l is close to a threshold level for $PGF_{2\alpha}$ secretion.

8. Oestrous signs during PG-induced oestrus with or without GnRH-induced ovulation

To our knowledge, no studies have been published on the intensity of external oestrous signs after PG treatments. However, many farmers believe that this treatment is followed by weak oestrous signs. This does not seem to be true according to our findings. The many studies show an enormous variation in the incidence of metoestral bleeding: from 0.93% (Quayam and Austin, 1983) to “in nearly all cows” (Youngquist, 1997). In practice, there seems to be a great deal of variation among herds. Although some of this variation can be explained by the variation in intensity of oestrous detection, real differences probably exist.

CONCLUSIONS

- Prematurely induced ovulation with GnRH treatment, at least when performed after PG-induced luteolysis, causes shortened luteal function, and thus, a short oestrous cycle, in some animals, either cows or heifers, in both field and controlled study conditions. This phenomenon was detected when the PG and GnRH treatments were given 24 hours apart, and the PG treatment was given during the dominant phase of the first follicular wave. This disturbance may lead to reduced fertility, as was observed in some earlier studies.

- We have demonstrated that the luteal regression seen in short oestrous cycles induced with cloprostenol and GnRH 24 h apart in normal cycling dairy heifers is related to premature release of $PGF_{2\alpha}$. This release closely resembles the release of $PGF_{2\alpha}$ during normal spontaneous luteolysis.

- In heifers with a short oestrous cycle, no disturbances or changes in the growth of the ovulatory follicle and ovulation preceding premature luteolysis, or in the growth of the CL and the dominant follicle of the first follicular wave of the short oestrous cycle could be detected, as compared to heifers with an oestrous cycle of normal length. However, early induction of ovulation with GnRH, 24 hours after PG treatment may have affected the subsequent growth of the first follicular wave in both groups of heifers with a short or a normal oestrous cycle. The follicle exceeded 6 mm one day earlier than in controls, which probably means that recruitment had occurred earlier in relation to ovulation, or that the follicle had been larger at recruitment. This difference remained almost unchanged during the growth phase.

- Exogenous GnRH given 0 to 48 hours after ovulation had no influence on P_4 concentrations during the subsequent oestrous cycle, nor on the length of the cycle. The only significant effect was a slight decrease in the size of the CL. These findings are unlikely to explain the reduced pregnancy rate after the GnRH treatment given 1 to 3 days after insemination, as reported elsewhere.

- PG treatment did not seem to have any effect either on the intensity of behavioural oestrous signs or metoestrous bleeding. GnRH treatment 24 h after PG treatment weakened the oestrous signs significantly.

- The oestrus synchronisation effect of PG on day 6, and perhaps also on day 7, after oestrus, may be poor, not only in cows, which has been clearly demonstrated, but also in heifers.

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