Studies on hormonal dynamics and ovarian response to ovulation-inducing treatments

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CONTENTS

INTRODUCTION

Profitability in dairy farming is greatly influenced by cow reproduction. In recent years, as dairy farms have grown larger, it has become more difficult to observe the estrus behavior of individual cows (Hany Abdalla et al., 2019). This is partly due to the shortage of manpower on dairy farms. Furthermore, community relations are critical in areas where large tracts of land are scarce, such as Japan. In terms of community relations, the dairy farmer's job is much more diversified than before, including environmental measures. Mechanization, such as the introduction of robots, is advancing to compensate for the labor shortage. However, robots are still prohibitively expensive for small-scale farmers with limited capital. As a result, methods such as administering hormones to synchronize estrus or constantly attaching a machine to the body of a cow and detecting estrus using data obtained from inside the body are being attempted (Hai Ho Dac et al., 2022). Cows in estrus take more steps and exhibit a higher level of arousal. The behavior of cows is measured daily using this acceleration sensor. Based on the alarms, ranch managers can attempt to artificially inseminate cows in heat. Given that there is a 90% or higher chance of insemination, the expertise and knowledge of the surgeon are crucial factors in artificial

insemination. Using semen from bulls with high conception rates will produce higher-quality embryos, while using semen from bulls with low conception rates will result in poor conception rates (Hany Abdalla et al., 2019). It is also claimed that insemination close to the date of ovulation increases the conception rate, whereas insemination during estrus decreases embryo quality (L.F.M. Pfeifer et al., 2022). According to previous reports, the manager's ability to accurately understand the estrus signs exhibited by cows and to perform well-timed insemination is critical for producing quality cows. Understanding the hormonal mechanism of estrus in cows is essential for determining the proper timing of insemination. Previous studies have revealed that the dynamics of hormones during estrus in cows are well understood. Elevated estrogen causes follicles to develop, and the primary follicle is selected（S.Reith 2007;). The follicle triggers ovulation with a luteinizing hormone (LH) surge, and ovulation produces the corpus luteum. The corpus luteum is maintained by progesterone, and pregnancy is maintained by progesterone derived from the corpus luteum during the early gestation period. When luteal-derived progesterone is present, estrogen levels do not rise, and the follicle closes. Cows, therefore, require LH to induce ovulation. As a result, if LH levels can be measured using methods other than blood sampling, it can help determine the appropriate timing for artificial insemination and fertilized egg

transfer. Estrus synchronization with hormone therapy in cows has been studied to solve various farm problems. The gonadotropic hormone is commonly used in estrus synchronization therapy to achieve estrus synchronization. However, there is concern about animal stress as a result of the high cost of chemicals and the need for injections in estrus synchronization methods. It is, therefore, crucial to be able to accurately recognize the signs of estrus and perform artificial insemination at the appropriate time. In this study, we investigated the possibility of more accurate artificial insemination timing by measuring LH from vaginal mucus to artificially inseminate the cows at the right time.

Cows are large animals with gestation periods of up to 285 days, making them difficult to manage as laboratory animals. It is also impossible to manage a large number of cows. As a result, other animals that are easier to manage had to be considered. Unlike cattle, which are in estrus all year, cats have unique reproductive physiology, including seasonal estrus and ovulation by insertion stimulation, and their gestation period is only 60 days, which is extremely short when compared to cattle (Root MV et al., 1995; Sparkes AH et al., 2006). Cats repeat the estrous cycle during the breeding season unless interrupted by pregnancy, pseudopregnancy, or disease.

The estrus cycle occurs at various intervals, with 14–21 days being the most common. Cats kept indoors are greatly affected by the brightness of the room, so they repeat their estrous cycle regardless of the season. In the Northern Hemisphere, the onset of estrus activity is earlier in January and February due to the longer h of daylight. Peak estrus activity is usually seen from February to April in the Northern Hemisphere. Normal estrus activity lasts from late October to November (Ticiana Franco Pereira da Silva et al., 2006). The mechanism by which the photoperiod influences the estrous cycle via the hypothalamic-pituitary-gonadal axis and the hormone melatonin is partially understood in cats. Short daylight h raise melatonin and prolactin levels while decreasing ovarian activity. Ovaries are readily retained in cows and can be confirmed via rectal examination. The presence of ovaries in the abdominal cavity can be confirmed by ultrasonography. Cats are also easier to manage because they are smaller and require lower doses of hormones. Various protocols using Follicle stimulating hormone（FSH）and pregnant mare serum gonadotropin have been reported for estrus induction in cats (Kutzler MA: et al., 2007). Ultrasonography can also confirm ovarian cysts in cats. The most effective treatment is yet to be discovered. Ovulation and luteinization may occur spontaneously. If the ovarian cyst is

accompanied by prolonged estrus and ovulation does not occur with spontaneous mating, hormonal induction may be attempted.

As a result, the dynamics of the ovary in cats are still poorly understood. A study was, therefore, conducted to clarify ovarian dynamics during hormone therapy in cats.

Chapter 1

Relationship between GnRH-induced LH increase profiles in the serum and vaginal mucus of Japanese Black beef cows

1. Introduction

Technological progress has led to the development and application of sensor-based monitoring systems that can continuously monitor and record detailed information for oestrus detection in cows (Saint-Dizier and Chastant-Maillard, 2012). The measurement of luteinizing hormone (LH) concentrations is laborious, costly, time-consuming, and requires sophisticated equipment, thus, it is not suitable for field conditions. Quantifying LH concentrations during oestrus improves the accuracy of predicting the optimal time of insemination to ensure conception. If an electronic device is developed for the measurement of LH concentrations, it can provide the necessary information for the optimum timing of insemination and embryo transfer with greater accuracy and efficiency. Intravaginal measurement is probably the most practical method of LH detection, other than blood, because of the labor and animal handling requirements. Therefore, the

present study investigated the relationship between gonadotropin-releasing hormone (GnRH)-induced LH increase profiles in the serum and vaginal mucus of cows.

2. Materials and methods

All animal handling and experimental procedures were performed in accordance with the Guidelines for Animal Experiments of the Tokushima University. All the animals were housed and maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee. Seven cows (Japanese black beef cow, 10–15 years old) with regular oestrous cycles were used for this experiment, which was conducted from July to December 2020. The cows were kept in a group of two in a concrete-floored paddock bedded with sawdust. The cows were fed Italian ryegrass hay and concentrate with water ad libitum. The diet was formulated to meet the nutritional requirements of non-lactating Japanese beef cows (Japanese feeding standard for beef cattle, 2008).

The insertion of a controlled internal drug-releasing device (PRID-Delta; Asuka Animal Health, Tokyo, Japan) containing 1.55 g progesterone and 10 mg estradiol benzoate

was conducted on day 0 (start of the protocol) in cows with unknown oestrous cycles; intramuscular administration of 500 μg of cloprostenol (Estrumate®; Intervet K.K, Tokyo, Japan) was performed on day 9, along with PRID-Delta removal; 100 μg of GnRH (Fertirelin acetate, Conceral®; MSD Animal Health, Tokyo, Japan) was administered (intramuscularly) on day 11. Samples for LH determination were collected immediately before GnRH administration and every 30 min from the start of GnRH administration for 6.5 h. Blood was collected from the jugular vein using an indwelling catheter. Briefly, intravenous 18 G catheters (NIPRO, Osaka, Japan) were connected to three-way valves (3 W-RC type; NIPRO, Osaka, Japan) and inserted into the jugular veins for blood sampling. Vaginal mucus was collected from the vagina using a kitchen sponge. Briefly, a kitchen sponge made of polyurethane (3.5 cm diameter, 7 cm length) with a handle (40 cm length) was inserted into the vagina using a vaginal speculum. The sponge was glued to the mucosal surface to absorb the mucus and was then placed in a 50 mL syringe, and the mucus (about 1–3 mL) was squeezed out into a 15-ml conical polypropylene tube. Both samples in the conical tubes were immediately chilled in ice in styrofoam box and stored in a refrigerator until centrifugation. Serum and vaginal mucus were separated by centrifugation at 3,000 rpm for 15 min at 4 °C and stored at –80 °C until assayed for LH concentrations. The LH concentrations of the

serum and vaginal mucus were determined using a double-antibody radioimmunoassay (RIA) according to the method described by Naniwa et al. (2013), using a bovine LH RIA kit provided by the National Hormone and Peptide Program (NHPP; Baltimore, MD, USA). In addition, for measuring LH concentrations in the mucus, samples were mixed well with the Vortex Mixer until the samples were homogenized and deliquesced, and then the supernatant was applied to the RIA. The lowest detectable concentration of LH in both samples was 0.049 ng/mL in a 100 μL both samples, and the intra- and inter-assay coefficients of variation were 10.4% at 0.56 ng/mL and 8.0% at 0.61 ng/mL, respectively.

3. Results

Our study was not designed to validate the treatment effects on ovulatory response, but ovulations in all cows treated by the synchronization program were observed by transrectal ultrasonography. LH concentrations in the serum and vaginal mucus versus time after intramuscular administration of GnRH are shown in Figure 1. The mean peak of serum LH concentrations (28.89 ± 8.47 ng/mL) observed after GnRH administration were approximately 40-

fold higher than vaginal mucus LH concentrations (0.69 ± 0.32 ng/mL). Serum LH concentration (range 8.45 – 74.28 ng/mL) peaked at 2.5 h post-GnRH administration with serum concentrations returning to near values of pre-GnRH administration after 6.5 h, whereas the peak of LH concentration (range 0.18 – 2.42 ng/mL) in vaginal mucus was observed 4.5 h after GnRH administration. The mean time interval between serum and mucus peaks averaged 1.92 ± 0.23 h (range 1.0 – 3.0 h). Moreover, the surge-like LH secretion was clearly identified in the serum of all cows, while the LH peak concentration in the vaginal mucus of some cows was not as distinct as in the serum.

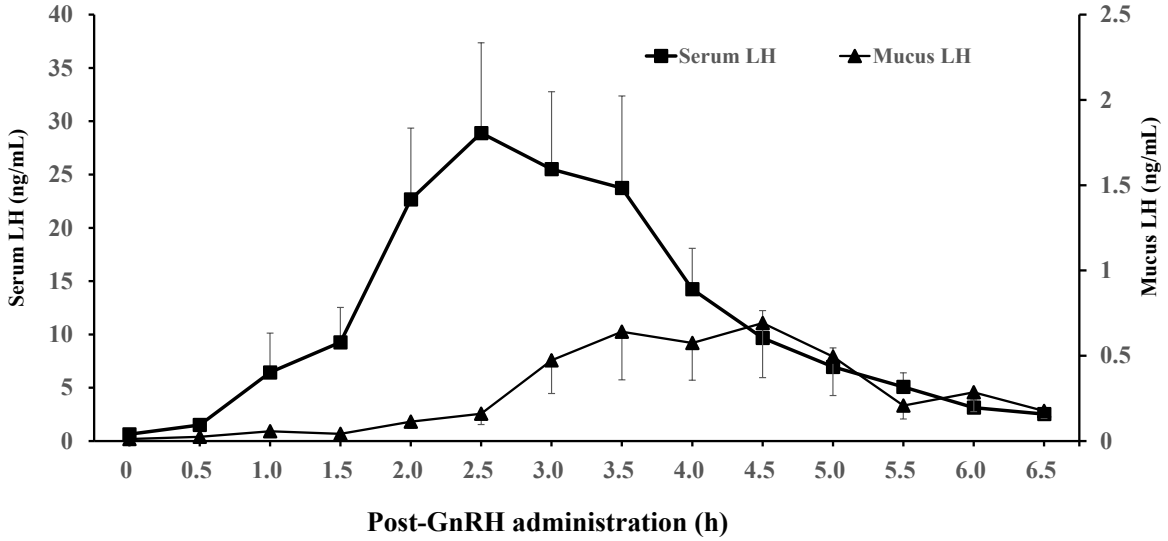


Figure 1: Mean (± SEM) concentrations of luteinizing hormone (LH) in serum and vaginal mucus from the start of gonadotropin-releasing hormone (GnRH) injection to 6.5 h. All cows received a PRID-Delta for 9 days, which was removed at the time of cloprostenol treatment. Two days after the cloprostenol treatment, cows received GnRH. Samples were collected immediately before GnRH administration and every 30 min up to 6.5 h after GnRH treatment. A peak LH concentration was observed at 2.5 h and 4.5 h in serum and vaginal mucus, respectively, after GnRH injection.

4. Discussion

Previous studies reported that cervical mucus has a strong relationship with progesterone concentration and ovulation time (Layek et al., 2013), and the oestrus-specific odor in mucus has been suggested to influence LH surges in cows in the same barn (Nordeus et al., 2012). Moreover, the vaginal electrical resistance is known to fluctuate with the stages of the estrous cycle and is closely related to the timing of ovulation (Canfield and Butler, 1989). These reports indicate that cervical mucus may be used as a tool to determine the proper time of insemination. Moreover, the pre-ovulatory LH surge has also been suggested as a more precise indicator of ovulation time (Larsson, 1987; Rajamahendran et al., 1989). In the present study, we observed that in all cows, the LH profile in vaginal mucus showed surge-like LH secretion after GnRH injection, similar to the serum LH profile. Moreover, the surge-like LH secretion appeared at 2.5 and 4.5 h for serum and vaginal mucus, respectively, and the surge-like LH secretion in vaginal mucus appeared about 2 h later than the surge-like LH secretion in serum. In previous studies, GnRH-induced LH increase was reported to appear 1.5 to 2 h after GnRH administration in dairy and beef cows (Colazo et al., 2008; Armengol-Gelonch et al., 2017; Wijma et al., 2017), which was similar to the time of surge-like LH increase in the serum observed in the present study. Moreover, LH peak concentrations in

the vaginal mucus were approximately 1/40th of those in the serum. The peak of LH concentrations in the vaginal mucus was not distinct in some cows. In the present study, the sponge sampling method might have a cleaning effect, resulting in a lower LH concentration. On the other hand, if the LH measuring device was left in the vagina for an extended period, the increase in LH concentration may have been delayed and consequently obscured, as secreted LH is likely to be added to the mucus present in the vagina. Therefore, these results indicate that precise electronic equipment for measuring LH concentrations needs to be developed to obtain the necessary information from vaginal mucus for the optimal timing of insemination. In conclusion, the results from the current trial confirmed the peak of LH secretion in vaginal mucus, which appeared about 2 h later than the peak of LH secretion in the serum. However, measuring the dynamics of the peak LH secretion from vaginal mucus requires an accurate measurement device because of the extremely low LH concentration in the vaginal mucus.

Chapter 2

Vaginal stimulation enhances ovulation of queen ovaries treated using a combination of eCG and hCG

1. Introduction

Understanding the physiology of feline reproduction might be beneficial for breeding both domestic and wild felid species, as well as for biomedical research. The reproductive physiology of feline animals is particularly unique, compared to other animal species and is characterised by long-day breeders. There is also spontaneous ovulation, but mechanical stimuli of the vagina by coitus induces ovulation under normal physiological conditions (Shille et al., 1983; Lawler et al., 1993; Ferre-Dolcet et al., 2020). Thus, the vaginal stimulation by coitus is important for the occurrence of ovulation. In domestic cats, combination regimens of equine chorionic gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) have often been used in artificial insemination (Swanson et al., 1995a; Roth et al., 1997). eCG is typically administered first to stimulate follicular growth, and hCG is administered several days later to induce follicular

maturation and ovulation. When combining a hCG treatment with vaginal stimulation, ovulation after natural breeding or artificial insemination is ensured (Roth et al., 1997). However, repeated treatment of eCG and hCG may causes an immunologically mediated refractoriness to ovarian stimulation (Swanson et al., 1995a).

In queens, follicular changes (growth, ovulation, or atresia) throughout the oestrous phase have been poorly documented because successful ultrasound examination of the ovaries relies on the use of adequate equipment (high-frequency probes) and experienced personnel. Thus, it restricts the progress made in our understanding of follicular development. It has been suggested that the current ability of ultrasound findings of ovaries is limited to follicular growth and ovulation during pro-oestrus and oestrus (Malandain et al., 2011). Moreover, ultrasound echogenicity of corpora lutea is variable and depends on the observation period, thus making sonographic detection difficult (Gatel et al., 2020). However, when ovaries are clamped at a subcutaneous site, their follicular growth can more easily be monitored using ultrasound imaging (Hirata et al., 2018). Moreover, the ovarian clamp at the subcutaneous site can provide easy access for repeated collection of oocytes for in vitro fertilization in endangered animals.

The objectives of the present study were to evaluate the effects of vaginal stimulation by a tomcat on the induction of ovulation in queens treated with a combination of eCG and hCG, in which the ovaries were clamped at a subcutaneous site for ultrasound imaging of follicular growth.

2. Materials and methods

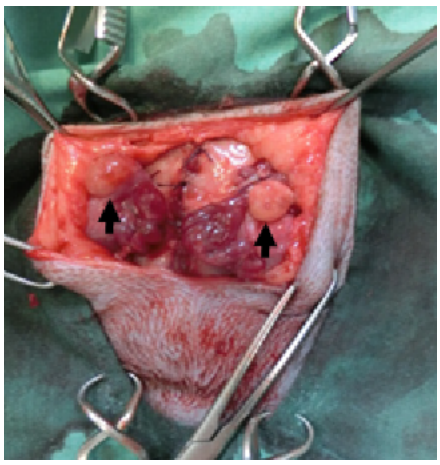
Animals

Four healthy domestic queens (aged 5 – 7 years; mean weight, 3.5 ± 0.5 kg) obtained from Kitayamarabesu Co. Ltd (Nagano, Japan) were used. The queens were individually housed in a single cage and were given standard commercial cat food once a day, and water ad libitum. The rooms where cages were placed were environmentally controlled with 12 h:12 h light:dark cycles at a temperature of 25 - 28°C. Laboratory animal management and experiments including anesthesia protocol performed for surgery were performed with the approval of the committee based on the guidelines of the Laboratory Animal Committee of the Faculty of Agriculture, Yamaguchi University. The four cats used in the experiment had been treated for research purpose only and continue to be bred after the experiment.

Ovary clamp at the subcutaneous site

Bilateral malacotomy of four queens was performed using a ventral-flank abdominal approach using routine techniques and materials, according to the methods described by Hirata et al. (2018). Briefly, the queen was mechanically ventilated with isoflurane in pure oxygen. Then, the uterine artery and vein were ligated and severed at the cranial tip of the uterine horn, after which the ovary was separated from the uterus. Each separated ovary, maintaining blood circulation from the suspensory ligament, was clamped at a subcutaneous site through the external abdominal oblique muscle. Each ovary was superficially placed on the external abdominal oblique muscle, making sure to not strangulate the ovarian blood supply (Figure 2). Finally, the subcutaneous layer and skin incision were closed.

Figure 2.



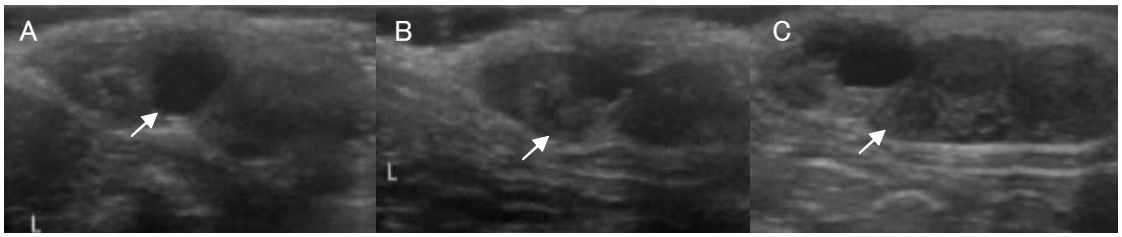
Hormonal treatment and vaginal stimulation

To induce oestrus and ovulation, a hormonal treatment was conducted using a simple crossover design. Four queens alternately received the two treatments with and without vaginal stimulation by a vasectomised tomcat after hCG treatment at 2-month intervals to approximate the normal interoestrous interval. Before the hormonal treatment, for each queen, ultrasonography results confirmed that the ovaries had no corpus luteum (CL). The queens were intramuscularly administered 150 IU of eCG (Kyoritu Seiyaku, Tokyo, Japan) (day 1). Each queen was given 250 IU of hCG (Kyoritu Seiyaku) on days 5 and 6 after the eCG treatment, with reference to a standard regiment with eCG and hCG in combination (Swanson et al., 1996; Villaverde et al., 2009). In the stimulation group, queens were mated with a vasectomised tomcat for 3 days after hCG injection on day 5.

Ovarian ultrasonography

Ovarian ultrasonography examinations were performed under sedation with an intramuscular injection of 1 mg/kg of ketamine hydrochloride (Daiichi Sankyo Co., Ltd., Tokyo, Japan). The development of the follicle and CL after inducing oestrus and ovulation was measured using an HS-2100V veterinary ultrasound imaging system equipped with a 5.0 - 10.0 MHz linear array transducer (Honda Electronics, Aichi, Japan). The ovaries were examined once a day from day 1 to 7, and on day 13, and a diagram of all follicles with diameters ≥ 3 mm and the CL was recorded for each ovary (Figure 3). The follicles were divided into three groups, according to their diameter: small follicle group with a diameter ≥ 3 mm and < 4 mm, middle follicle group, with a diameter ≥ 4 mm and < 5 mm, and the large follicle group, with a diameter ≥ 5 mm. The ovulation rates were calculated by dividing the number of CL observed on day 13 by the total number of follicles observed on day 5.

Figure 3.



Hormonal assay

Blood samples were collected from the femoral vein on days 1, 5, 7, and 13 after eCG treatment (day 1); the samples were collected in a 2.5-mL syringe under sedation and transferred into 1.5-mL microtubes. The microtubes were centrifuged at 1,500 × g for 30 min. The serum was separated from clot and stored at –30°C until the sample was assayed for hormonal concentrations. The serum concentrations of oestradiol and progesterone were measured using a chemiluminescent enzyme immunoassay using commercial kits (Immulite 2000; Siemens Healthcare K.K., Tokyo, Japan).

Statistical analyses

Differences between queens with and without vaginal stimulation by a tomcat regarding the ovulation rates and hormonal concentrations were evaluated using an independent Student's t-test by StatView software (Abacus Concepts, Berkeley, CA, USA). Differences with a P value of ≤ 0.05 were considered statistically significant.

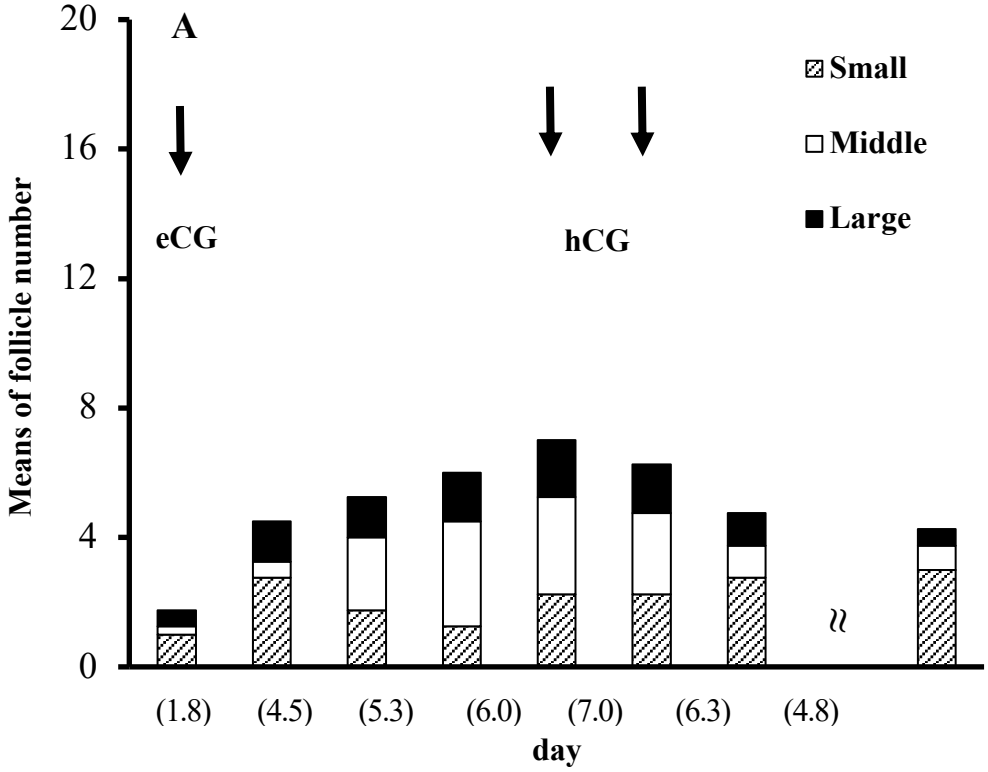
3. Results

Follicles in ovaries clamped at a subcutaneous site were visualised as well-defined, anechoic cavitary structures with diameters ≥ 3 mm. The mean numbers of follicles with a small, middle, and large size gradually increased with the eCG treatment and decreased after hCG injection, irrespective of vaginal stimulation by a vasectomised tomcat (Figure. 4). The total number of follicles on day 5 was significantly higher ($P < 0.01$) in the vaginal stimulation group (12.3 ± 1.0) than in the control group without stimulation (7.0 ± 0.7). In the vaginal stimulation group, moreover, a higher total number of CL was observed on day 13 (8.5 ± 0.5) compared with that in the control group (3.0 ± 0.4) ($P < 0.01$). The ovulation rate of follicles observed on day 5

was significantly higher ($P < 0.01$) in the vaginal stimulation group (70.0 ± 3.3%) than in the control group (42.6 ± 2.7%).

As shown in Figure 5, the serum concentrations of oestradiol and progesterone during the hormonal treatments did not differ between the two groups. The mean concentrations of oestradiol in both the stimulation and control groups increased from 59.7 and 37.9 pg/mL (day 1) to 72.6. and 77.3 pg/mL (day 5) after eCG treatment, respectively. Moreover, the mean concentrations of progesterone also increased from 0.3 and 0.6 ng/mL (day 5) and 0.3 and 0.4 ng/mL (day 7) to 11.5 and 20.2 ng/mL (day 13) due to the hCG injection and both the hCG injection and vaginal stimulation, respectively.

Figure 4.


A
Small
Middle
Large
eCG
hCG
Means of follicle number
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12
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4
0
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(4.5)
(5.3)
(6.0)
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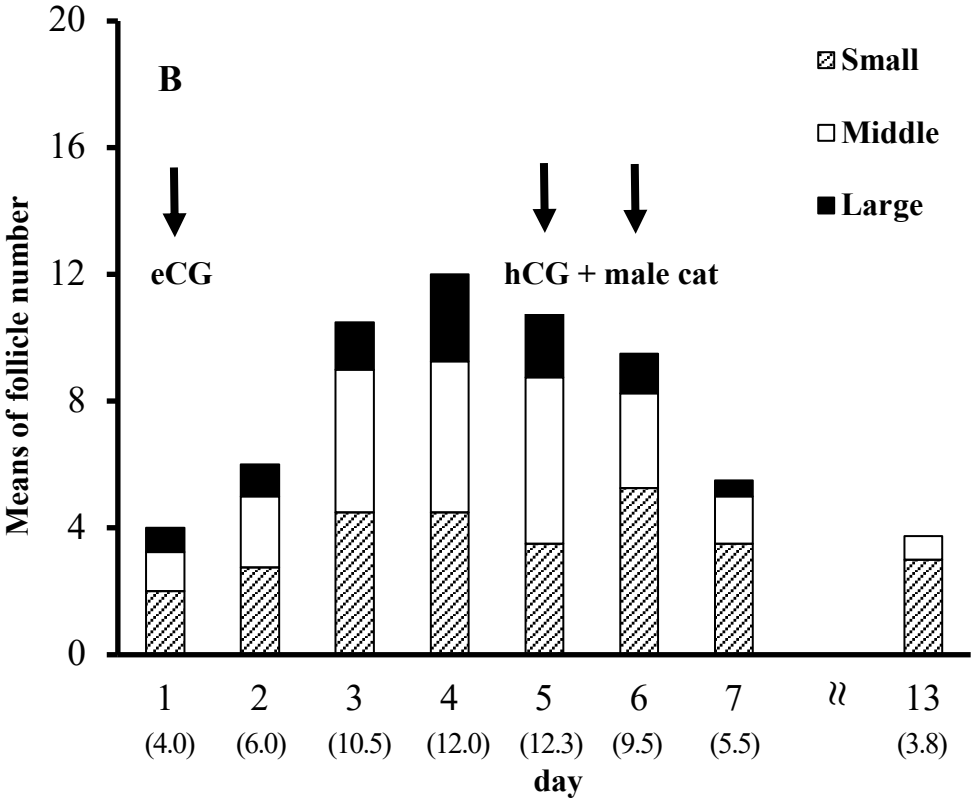
day


B
Small
Middle
Large
eCG
hCG + male cat
Means of follicle number
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5
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(12.3)
(9.5)
(5.5)
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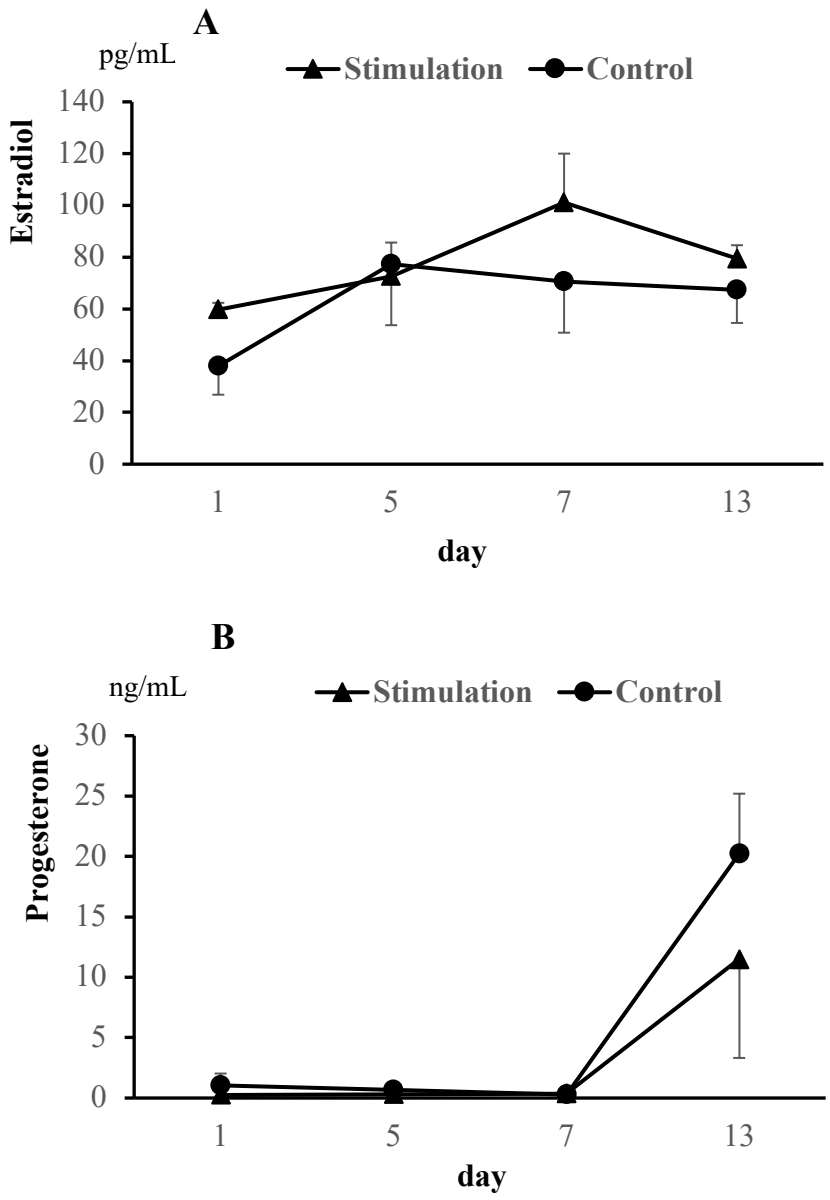
day

Figure 5.


A
pg/mL
Stimulation
Control
Estradiol
140
120
100
80
60
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Progesterone
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25
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day

4. Discussion

Ultrasound is widely used in the screening of pregnant companion animals. However, a detailed ultrasound description of follicular growth in queens has been far less reported than that of other species, such as cows for instance (Adams et al., 2008). Transabdominal ultrasonographic examination of the reproductive tract in queens has been performed from the ventral abdomen in dorsal recumbency under sedation (Gatel et al., 2020). Recently, high frequency probes were used to study the ovaries, and the relatively small size of follicles could be measured (Malandain et al., 2011; Gatel et al., 2020); however, the ultrasound detection rate from the ventral abdomen has been reported to be good for follicles, but poor or moderate for CL, even when high-frequency probes are used (Gatel et al., 2020). In the present study, therefore, the growth of follicles and CL was observed using ultrasound imaging of the ovaries clamped at a subcutaneous site. Furthermore, because ovulation could be induced by vaginal stimulation after the follicles reached a diameter of 3 mm (Malandain et al., 2011), the number of follicles with a diameter of 3 mm or more was recorded, according to the diameter size. In a previous study, mating was performed on Day 3 of natural oestrus in queens (Concannon et al., 1980) However, in the present study, oestrus was

induced by eCG administration and queens were subsequently mated with a vasectomized tomcat after hCG injection on day 5. As a result, we observed that eCG treatment increased the number of follicles, regardless of the specific follicle size, hCG injection induced ovulation of developed follicles, and some follicles have regressed without ovulation, irrespective of vaginal stimulation. These results were supported in part by the experiment of Ferre-Dolcet et al. (2020) who reported that GnRH-treated queens between the second and fourth days of oestrus had about twice ovulation rates compared with the untreated queens. However, we found that the combination of hCG injection with vaginal stimulation by a vasectomised tomcat increased the ovulation rate of follicles, in which the vaginal stimulation increased the ovulation rates by about 27%. Goodrowe and Wildt (1987) demonstrated that the excessive follicle number by porcine FSH (FSH-p) treatment cannot be reduced with any of the hCG or GnRH treatments, but combination of hCG injection with copulatory stimuli synergistically enhances the ovulatory response of queens treated with FSH-p. Therefore, our findings indicate that, in queens with vaginal stimulation, follicular development was initiated by eCG, whereas ovulation was presumably enhanced by both vaginal stimulation-induced endogenous LH surges and hCG injection-induced exogenous LH surges. However, our present results are not consistent with the findings of Roth et al. (1997) who reported

that when the numbers of CL were observed in ovaries obtained by ovariohysterectomy, the combination of hCG injection and natural mating did not increase the ovulation rates in queens treated with eCG. It has been reported that the serum concentrations of eCG after intramuscular injection are highly variable in domestic cats with even similar body weights (Swanson et al., 1996). Moreover, Yu et al. (2010) have suggested that the dose of eCG influences ovarian activity and embryo production in queens. In the present study, the number of middle and small follicles in the vaginal stimulation group was slightly higher than that in the unstimulated control group, indicating that the number of follicles before the start of the hormonal treatment might affect the ovulation rates. Therefore, the discrepancy concerning the vaginal stimulation effect remains to be explained, but it might, in part, be due to the influence of eCG used for ovarian stimulation and the status of ovarian follicles.

When administered prior to hCG, eCG stimulates follicle growth and potentiates the response of early antral follicles to the intrinsic folliculogenic and luteotrophic effects of hCG (Swanson et al., 1997). In the present study, we observed that, in all queens, serum concentrations of oestradiol and progesterone increased after eCG treatment and hCG injection, respectively. However, despite the higher total number of follicles and CL found in the vaginal stimulation

group, the serum concentrations of oestradiol and progesterone during the hormonal treatments did not differ from those in the non-stimulated group. In a previous study, it was suggested that there is a lack of correlation between the serum oestradiol-17 concentration and the presence of distinct ovarian follicles, whereas serum progesterone has a strong positive correlation with the CL mass (Swanson et al., 1995b). In the present study, we evaluated the concentrations of oestradiol and progesterone during hormonal treatment in four queens using a crossover design. In cats, an extragonadal origin has been suggested to affect progesterone concentrations due to stress induced by restraint and handling even under anaesthesia (Howard et al., 1992; Chatdarong et al., 2006). However, considering a fact that only a limited number of samples were used for evaluation of the hormonal concentrations, there might be no significant differences in the progesterone concentrations because of the small number of samples and unknown factors due to a repeated use. In conclusion, the observation of follicular development in the ovaries clamped at a subcutaneous site using ultrasonography indicates that the combination of hCG injection with vaginal stimulation by a vasectomised tomcat may increase the ovulation rate of follicles. However, further studies are needed to clarify the hormonal concentrations and to determine the vaginal stimulation effects because of a small number of examined animals.

OVERALL DISCUSSION

Estrus timing is critical when considering the timing of artificial insemination in cows. There are currently two methods to determine estrus behavior: one is to have the farm manager check the estrus behavior of cows, and the other is to check the estrus through biological data using a machine attached to the cow's body (Hai Ho Dac et al., 2022).We also considered methods other than blood to accurately understand the timing of estrus. The purpose of Chapter 1 was to examine the relationship between gonadotropin-releasing hormone (GnRH) and the LH increase profile in cow serum and vaginal mucus. Samples for LH measurement were collected from Wagyu cows just before and every 30 minutes for the next 6.5 h after the start of GnRH administration. Serum LH concentrations peaked 2.5 h after GnRH administration and returned to the same level as before administration at 6.5 h, while vaginal mucus LH concentrations peaked at 4.5 h after GnRH administration. These results indicate that the peak of LH secretion in vaginal mucus occurred approximately 2 h later than the peak of LH secretion in serum. This difference in peak time may affect the timing of LH ovulation into follicles in cow ovaries.

In Chapter 2, we aimed to confirm the ovarian dynamics in cats. In cows, it is relatively easy to confirm ovarian dynamics by rectal examination and other methods using

echocardiography. Ovarian dynamics during treatment with gonadotropins are extremely important for understanding the efficacy and appropriate timing of hormonal treatment. It is, however, still poorly understood in cats (Gatel et al., 2020). Follicular changes during hormonal treatment have not been well documented in cats because of the deep location and small size of the ovaries. We believed that we could better understand ovarian dynamics by first relocating the deep ovaries to the body surface. We conducted experiments using cats with ovaries relocated to the body surface as an experimental model to confirm the ovarian dynamics. We used the estrus synchronization program commonly used in cattle to create a schedule for the cat. We examined ovarian kinetics in cats treated with a combination of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) (Swanson et al., 1995a; Roth et al., 1997). Cats have a special reproductive physiology in which insertion stimulation induces ovulation. As a result, the effect of insertion stimulation by male cats on ovulation induction was also evaluated. Hormonal treatments were performed using a simple crossover design. Four cats were treated with eCG 150 IU (day 1) and hCG 250 IU (days 5 and 6). Half of the cats were allowed to mate with vasectomized male cats for three days following hCG administration. The mating was visually confirmed. Ultrasound imaging of subcutaneously clamped ovaries was performed once daily from days 1–7

and on day 13, and serum estradiol and progesterone concentrations were examined on days 1, 5, 7, and 13. The average number of follicles gradually increased with eCG administration and decreased after hCG injection. The ovulation rate of follicles was significantly higher in the vaginal stimulation group (70.0%) than in the control group (42.6%). Serum concentrations of estradiol and progesterone did not differ between the two groups during hormone treatment. Ultrasound images of subcutaneously clamped ovaries revealed that eCG and hCG administration promoted follicle development and corpus luteum formation, respectively. hCG injections combined with vaginal stimulation in a vasectomized cat increased the follicle ovulation rate. These findings imply that the experimental model of ovarian transfer to the body surface may be used to understand ovarian dynamics in other animal species.

In conclusion, technological advances have made it possible to measure LH concentrations, which were difficult to measure in the field in Chapter 1. We have gained the necessary understanding of the dynamics of serum gonadotropins in cattle and the appropriate timing of insemination. Until recently, artificial insemination was frequently performed based on the experience and intuition of the technician performing the insemination. Inexperienced technicians struggled to understand when cows were in estrus and when the best timing for artificial

insemination was. Understanding the proper timing of artificial insemination is a critical factor in improving conception rates on farms. When fertility is improved, it has a substantial impact on farm management (Hany Abdalla et al., 2019). Reducing the number of inseminations per cow before conception can prevent a great deal of economic loss. Understanding proper insemination timing is also important for other animals, and further research for other species is required. However, there are many animal species in the world, and it would be difficult to conduct experiments on each of them. Reproductive physiology frequently varies among animal species. Seasonal and annual estrus differ greatly. Additionally, the living environment and food habits of each species differ. As a result, we considered using cats as an easy-to-manage experimental model. This is because seasonal estrus can be controlled by adjusting the amount of light in the living environment. In Chapter 2, we examined the ovarian dynamics of cats, which have not been well elucidated. Cats have a unique reproductive physiology of ovulation that involves seasonal estrus and insertion stimulation (Shille et al., 1983; Lawler et al., 1993; Ferre-Dolcet et al., 2020). By relocating the cat's ovaries to the body surface using echo, we succeeded in confirming ovarian dynamics, which had been difficult to confirm due to their deep location in the abdominal cavity. Understanding ovarian dynamics during hormone treatment in cats as reproductive physiology

may have brought us closer to understanding feline animals with special reproductive physiology. We also believe that subcutaneous implantation of ovaries has provided a model for repeated retrieval of oocytes for in vitro fertilization in cats. If this were possible, it was hoped that the generation of IVF eggs could be facilitated in other animal species. Furthermore, by using cats, which are smaller than cows, as an experimental model, we were able to understand the dynamics of hormones in the body with a small amount of gonadotropic hormone. In the future, this model may be used to predict the type of hormone dynamics caused by a gonadotropic hormone in cows. This research has advanced our understanding of efficient artificial insemination timing and the reproductive physiology of cats, which had not been fully elucidated. Furthermore, there are many cat species in the world, including lions. It would be very gratifying if our research could contribute to the preservation of other cat species in addition to cattle and other livestock.

The prediction of gonadotropic hormone disposition in various animal species is critical for the conservation of species. Our experimental model using cats could be used with many animal species. In cattle, we found the model useful for understanding the exact timing of reproduction. We hope that our research will lead to improved reproductive performance in cattle and efficient

ranch management. Improving the reproductive performance of cattle may contribute to the conservation of various animal species.

SUMMARY

Over the years, humans have developed a relationship with cattle and other livestock. A systematic rise in livestock production and efficient reproduction have been necessary in this context. Reproduction is critical for various animal species, including livestock. Multiple studies have been conducted to improve the reproductive performance of livestock and achieve efficient reproduction. We examined the time of appearance of LH in serum and vaginal mucus after administration of gonadotropins, which are used daily in bovine reproductive practice. Serum LH was highest at 2.5 h after injection and returned to pre-injection levels at 6.5 h. LH in vaginal mucus was highest at 4.5 h following injection. From the viewpoint of animal welfare, it appears necessary to develop a non-invasive model other than blood; further development of a machine for more accurate measurement is desirable in the future.

In cats, we also developed a model in which the ovaries were surgically transferred subcutaneously. Using this model, we confirmed ovarian dynamics using gonadotropic hormone and examined appropriate ovulation induction in cats with special reproductive physiology. It was now possible to confirm ovarian kinetics in cats using ultrasound and ovulation induction using

insertion stimulation, which had previously been impossible. However, as the number of animals examined was small, additional research studies on hormone concentration and volume are required in the future.

With these two studies, we were able to confirm the response of gonadotropic hormones in different animal species. The reproductive physiologies of animals vary greatly. We will be glad if our research plays a role in improving fertility in each animal species and contributes to farm management and the conservation of various endangered animal species.

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