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Abstract

Exogenous gestagenic products have been used widely for many years. After establishment of developing the products using new technique not only of the administration route for absorption, that is through vaginal mucosa, but also of deliberation of gestagen from the device, gestagenic compounds have been able to be exposed sufficiently for certain periods with less stressful and no damage for skin or tissue compared with injectable products in 1980s.

The Controlled Internal Drug Release (CIDR) has been developed in 1980s as one of the exogenous progesterone (P_4) products using technique of absorption through vaginal mucosa in cattle. The CIDR that is available in Japan, is a T-shaped form containing 1.9 g of natural P_4 and this is one of the valuable tools to solve certain reproductive disorders. The plasma P_4 concentration peaks within 1-2 hours rapidly after the

insertion and declines smoothly after the removal. During the intravaginal insertion, its plasma P_4 concentration maintains as artificial luteal phase.

Reproductive performance in cattle has decreased recently. The CIDR has been used by itself or by combination with other hormonal substances for treatment of reproductive disorders to improve the performance as a part of the short term perspective. The usages are based on the role of P_4 itself from the inserted CIDR and also based on the human-side's reason of increased detected estrus by producers due to more concentrated and stronger estrous expressions.

The specific objectives of this investigation were to determine for the first, whether the usage of CIDR as a part of the fixed timed artificial insemination (TAI) protocols was efficacious and beneficial regimen in Holstein dairy heifers and as the second, whether the usage of CIDR for cystic ovarian disease (COD) in Japanese Black cows was efficacious and beneficial treatment program.

In the first study, the reproductive performance of two types of TAI protocols with or without CIDR was evaluated in a commercial herd of Holstein dairy heifers. A total of 74 heifers with 14.4 ± 0.2 months of age (average ± SEM) were allocated to two groups; Ovsynch (n=44) and estradiol benzoate (EB) used Heatsynch (EB-Heatsynch, n=30), and each group was additionally divided into two subgroups with CIDR insertion (CIDR-treated group) from day 0 to 7 (n=36) and without CIDR (No-CIDR-treated group) (n=38). Blood was collected for P_4 analysis and findings of ovaries were monitored by ultrasonography. Heifers in CIDR-treated group resulted in higher pregnancy rate as compared with No-CIDR-treated group (63.9% vs 21.1%, $P<0.01$). Heifers with functional corpus luteum (CL) on day 0 resulted in significantly higher pregnancy rate in CIDR-treated group than No-CIDR-treated group (67.9% vs 13.0%, $P<0.01$). The CIDR insertion suppressed the intermediate ovulation during the first 7 days and during the period from the second gonadotropin releasing

hormone (GnRH) or EB administration to TAI as compared with No-CIDR-treated group (first 7 days: 33.3% vs. 52.6%; $P<0.05$, before TAI: 11.1% vs. 37.0%; $P<0.05$). In conclusion of the first study, the selected TAI protocols with CIDRs provided acceptable pregnancy rate in heifers compared with original TAI protocols without CIDRs and contributed to the economical improvement by shortening the average age of first calving approximately for 2.5 months as compared with the previous management without TAI protocols in the same commercial herd.

Secondly, the value of the CIDR treatment combined with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) against COD was evaluated in commercial Japanese Black cows through the observation by ultrasound and measuring plasma P_4 concentrations. The treatment strategies against COD are different for the follicular cyst (FC) and luteal cyst (LC). GnRH for the treatment of FC and $PGF_{2\alpha}$ for LC should be selected; however, it is difficult to discriminate an FC from LC, particularly under field

conditions because 1. The ovaries always change; therefore it is very difficult for veterinarians when they should nail down to select alternative treatment, 2. Measuring plasma P_4 concentration takes certain time, 3. It is difficult to diagnose COD by rectal palpation properly, 4. Even though using ultrasonography, the diagnosis was based on the morphological stand point without functional relevance. Therefore, it was necessary to develop new strategy to minimize the risk of selecting incorrect treatment.

Experiment 1 was preliminarily conducted to evaluate simply clinical efficacy of the CIDR combined with $PGF_{2\alpha}$ against COD by two insertion periods of CIDR that were 7-day and 14-day of insertion from day 0. A total of 28 Japanese Black cows with COD that were $\geq$40 days postpartum and anestrous after calving, that were categorized by plasma P_4 concentrations on day 0 (=CIDR insertion) and were utilized. As a result, in the cows with plasma P_4 <1.0 *ng/ml* on day 0, CLs were observed at 14 days after CIDR removal in 87.5% of the

CIDR 7-day insertion group and in 100% of the CIDR 14-day insertion group. In the cows with plasma P_4 $\geq$1.0 *n*g/m*l* on day 0, CL was formed in 77.8% and 50.0%, respectively. Thus, the formations of CLs were high in both CIDR 7-day and 14-day insertion groups, and there were no significant differences in the all reproductive parameters ($P>0.05$). Therefore, the following evaluation studies on the efficacy of CIDR insertion against COD was performed based on its 7-day insertion.

Experiment 2 was conducted to group cysts into 4 patterns based on alteration of plasma P_4 concentrations on day-7 and day 0 at 1 week interval of observation with 1.0 *n*g/m*l* as the cut-off level by ultrasonographic examination of 28 cows with COD that were $\geq$40 days postpartum and anestrous after calving. All the cows were administered with CIDR from day 0 to day 7 and $PGF_{2\alpha}$ on day 7 at CIDR removal.

In Experiment 3, a total of 55 cows under the same conditions as in Experiment 2 were utilized, and

the same regimen as in Experiment 2 was performed without 1 week of observation before CIDR treatment. In Experiment 2, the average size of cysts in both cows with plasma P_4 concentrations $\geq$1.0 *n*g/m*l* on both day -7 and day 0 and cows with plasma P_4 concentrations <1.0 *n*g/m*l* on both day -7 and day 0, decreased significantly during 7 days of CIDR insertion ($P<0.05$). As an overall result in Experiment 2 and 3, 92.9% of CLs on day 21 were highly formed in Experiment 2 and 83.6% in Experiment 3. The conception rates within 60 days after CIDR removal were also satisfactory high and were 71.4% and 54.5%, respectively. There were no differences in any overall reproductive parameters between Experiments 2 and 3 ($P>0.05$). The average days between CIDR removal and conception were 24.4 ± 5.3 and 24.0 ± 6.5 days (average ± SEM), respectively ($P>0.05$); therefore, the interval to conception of the cows in Experiment 3 were at least 7 days shortened compared with Experiment 2. In conclusion, treatment with a CIDR and $PGF_{2\alpha}$ against COD could minimize the

risk of selecting incorrect treatment firstly and provided sufficient reproductive performance in Japanese Black cows with COD. The positive economic impact of 1 week observation for producers was not found out from the results in these studies.

Experiment 4 was conducted to compare the efficacy of CIDR for 7-day insertion from day 0 followed by $PGF_{2\alpha}$ on day 7 (=CIDR removal) (n=13) with that of GnRH administration on day 0 combined with $PGF_{2\alpha}$ on day 7 (n=10) against COD. The cows with plasma P_4 concentrations <1.0 *n*g/m*l* both on day -7 and day 0 were utilized. As a result, no ovulation of any cystic follicles was observed in both groups; however, ovulation of a coexistent follicle was observed in the GnRH group but only in 30% of the cows. There were not significant differences on any reproductive parameters between the 2 groups ($P>0.05$). In conclusion from Experiment 4, CIDR insertion for 7 days followed by $PGF_{2\alpha}$ administration could be replaceable regimen of GnRH protocol; however, the study should be

continued to collect more number of samples.

In general conclusion of this investigation, the author found out that;

1. The CIDR was a cost-benefit and a valuable tool for improvement of reproductive performance when using TAI protocol in heifers due to suppression of intermediate ovulation.
2. The CIDR treatment for COD could provide the opportunity of AI at the detected heat that the treated cows expressed even though cystic follicles remained, and its conception rate was acceptable economically because using the CIDR and $PGF_{2\alpha}$ program could minimize the risk of selecting incorrect treatment firstly for COD in Japanese Black cows and the program also could contribute to the economic benefit for producers.

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Chapter 1

General introduction

1.1. Gestagenic products

The exogenous gestagenic compounds have been used for treatment of several reproductive disorders or for control of estrous cycle by itself or by combination with non-gestagenic compounds in cattle for many years. The gestagenic compounds have an important role on negative feedback on LH pulse frequency as the same as endogenous P_4 at the sufficient level of the concentrations [10-11, 24, 103].

The gestagenic products that are currently available in veterinary medicine for cattle are injectables, feed additives and intravaginal inserts worldwide [81]; however, only injectables and intravaginal inserts are currently approved in Japan. The injectables have been used for treatment for

disorder of implantation of fertilized ovum, habitual abortion, follicular cyst, the cattle with difficulties of pregnancy, improvement of conception in cattle with silent heat and prevention of retained fetus placenta. However, the intravaginal inserts have been widely used more than the injectables for estrous synchronization, ovarian quiescence and silent heat. The advantages are; 1. These devices can avoid damage to skin or tissue which is sometimes associated with injections, 2. The administration is less stressful to the animal and these allow termination of delivery at removal [80], 3. These devices can provide consecutive release of gestagen during the inserts. These advantages of the intravaginal gestagenic administration can set cattle be under artificial gestagenic situation more certainly and more consecutively than injection of gestagenic compounds.

1.2. Developmental background of intravaginal

insert device

In 1960s, Robinson's research is the first one on intravaginal administration of gestagenic compounds using polyurethane sponge in ewe [87]. In 1970s, the sponges have been developed for cattle and horse; however, various retention rate and vaginal discharge due to irritation had become issues to be solved [80]. Then, silicon rubber has been used for a new device in development of Progesterone Releasing Intravaginal Device (PRID), firstly [88-89]. On the other hand, Controlled Internal Drug Release (CIDR) device has been developed in 1980s and it was firstly marketed in New Zealand in 1987 [79].

1.3. Controlled Internal Drug Release (CIDR)

The CIDR is a T-shaped intravaginal device comprising a pre-molded annealed T-shaped nylon

spine coated with a 19 g silicone polymer uniformly impregnated with 1.9 g (10% w/w) of USP grade micronized P_4 [79-80] (Figure 1.1) and this is one of the valuable devices for solving several reproductive issues in cattle by providing an artificial luteal phase during its insertion by consecutive delivery of P_4. The CIDR has been used for the treatment of anestrus, advancing estrous cycle in pre-pubertal heifers and programming estrous synchronization worldwide. The approved claim in Japan is for synchronization of estrous cycle and for treatment of ovarian quiescence and silent heat. There are two brands of the CIDR in Japan, one is "Eazi-Breed" distributed by Livestock Improvement Association of Japan, and another one is "CIDR 1900" by Pfizer Animal Health; however, these are the same products from Pfizer Animal Health and it is just only for that the product name is different.

The release of P_4 from the CIDR is constant in vitro and this change is proportional [79, 81]. Macmillan, *et al.* [60] reported that after a CIDR was

inserted for 15 days, the device retained 0.85 g of P_4 and this amount was not different from the content of the each CIDR when three devices were inserted at once for the same period. On the other hand, plasma P_4 concentrations following insertion of a CIDR have been studied in both ovariectomized [9, 59, 79, 81, 104] and entire cattle [13, 15, 43, 60, 94]. In vivo, plasma concentration profiles during the CIDR insertion vary in cattle [58, 74] and the results suggested that individual cattle had specific profiles in their plasma P_4 concentrations to metabolize [74]. The variation was due to individual cattle, not related to the amount of P_4 released from the CIDR [81].

The CIDR has been administered for between 7 and 15 days; however, shorter insertion periods have become common by combination usage with $PGF_{2\alpha}$ or estradiol. One of the reasons is that the treatment with low concentrations of P_4 results in the development of persistent ovarian dominant follicles in cows displaying normal estrous cycles [83, 98]. These follicles are

characterized by an increase in size and production of estradiol, suppression of the development of subordinate follicles and are associated with an increase in frequency of release of LH pulses [93-95, 98]. However, fertility following ovulation of dominant follicles which have persisted for ≥10 days is significantly reduced [5, 61]. Plasma P_4 concentration during insertion of the CIDR declines with course of time [79, 81] (Figure 1.2). This means that longer insertion period of the CIDR alone can increase the risk of existence of persistent follicle due to lower P_4 during the later part of the insertion period and this can result in lower performance of fertility [27, 83, 94]. Therefore, the CIDR has been accepted by the field to insert in shorter period in order to minimize the risk of persistent follicles. However, shorter period of insertion by itself may provide lower heat detection or expression because endogenous P_4 may be secreted from CL and the estrus may not be observed even after removal of the CIDR. Therefore, $PGF_{2\alpha}$ or estradiol as luteolytic factors

should be utilized as combined products to regress CL or luteal structures.

1.4. Profile of plasma P_4 concentrations during CIDR insertion

Ovariectomy removes the endogenous source of P_4; therefore the plasma P_4 to be measured can only have come from the CIDR device. There are several reports on the plasma P_4 concentrations during the CIDR insertion in ovariectomized cattle [9, 59, 79, 81, 104].

The author's research group also conducted the study using ovariectomized cows which were a Japanese Black, 350 kg body weight and a Japanese Brown, 450 kg body weight with 2 replications, respectively. The blood samples were collected to measure concentrations of plasma P_4 from jugular vein at 0, 0.5, 1, 2, 3, 6 and 12 hours on day 0 after insertion

of a CIDR and once per day from day 1 to day 6 and 0, 0.5, 1, 2, 3, 6 and 12 hours on day 7 after removal of the CIDR. The maximum average concentration of plasma P_4 was 11.4 ± 5.4 *ng/ml* (mean ± SEM) and the minimum average concentration was 5.1 ± 0.5 *ng/ml*. The alteration of the average plasma concentrations of P_4 during 7-day CIDR insertion is shown in Figure 1.2. The reported average concentrations of P_4 varied but the alterations are similar among the breeds and between cows and heifers. The concentrations of plasma P_4 for 7-day insertion of the CIDR peak within 1-2 hours after the insertion and it is sustained for about 2-3 days and then declines or diminish its level of the concentration very slightly for remaining 4 days until removal [79, 81] (Figure 1.2). Peak concentrations are maintained because of reduced levels of endogenous metabolizing enzymes specific to P_4 in the ovariectomized cattle [81]. Increased concentrations of P_4 in the blood following insertion of the device induces enzyme production and fall in plasma

concentrations after 2-3 days due to the increased ability of the cattle to metabolize P_4 [81]. Apparent steady-state concentrations occur over the last four days of insertion because P_4 is being delivered at a rate from the device similar to that which the body is clearing P_4 from the blood [81].

1.5. CIDR for improvement of reproductive performance

Recent reports have described that the reproductive performance in cattle has decreased [26, 51, 54-56]. The genetical improvement has provided higher performance of milk yield and meat productions by individual cattle, and therefore the appropriate management should be accommodated in order to cope the nutritional requirement at the same time. However, physiological and environmental stresses, inadequate nutrient intake, low body condition before and after the

parturition, higher quantities of lactation, and intensive management systems impair aspects of reproductive performance in both dairy and beef cattle [82, 101], which have resulted in the poor heat expression and/or detection, anestrus, low conception rates, and increased embryo mortality [100]. Four primary mechanisms that depress fertility in cows are anovulatory [109] and behavioral anestrus (failure to cycle and display estrus), suboptimal and irregular estrous cyclicity (this category includes ovarian disease and subnormal luteal function after breeding), abnormal preimplantation embryo development (may be secondary to poor oocyte quality), and uterine/placental incompetence [56].

New technology has advanced the development of various protocols to improve the pregnancy rates by synchronizing follicle development with occurrence of CL regression, precisely controlling the time of ovulation, application of a TAI and improving embryo survival [101]. The CIDR has been used by itself or by

combination with other hormonal substances for treatment of reproductive disorders as a part of short term perspective. The treatment of anestrus in both pre-pubertal and pubertal heifers [34, 57], postpartum anestrus [22, 71, 84, 110], repeat breeder [1, 50, 96], estrous synchronization with detected artificial insemination (AI) and/or with several TAI protocols [3, 22, 46, 49, 52-53, 70, 83, 86, 90-91, 106], embryo transfer [16, 47, 51, 76, 96], anovulatory disease including cystic ovarian disease (COD) [2, 11, 20, 36, 39, 41, 102, 103, 112] have been reported. So-called "re-synchronization" protocol has also been utilized widely after AI in order to detect non-pregnant cattle faster before general method for pregnancy check by veterinarians [14, 17, 97, 105]; however, issues on conception rate and embryo survival have still been under the evaluation [19, 29, 33]. These usages are based on the role of P_4 itself from the inserted CIDR and also based on increased estrous detection by producers due to more concentrated [17, 22, 57, 105] and stronger

estrous expressions [106]. In terms of induction of puberty in heifer, LH secretion is suppressed during CIDR insertion and after the removal, LH secretion increases and puberty is hastened [21]. The action of the CIDR to induce resumption of normal estrous cycles has been attributed, in part, to its effect of P_4 to increase LH secretion both during and after treatment in anestrous females [21]. P_4 treatment increased LH secretion during P_4 exposure in postpartum beef [35] and seasonal dairy [83] cows.

The reproductive disorders are one of the key causes of culling [63]. Therefore, it is obvious that preventive activities which are improvement of environmental issues, management issues and appropriate nutritional approach are very important measures; however, there is limitation which is to take time to solve or improve these issues.

1.6. Objective of the studies

As a treatment tool or leverage for improving reproductive performance from short term perspective in cattle, the CIDR is very valuable device. Many reports described usefulness of the CIDR for increase of reproductive performance but even the further studies are necessary to establish its efficacious and valuable usage, and to find out the events theoretically. The specific objectives of the studies were to determine:

1) whether the usage of the CIDR as a part of the TAI protocols to be efficacious and beneficial treatment regime in Holstein dairy heifers

The several TAI protocols such as Ovsynch (GnRH on day 0 and 9, $PGF_{2\alpha}$ on day 7, and TAI is performed 16-20 hours after 2nd GnRH injection) or Heatsynch (GnRH on day 0, $PGF_{2\alpha}$ on day 7, estradiol cypionate on day 8, and TAI on day 10), have been developed since 1995, started from the famous research

work by Pursley, *et al.* [77]; however, these protocols have not been used in heifers due to lower pregnancy rate compared with AI at detected estrus [46, 78]. This impaired fertility may be caused by early maturation of follicles compared with lactating cows [77], and premature estrus or ovulation [23, 49, 90-91]. Recently, it was reported that either premature estrus or ovulation could be prevented by exogenous P_4 administration in cows [49, 90-91]. Therefore, the comparative studies using two TAI protocols with or without CIDR were conducted in a commercial dairy farm to find out the usefulness of CIDR with observation of ovaries by using ultrasonography.

2) whether the usage of the CIDR for cystic ovarian disease (COD) in Japanese Black cows to be efficacious and beneficial treatment regime

There are two types of cysts, follicular (FC) and luteal cysts (LC) and the treatment strategies are

different depend on the FC or LC. However, there is limitation to select correct treatment procedure because it is difficult to discriminate an FC from an LC [65], particularly under field conditions even though using ultrasonography or measuring P_4, that is to say, there risks selecting incorrect treatment procedure. This selected incorrect treatment will lead to the negative profit of producers directly. On the other hand, CIDR insertion can reduce the LH pulse frequency and induce atresia of cystic follicles and normal estrus can be resumed after removal of the CIDR [11, 103]. A CIDR may also be able to restore the ability of the hypothalamo-pituitary axis to generate an LH surge in response to an increase in circulating estradiol [40]. Therefore, CIDR may be able to apply for the treatment both follicular and luteal cysts. Recently, COD has become one of the important issues in Japanese local beef cows, but the data was still very limited. Therefore, an easier, more practical clinical treatment protocol against COD should be developed. For this

objective, the four studies with or without CIDR were conducted.

1.7. Tables and Figures

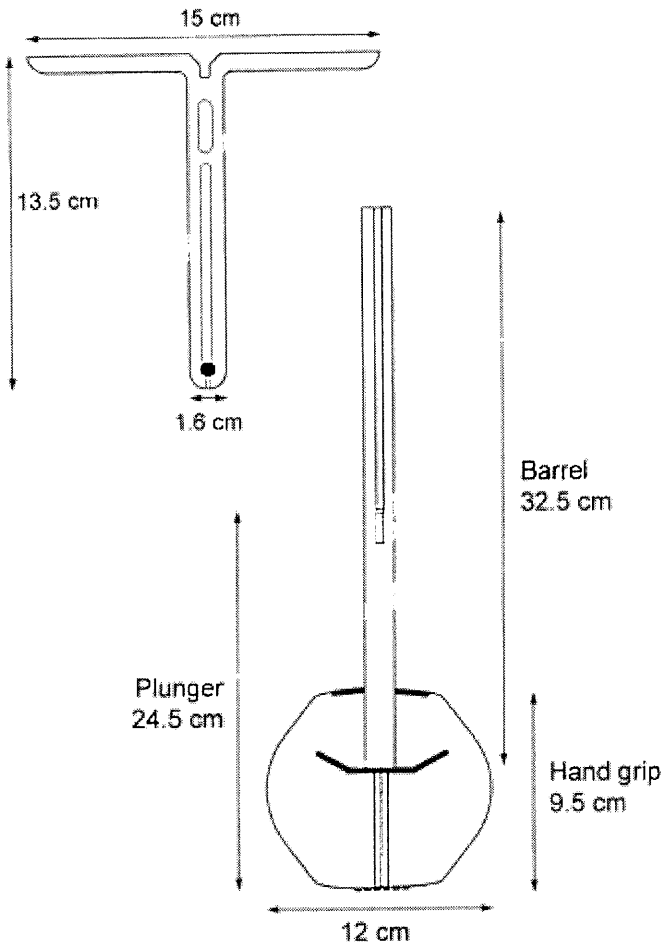

Figure 1.1. CIDR device (left upper) and the applicator for "heifers" (right lower) to insert a CIDR [80].

Note: Two types of applicators for cattle use are available, which are for "heifers" and for "cows". The differences between the applicators are the length of barrel.

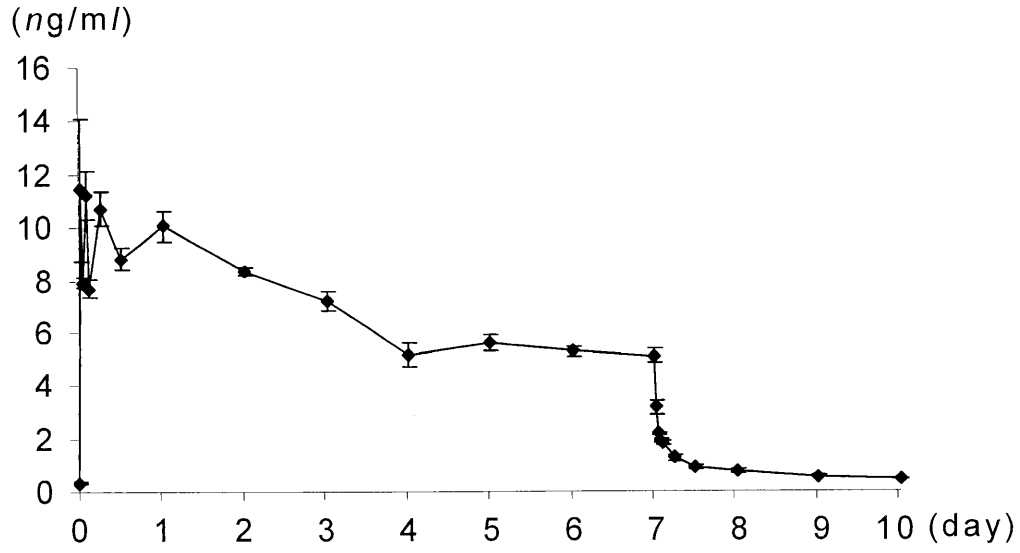

Figure 1.2. Plasma P_4 concentrations (mean±SEM) during a 7-day insertion of the CIDR device in ovariectomized Japanese Black and Japanese Brown cows (two cows in duplicate; n=4).

Chapter 2

Usage of CIDR for timed AI protocols in heifers

(The parts of this study was published in The Journal of Veterinary Medical Science, entitled Efficacy of Intravaginal Progesterone Administration as an Additional Treatment on Two Types of Timed AI Protocols in a Commercial Herd of Holstein Heifers, 70 (3): 243–249, 2008.)

2.1. Introduction

Synchronization of ovulation and a TAI protocol in cattle were introduced firstly in 1995 [77]. The protocol consists of GnRH on day 0 and 9, $PGF_{2\alpha}$ on day 7, and TAI is performed 16-20 hours after 2nd GnRH

injection [77]. After introduction of this protocol [77], several methods such as Co-synch (GnRH on day 0, $PGF_{2\alpha}$ on day 7, and GnRH and TAI on day 9) [37], Heatsynch (GnRH on day 0, $PGF_{2\alpha}$ on day 7, estradiol cypionate on day 8, and TAI on day 10) [69] and their modifications also have been developed. These TAI protocols including Ovsynch have been widely used in the world, mainly in dairy and beef cows due to the management advantages such as deletion of estrous detection and implementation of time mannered breeding programs. However, these TAI protocols have not been utilized in heifers due to impaired pregnancy rate compared with AI at detected estrus [46, 78]. One of the major reasons in heifers that estrus was often observed before TAI and thus it failed in fertile inseminations [46, 85-86]. This may occur due to early maturation of follicles compared with lactating cows [77] and results in premature estrus or ovulation [23, 49, 90-91]. Additionally, high frequency of three-wave cycles observed in heifers [92] puts the

synchronization program rather difficult. Recently, it was reported that either premature estrus or ovulation could be prevented by the administration of a CIDR in cows [49, 90-91]. However, the field application of this protocol to Holstein heifers has been limited.

Regarding estrous synchronization, CIDR has been used on its alone for a short time; however, longer period of insertion resulted in impaired conception rate, particularly in lactating Holstein cow due to the persistent follicle by low concentration of P_4 [27, 83, 94]. Therefore, recently the CIDR has been inserted for 7 days with combination of $PGF_{2\alpha}$ treatment at CIDR removal.

Recently, there is a commercial dairy farm where their management issue on estrous detection has been impaored in heifer; therefore TAI protocol should be considered to use to improve reproductive performance in heifer, too. Thus, the purpose of this study were: 1) to investigate the reproductive performance by using TAI protocols with CIDR, which were Ovsynch or

Heatsynch using estradiol benzoate (EB-Heatsynch), and 2) to evaluate economic benefit of the protocols.

2.2. Materials and Methods

A total of seventy-four Holstein heifers in a commercial dairy farm which is raising 120 heads of lactating cows regularly in Kagoshima prefecture were used from June 2004 to September 2006.

Until April 2004, the farm where the present study was conducted, raised heifers inside the main shed together with lactating cows. However, installing heifers were moved to the new shed and maintained separately from lactating cows. In the annex, estrous detection of heifers had not been performed successfully by farm owners due to the limitation of labor. Average age of first calving in heifers before and after moving delayed from 27.2 ± 4.6 months (average ± SEM) to 28.4 ± 4.0 months.

The heifers, 14.4 ± 0.2 (12-22) months of age (average ± SEM, range), 350-400kg of estimated body weight at the initial treatment were checked out health condition, and their body condition scores (BCS) were recorded by using 1 to 5 scaled scoring system with 0.25 points of scale, where 1: emaciated and 5: obese [32]. The heifers were maintained in loose barn with stanchion confinement at feeding or routine health check. Reproductive organs, such as uterus and ovaries were monitored using a handy-type ultrasonography equipped with transrectal 7.5-MHz linear–array transducer (SonoSite® 180plus, SonoSite, Inc., WA, USA). The size of uteri and ovaries of the experimental heifers were normal as mature and we recognized that all experimental heifers were pubertal. The experimental heifers were randomly allocated by age, BCS, estimated body weight and existence of CL using ultrasonography to one of two different TAI protocol groups, and then were divided additionally into each of two subgroups, which were treated with CIDR

(CIDR-treated) or without CIDR (No-CIDR-treated) (CIDR® 1900, Pfizer Animal Health, Tokyo). All animals were inseminated once along the protocols shown in Figure 2.1; Ovsynch group (n=22), all animals in this group received 100 μg of GnRH (Fertirelin acetate: Conceral®, Schering-Plough Animal Health, Tokyo, Japan) on day 0, 25 mg of $PGF_{2\alpha}$ (Tromethamine dinoprost: Pronalgon® F, Pfizer Animal Health, Tokyo, Japan) on day 7 and 100 μg of GnRH on day 9, and were inseminated 20 hours after the second GnRH injection. EB-Heatsynch group (n=16) received 100 μg of GnRH on day 0, 25 mg of $PGF_{2\alpha}$ on day 7 and 1 mg of estradiol benzoate (EB, Ginandol®, Sankyo-Yell Pharmaceuticals, Tokyo, Japan) on day 8, and was inseminated 30 hours after the EB injection. The CIDR was inserted intravaginally to 22 heads in the Ovsynch + CIDR and 14 heads in the EB-Heatsynch + CIDR group for the initial 7 days of each protocol.

During the study, the heifers were clinically examined by rectal palpation and ultrasonography on

day 0 (GnRH injection), day 7 ($PGF_{2\alpha}$), day 8 (EB injection) or day 9 (2^{nd} GnRH injection), day 10 (TAI), day 24 (check of CL), day 38 (early pregnancy check) and day 55-65 (final pregnancy check). Blood samples were collected from caudal vein on day 0, 7, 8 or 9 and 24 with heparinized syringe, then centrifuged at 1,500 *g* for 15 min at 4 °C and stored at –20 °C until assay. Plasma concentrations of P_4 were measured by the homologous double-antibody radioimmunoassay (RIA) method described by Taya, *et al.* [99].

All statistical analysis was applied by using JMP™ software ver. 5.1.1 (SAS Institute Japan Inc., Tokyo, Japan). Initial month of age, average BCS, P_4 concentrations and follicle diameters were assessed by Bartlett's test for equality of variances, and then analyzed by ANOVA or Welch's test in case of inequality of variance. In case that significant differences were detected and then comparative tests for average value were performed. The multiple comparison of the month of age for first calving and P_4 concentrations were

evaluated by using Tukey-Kramer HSD test. The comparison for time-course alteration of P_4 concentrations was assessed by paired *t*-test. The other data for frequency were analyzed by Chi-square test and Fisher's exact probability test in case of comparison of frequency in the two groups.

2.3. Results

During the experiment, the retention rate of CIDR for 7 days in heifers was 100% (36/36).

The experimental heifers with functional CL characterized by ultrasonography with plasma P_4 level $\geq$1.0 *n*g/m*l* was detected in 51/74 (68.9%) heifers on day 0; however, the percentage of heifers with functional CL on day 0 in Ovsynch group was significantly higher than in EB-Heatsynch group (81.8% vs 50.0%, respectively, $P<0.01$, Table 2.1).

The pregnancy rate and formation of CL after 14

days from TAI were shown in Table 2.2. The pregnancy rate in CIDR-treated heifers were significantly higher as compared to No-CIDR-treated group both in Ovsynch group (68.2% vs 22.7%, $P<0.01$) and in EB-Heatsynch group (57.1% vs 18.8%, $P<0.05$). Existence of functional CL either on day 0 or day 7 resulted in significantly higher pregnancy rate in CIDR-treated group than No-CIDR-treated group in both Ovsynch group on day 0 and day 7, and in EB-Heatsynch group on day 0 (Ovsynch group; day 0: 65.0% vs 12.5%, $P<0.01$, day 7: 70.0% vs 22.2%, $P<0.01$, EB-Heatsynch group; day 0: 75.0% vs 14.3%, $P<0.05$). Formation of CL after 14 days from TAI was observed in 69 of 74 heads (93.2%) in total and there were no significant differences among the groups ($P>0.05$).

P_4 concentrations in between CIDR-treated group and No-CIDR-treated group in both Ovsynch and EB-Heatsynch group did not differ significantly ($P>0.05$) even by pregnant ($P>0.05$, Table 2.3).

The findings on ovulation during the experimental

period and P_4 concentrations on day 0 and 7 by existence of CL on day 0 were shown in Table 2.4. The percentage of heifers with intermediate ovulation during initial 7 days was significantly lower in heifers with CL on day 0 compared to heifers without CL on day 0 (29.4% vs 73.9%, $P<0.01$). This tendency was observed in both CIDR-treated (21.4% vs 75.0%, $P<0.01$) and No-CIDR-treated groups (39.1% vs 73.3%, $P<0.05$). However, total frequency of ovulations in heifers during initial 7 days was significantly lower in heifers in CIDR-treated group compared to No-CIDR treated group (33.3% vs 52.6%, $P<0.05$). Plasma P_4 concentrations on day 7 by existence of CL on day 0 tended to be higher in heifers with functional CL on day 0 than in heifers without functional CL on day 0; however, there were no significant differences both in CIDR-treated group and No-CIDR-treated group (CIDR-treated group: 5.1 ± 0.7 *n*g/m*l* vs 3.7 ± 1.4 *n*g/m*l*, $P>0.05$, No-CIDR-treated group: 5.3 ± 1.1 *n*g/m*l* vs 3.0 ± 0.9 *n*g/m*l*, $P>0.05$). Furthermore, during the period

from EB or 2nd GnRH treatment to TAI, intermediate ovulation were observed in 54 heifers and the percentage of heifers in CIDR-treated group was lower than No-CIDR-treated group (11.1% vs 37.0%, $P<0.05$). The average diameters of dominant follicles on day 7 differed neither between CIDR-treated group (n=35) and No-CIDR-treated group (n=36) (11.7 ± 0.5 mm vs 11.5 ± 0.4 mm, average ± SEM, $P>0.05$) nor between heifers with pregnant (n=31) and non pregnant (n=40) (11.3 ± 0.5 mm vs 11.8 ± 0.4 mm, $P>0.05$). Follicles in the remained three heifers were not found on day 7.

As a result in overall approach of the study, comparing with the past data of the farm, the average age of first calving tended to be shortened by 1.2 months; 27.2 ± 4.6 months before April 2004 raised in the main house (n=28: final AI was performed from January 2003 to April 2004) or by 2.5 months; 28.4 ± 4.0 months during April to June 2004 in the annex before innovation (n=26: final AI was performed from April 2004 to June 2004), as compared to 25.9 ± 4.0 months

after implementation of TAI protocols (P=0.08).

2.4. Discussion

Holstein heifers in the commercially operated farm were used in the present study. In the farm where estrous observation was insufficient due to the issues of separated location of newly built rearing shed and shortage of labors. Thus those conditions had impaired the reproductive performance compared to the past when heifers had been maintained together with lactating cows in the main shed. In order to cope with lowered reproductive performance, two types of TAI protocols with additional CIDR insertion for 7 days were conducted in the present study.

On day 0 when ovarian findings were determined with ultrasonography and plasma P_4 concentrations, 68.9% of heifers had a functional CL. This indicates that these heifers were already in cycling of estrus.

The other heifers also had normal size of ovaries and uteri and had follicles $\geq$ 8 mm; therefore, all the experimental heifers subjected in the present study were pubertal at the initial treatment. However, there were significant difference between Ovsynch group and EB-Heatsynch group on existence of functional CL on day 0 ($P<0.01$); therefore, we can not describe the comparison on efficacy between Ovsynch and EB-Heatsynch group. This finding came from the gap between ultrasonographically observed CL and their P_4 concentrations on day 0.

The pregnancy rate was significantly higher in CIDR-treated group as compared to No-CIDR-treated group in both Ovsynch group ($P<0.01$) and EB-Heatsynch group ($P<0.05$). Both CIDR-treated groups with functional CL on day 0 and/or day 7 showed relatively higher pregnancy rate as compared to No-CIDR-treated group. This result indicated that one reason of the higher pregnancy rate in CIDR-treated group were probably due to existence of CL on day 0.

The most appropriate timing to start with the Ovsynch protocol for pregnancy is the early luteal phase, between day 5 and 10 of the estrous cycle [62]; however, in the present study, the initial day of the TAI protocol in the estrous cycle in each heifer was not determined.

On the other hand, P_4 concentrations maintained relatively high during 7 days of Ovsynch with CIDR protocol may result in the increased pregnancy rate compared Ovsynch without CIDR group in Japanese Black cows [46, 91]; however, there were no significant differences in the average plasma P_4 concentrations on day 0 or day 7 both in heifers with or without pregnant in CIDR-treated group in the present study.

However, one of the advantages of a CIDR for pregnancy rate were probably due to the preventive effect against early ovulation in case that initiation of the Ovsynch protocol after day 15 of the estrous cycle caused premature ovulation prior to insemination, reported by Moreira *et al.* [62]. During initial 7 days, heifers without CL on day 0 ovulated intermediately

after 1st GnRH treatment with higher percentage (CIDR-treated group: 75.0%, No-CIDR-treated group: 73.3%) and newly formed CL was observed. By contrast, in heifers with CL on day 0, intermediate ovulation was limited (CIDR-treated group: 21.4%, No-CIDR-treated group: 39.1%). This result may due to that CIDR insertion with GnRH treatment induces intermediate ovulation of the existing dominant follicle [3] and forms new CL [4]. However, on the whole, CIDR insertion suppressed the intermediate ovulation during initial 7 days induced by GnRH treatment as compared to No-CIDR-treated group in the present study (CIDR-treated group: 33.3%, No-CIDR-treated group: 52.6%, $P<0.05$). GnRH induced the intermediate ovulation even during the period of CIDR insertion. Ando, *et al.* reported that the ovulation was not induced during CIDR insertion without GnRH treatment at CIDR in [4]. These results indicated that CIDR insertion with GnRH treatment on day 0 probably does not affect intermediate ovulation induced by GnRH in heifers

without functional CL on day 0; however, CIDR tends to suppress intermediate ovulation in heifers with functional CL on day 0 together with existent CL. This may due to that high P_4 concentrations from both CIDR and CL reduce GnRH-induced LH concentrations and ovulatory responses [18]. In the present study, P_4 concentrations on day 7 in heifers with functional CL on day 0 in both CIDR-treated group and No-CIDR-treated group were tended to be higher than in heifers without functional CL on day 0.

During the period from EB or 2nd GnRH injection to TAI in CIDR-treated group, ovulation was observed at significantly lower frequency as compared to No-CIDR-treated group (11.1% vs 37.0%, $P<0.05$). This suppression of the CIDR against premature follicle during this period might be one of the reasons why the results showed the higher pregnancy rate in group with CIDR. However, the diameters of dominant follicles on day 7 were neither different between CIDR-treated group and No-CIDR-treated group nor between pregnant

or non pregnant heifers. Kim, *et al.* reported similar results that the diameters of dominant follicles on day 7 and day 9 both in the TAI protocols of Ovsynch and CIDR with EB capsule for 7 days combined with GnRH treatment on day 9 were not different and described that these might not be influenced to the pregnancy rate [49]. Furthermore, plasma P_4 concentrations after ovulation by 2^{nd} GnRH treatment was not significantly different between CIDR-treated group and No-CIDR-treated group ($P>0.05$); therefore, the CIDR might affect the growth speed of follicle but this effect for pregnancy rate was not clear in the present study.

From viewpoint of economic impact for the farm, the approach using TAI protocol in the present study could provide successfully clear cost-benefit performance for the farm by shortening average first calving age by 2.5 months from previous situation in the annex and 1.2 months from original situation in the main house even in consideration of the cost of hormonal products and veterinary fee. "Opportunity cost" is an

economic term and is defined as the cost of something in terms of an opportunity forgone (and the benefits that could be received from that opportunity) or the most valuable forgone alternative. The cost obviously depends on the situation of veterinary activities, cost of AI, cost of drug and management style and procedure; however, we set these assumptions per head for calculation as follows; veterinary activities including AI: 5,000 yen, cost of drug: 5,000 yen, daily loss including cost of feed: 500 yen. The formula is; 500 yen multiply 1.2 months (= 36 days) minus 5,000 yen (veterinary fee) and minus 5,000 yen (cost of drug) equal approximately 8,000 yen; therefore in the present study, shortened 2.5 months and 1.2 months of the opportunity costs are simply equivalent to 16,000 yen and 8,000 yen per head respectively; therefore estimated benefit for a farm with 100 heads of heifers will be approximately 0.8 to 1.6 million yen by treatment with TAI protocol with single use of CIDR.

In conclusion, CIDR based TAI protocols for

heifer could provide applicable high pregnancy rate compared to without CIDR group (63.9% vs 21.1%, $P<0.01$). GnRH induced the intermediate ovulation in heifers without CL; however, CIDR insertion suppressed either the ovulation during the 7 days insertion or the period from EB/2nd GnRH treatment to TAI compared to without CIDR group. In CIDR group, heifers with existence of functional CL on day 0 and/or day 7 resulted in higher pregnancy rate; however, the remarkable difference of plasma P_4 concentrations in heifers between pregnant and non-pregnant was not clarified in the present study. Finally, the study could contribute the economical improvement for the farm by way of shortening calving age for 2.5 months from previous situation and 1.2 months from original situation.

2.5. Acknowledgements

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2.6. Tables and Figures

Table 2.1. The characteristic of the experimental heifers by TAI protocol.

Group (n)	CIDR (n)	Initial month of age (mean ± SEM)	Average BCS (mean ± SEM)	Functional CL on Day 0, % (n/n)
Ovsynch (n=44)	+ (n=22)	14.5 ± 0.4	2.93 ± 0.03	90.9% [a] (20/22)
	- (n=22)	15.0 ± 0.6	2.92 ± 0.05	72.7% (16/22)
	Total	14.8 ± 0.4	2.93 ± 0.03	81.8% [A] (36/44)
EB-Heatsynch (n=30)	+ (n=14)	15.1 ± 0.5	2.91 ± 0.07	57.1% [b] (8/14)
	- (n=16)	13.9 ± 0.3	2.89 ± 0.05	43.8% (7/16)
	Total	14.4 ± 0.3	2.90 ± 0.04	50.0% [B] (15/30)

Superscripts mean significant difference. ab: $P<0.05$, AB: $P<0.01$

Table 2.2. Comparison of reproductive parameters by TAI protocol.

Group (n)	CIDR (n)	Pregnant %, (n/n)	Pregnancy rate by existence of functional CL %, (n/n)		Formation of CL on Day 24 %, (n/n)
			Day 0	Day 7	
Ovsynch (n=44	+ (n=22)	68.2% [A] (15/22)	65.0% [C] (13/20)	70.0% [E] (14/20)	100.0% (22/22)
	- (n=22)	22.7% [B] (5/22)	12.5% [D] (2/16)	22.2% [F] (4/18)	95.5% (21/22)
EB-Heatsynch (n=30	+ (n=14)	57.1% [a] (8/14)	75.0% [c] (6/8)	54.5% (6/11)	85.7% (12/14)
	- (n=16)	18.8% [b] (3/16)	14.3% [d] (1/7)	20.0% (2/10)	87.5% (14/16)

Superscripts mean significant difference as follows; ab, cd: $P<0.05$, AB, CD, EF: $P<0.01$

Table 2.3. Comparison of plasma P_4 concentrations by TAI protocol.

Group (n)	CIDR (n)	Pregnant (n)	P_4 concentrations (*n*g/ml, mean ± SEM)					
			Day 0		Day 7		Day 8/9	Day 24
Ovsynch (n=44)	+ (n=22)	+ (n=15)	5.5 ± 0.9	4.3 ± 1.0	6.6 ± 0.9	6.6 ± 1.0	0.3 ± 0.1	7.8 ± 1.0
		- (n=7)		7.9 ± 1.4		6.4 ± 1.9	0.1 ± 0.0	6.3 ± 1.2
	- (n=22)	+ (n=5)	4.3 ± 0.9	3.9 ± 2.1	4.8 ± 1.0	2.0 ± 0.7	0.1 ± 0.1	8.5 ± 1.2
		- (n=17)		4.4 ± 1.0		5.9 ± 1.3	0.4 ± 0.1	7.3 ± 1.1
EB-Heatsynch (n=30)	+ (n=14)	+ (n=8)	3.0 ± 1.0	2.9 ± 0.8	2.4 ± 0.5	2.1 ± 0.7	0.4 ± 0.3	3.1 ± 0.6
		- (n=6)		3.2 ± 2.3		2.9 ± 0.7	1.7 ± 1.5	4.2 ± 2.8
	- (n=16)	+ (n=3)	1.9 ± 0.7	2.4 ± 2.2	3.7 ± 1.1	5.2 ± 4.3	0.7 ± 0.5	5.9 ± 2.2
		- (n=13)		1.8 ± 0.7		3.3 ± 1.0	0.3 ± 0.1	6.0 ± 2.2

There are no significant differences in the same day in the same group.

Table 2.4. Number of heifers with intermediate ovulation and plasma P_4 concentrations in CIDR-treated or No-CIDR-treated group.

Period	Functional CL on Day 0	CIDR-treated	No-CIDR-treated	Overall
Day 0 - Day 7	+ (n=51)	21.4% [A] (6/28) P_4 on day 0 (*n*g/ml): 5.6±0.7 [E] P_4 on day 7 (*n*g/ml): 5.1±0.7	39.1% [a] (9/23) P_4 on day 0 (*n*g/ml): 5.5±0.7 [G] P_4 on day 7 (*n*g/ml): 5.3±1.1	29.4% [C] (15/51)
	- (n=23)	75.0% [B] (6/8) P_4 on day 0 (*n*g/ml): 0.2±0.1 [F] P_4 on day 7 (*n*g/ml): 3.7±1.4	73.3% [b] (11/15) P_4 on day 0 (*n*g/ml): 0.3±0.1 [H] P_4 on day 7 (*n*g/ml): 3.0±0.9	73.9% [D] (17/23)
	Total (n=74)	33.3% [c] (12/36)	52.6% [d] (20/38)	-
Day 8/9* - TAI	(n=54)	11.1% [e] (3/27)	37.0% [f] (10/27)	-

*: The heifers in EB-Heatsynch group were administered with EB on Day 8 and heifers in Ovsynch group were administered with GnRH on Day 9.

1) Superscripts mean significant difference as follows; ab, cd, ef: $P<0.05$, AB, CD, EF, GH: $P<0.01$

2) P_4 (*n*g/ml): Mean ± SEM

3) The findings of ovaries at TAI were recorded in 54 of 74 heifers (CIDR-treated group: 27/36, No-CIDR-treated group: 27/38).

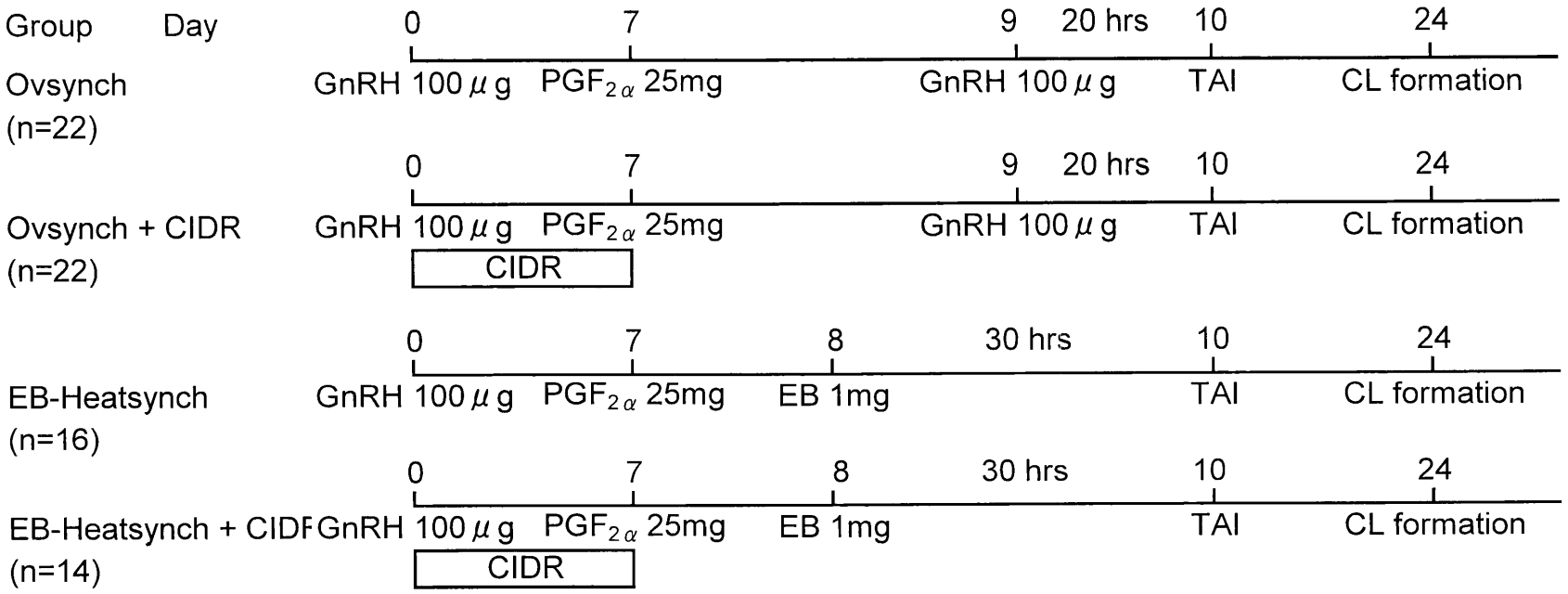

Figure 2.1. Schematic diagram of treatment protocols.
GnRH: fertirelin acetate, $PGF_{2\alpha}$: tromethamine dinoprost,
EB: estradiol benzoate, TAI: Timed artificial insemination.
CL formation: observation of CL formation

Chapter 3

Usage of CIDR for cystic ovarian disease

(The parts of this study was published in The Journal of Veterinary Medical Science, entitled Efficacy of Intravaginal Progesterone Administration Combined with Prostaglandin $F_{2\alpha}$ for Cystic Ovarian Disease in Japanese Black Cows, 70 (10): 1077–1083, 2008.)

3.1. Introduction

Cystic Ovarian Disease (COD) is one of the important costly issues for reproductive performance in cattle due to longer infertility as the condition persists [36]. Generally, there are two types of cysts, follicular (FC) and luteal cysts (LC); however, 20% [72] to nearly 70% [108] of cystic ovaries can recover spontaneously. A broad range of treatment strategies against COD have

been carried out in the field and reviewed [7, 73]. Short-term treatments have been approached from the perspective of manual rupture, administration of products for improvement of liver function or use of exogenous hormones, GnRH, hCG or estradiol, which is now the most popular strategy for treatment of an FC [7, 73]. Exogenous P_4 treatment, either intravaginally or intramuscularly has also been reported for COD treatment either alone or with GnRH or estradiol [2, 11, 20, 36, 39, 41, 45, 67, 102-103, 112]. On the other hand, follicles with luteinized structures or an LC should be treated with $PGF_{2\alpha}$, if diagnosed properly [73, 107].

CIDR insertion can reduce the LH pulse frequency and induce atresia of cystic follicles and normal estrus can be resumed after removal of the CIDR [11, 103]. A CIDR may also be able to restore the ability of the hypothalamo-pituitary axis to generate an LH surge in response to an increase in circulating estradiol [40]. Therefore, the CIDR could apply for the treatment of COD.

The treatment strategies are different for the FC and LC; however, it is difficult to discriminate an FC from LC [65], particularly under field conditions. The definition of an FC is described as persistence at least one follicle $\geq$25 mm or multiple follicles even <20 mm in diameter for more than 7 to 14 days with the absence of a CL in the ovary [107]. In Japan, diagnosis of an FC requires practitioners to re-examine the ovaries at intervals of 7 to 14 days by rectal palpation, and it should also be examined by measurement of the P_4 concentration in blood or milk and/or by ultrasonography in cases that are difficult to diagnose by re-examination using rectal palpation [107]. An LC is a cyst that has a thicker wall and where some degree of luteinization has occurred [44, 107]; the examination procedure is defined pursuant to the diagnosis guidelines for an FC, and clinicians should re-examine it at intervals of 14 to 20 days [107]. Therefore, to diagnose COD properly, it is necessary for clinicians to basically obtain ovarian findings from rectal palpation,

measurement of the P_4 concentrations in blood or milk and ultrasonography; however, these processes take time.

In practice, rectal palpation is obviously the basic diagnostic method for COD; however, its accuracy has been reported to vary as results range from 41 to 84% [6, 8, 25, 48, 65, 68]. On the other hand, successive measurement of the P_4 concentrations for 15 days results in more accurate diagnosis of an FC rather than measurement for 7 days [64, 68]. In addition to this, it takes certain amount of time for laboratory work in order to obtain the hormonal results. Recently, ultrasonography has been used to differentiate the type of cysts, and it has been shown to be superior to rectal palpation for detecting cysts [31]. Handy-type ultrasonography has recently been used for on-farm practice; however, it is not in wide enough use yet for general practice.

Thus, clinicians always face realistic limitations in proper differentiation of COD, and it is not easy in

common veterinary practice under field conditions to select the correct treatment strategy for COD. On the other hand, sequential examination might not be practical because of the health schedule for the herd [36] or from the perspective of the financial strain on farmers to continue raising open cows at least for a couple of weeks more after a clinician's first visit, even excepting the cost of hormonal assay examinations. Furthermore, incorrect diagnosis of COD could result in a severe burden on farmers.

On the other hand, although COD has generally been recognized as a disease in dairy cows, it has recently become one of the important issues in beef cows. Todoroki *et al.* [102] reported that approximately 20% of Japanese Black cattle previously used as donors for embryo transfer are affected by FCs due to receipt of repeated superovulation treatments. Even under commercial field conditions for Japanese Black cows in the southern area of Kyushu, Japan, the actual incidence of COD is officially unknown; however, the

authors speculate that the percentage of anestrous cows diagnosed with COD in routine field activities and that are more than 40 days postpartum is approximately 8% to 10% of Japanese Black cows. This percentage is in the third rank in the category of reproductive disorders and is followed by ovarian quiescence and persistence of the CL in Japanese Black cows.

As the treatment for COD in Japanese Black cow, the study that has been reported on the efficacy of the CIDR is very limited. Todoroki, *et al.* [103] reported the excellent efficacy of the CIDR against COD for 14 days in donor cows of Japanese Black breed. In terms of the period of gestagen exposure against treatment of COD in cattle, it has been reported using serial daily P_4 administration for 10 to 14 days [67] traditionally and the periods with 7 to 14 days of exposure have been reported; however, there is a few reports in Japanese Black cow.

As described above, therefore, an easier, more practical clinical treatment protocol, not only for

clinicians, but also for farmers against COD should be developed to minimize the risk of incorrect treatment. From this perspective, the authors conducted the following four experiments in the present study; Experiment 1 was a preliminary study to evaluate the clinical efficacy of a CIDR combined with $PGF_{2\alpha}$, particularly on the insertion period of a CIDR for 7 days or 14 days against clinically recognized COD. Experiment 2 was conducted to group COD cysts based on the pattern of plasma P_4 concentrations and observation using ultrasonography from two examinations at intervals of 7 days and to evaluate the efficacy of a CIDR with 7-day insertion combined with $PGF_{2\alpha}$. Experiment 3 was conducted to expand the results of Experiment 2 in order to evaluate the reproductive efficacy of 7-day advanced treatment compared with the period of Experiment 2 by using the same treatment protocol as in Experiment 2. Experiment 4 was on going comparative study with the current GnRH treatment against COD.

3.2. Materials and Methods

Commercial Japanese Black cows raised in both Kagoshima and Miyazaki prefectures were utilized in these studies. Experiment 1 was conducted in 2002 to 2003, Experiment 2 and 3 were conducted in 2004 to 2006, and Experiment 4 was conducted in 2006 to present. All cows were ≥40 days postpartum (43 to 507 days postpartum) and already separated from their calves at the start of the study. Estrus had not been detected by the owners or staff at each respective farm after calving, and the chief complaint was anestrus at the time of the first veterinary visit. These cows were palpated and/or examined by ultrasonography equipped with a transrectal 7.5 MHz linear–array transducer (Aloka SSD-630, Tokyo, Japan for Experiment 1 and SonoSite® 180 Plus, SonoSite, Inc., WA, USA for Experiment 2 - 4). A single cyst ≥25 mm in diameter or

at least one follicle ≥18 mm in diameter in the case of multiple cysts were examined in their ovaries.

Experiment 1: A total of 28 Japanese Black cows (year of age: 8.3 ± 0.7, parity: 6.0 ± 0.7, mean ± SEM) at 28 commercial farms that were categorized by plasma P_4 concentrations on day 0 were utilized (Figure 3.1). No CLs were observed in the ovaries on day 0. Blood samples were collected on day 0 and at 14 days after a CIDR removal from the caudal vein with a heparinized syringe and centrifuged at 1,500 *g* for 15 min at 4 °C and stored at –20 °C until assayed. The plasma concentrations of P_4 were measured by the homologous double-antibody radioimmunoassay (RIA) method described by Taya, *et al.* [99]. The health conditions and body condition scores (BCS) of the cows were checked and recorded using a 1 - 5 scaled scoring system with a 0.25-point scale, where 1 was emaciated and 5 was obese [32]. All cows were divided into 2 patterns based on their plasma P_4 concentrations with 1 *n*g/m*l* as the cut-off concentration on day 0.

A CIDR (Eazi-Breed™, Livestock Improvement Association of Japan, Tokyo, Japan) was intravaginally inserted into the cows for 7 days or 14 days from day 0 on the first visit, and 25 mg $PGF_{2\alpha}$ (Tromethamine dinoprost: Pronalgon® F, Pharmacia Animal Health (current Pfizer Animal Health), Tokyo, Japan) was injected intramuscularly at the time of CIDR removal. After removal of the CIDRs, the cows were observed for estrus twice daily by the owners or staff at each respective farm and were artificially inseminated after detection of estrus according to the common practices of licensed artificial inseminators. Formation of a CL 14 days after CIDR removal (day 21 in the CIDR 7-day insertion group and day 28 in the CIDR 14-day insertion group) was evaluated by ultrasonography and plasma P_4 concentrations. Pregnancy was checked by rectal palpation according to common practical methods at 45 to 55 days after AI.

Experiment 2: Anestrous cows with a single cyst $\geq$25 mm in diameter or at least one follicle $\geq$18 mm in

diameter in the case of multiple cysts in their ovaries at the first visit, were examined by rectal palpation and ultrasonography on day -7 and day 0 (day 0 = CIDR insertion) and a total of 28 cows at 8 farms was utilized. The sizes of the cysts on day 0 were stable or increased compared with the findings on day -7. No CLs were observed in the ovaries on day -7 and day 0. Blood samples were collected on day -7, 0, 7 and 21 from the caudal vein with a heparinized syringe and the process was the same as Experiment 1. The health conditions and body condition scores (BCS) of the cows were checked by using the same method of Experiment 1. All cows were divided into 4 patterns based on their plasma P_4 concentrations with 1 *n*g/m*l* as the cut-off concentration on day -7 and day 0. The patterns of the plasma P_4 concentrations of the cows were as follows: <1.0 *n*g/m*l* on both day -7 and day 0 (pattern Ⅰ; 13 cows); <1.0 *n*g/m*l* on day -7 and ≥1.0 *n*g/m*l* on day 0 (pattern Ⅱ; 2 cows); ≥1.0 *n*g/m*l* on day -7 and <1.0 *n*g/m*l* on day 0 (pattern Ⅲ; 3 cows); and ≥1.0 *n*g/m*l* on

both day -7 and day 0 (pattern Ⅳ; 10 cows).

A CIDR (CIDR® 1900, Pfizer Animal Health, Tokyo, Japan) was intravaginaly inserted into all cows for 7 days from day 0, and 25 mg $PGF_{2\alpha}$ (Tromethamine dinoprost: Pronalgon® F, Pfizer Animal Health, Tokyo, Japan) was injected intramuscularly at the time of CIDR removal (Figure 3.2). Cysts were examined on day 0 and day 7 (day 7 = CIDR removal) by ultrasonography. Each observation, artificial insemination, evaluation procedure and parameter was the same as in Experiment 1. Formation of a CL at 14 days after CIDR removal (day 21) was evaluated by ultrasonography and plasma P_4 concentrations. Pregnancy check was also performed by the same method of Experiment 1 (Figure 3.2).

Experiment 3: A total of 55 Japanese Black cows at 36 commercial farms that were categorized by plasma P_4 concentrations on day 0 were utilized (Figure 3.6). A CIDR (CIDR® 1900, Pfizer Animal Health, Tokyo, Japan) was intravaginaly inserted into the cows for 7

days from day 0 on the first visit, and 25 mg $PGF_{2\alpha}$ (Tromethamine dinoprost: Pronalgon® F, Pfizer Animal Health, Tokyo, Japan) was injected intramuscularly at the time of CIDR removal. Each observation, artificial insemination, evaluation procedure and parameter was the same as in Experiment 2.

Experiment 4: In addition to Experiment 2, a total of 10 at 10 commercial farms of Japanese Black cows that were categorized by plasma P_4 concentrations with < 1.0 *ng/ml* on both day -7 and day 0, i.e. this group was in the same group that was categorized as in pattern I in Experiment 2, were utilized to compare the results of the CIDR group in pattern I in Experiment 2 (Figure 3.7). These cows were administrated with 100 μg GnRH (Fertirelin acetate: Conceral®, Schering-Plough Animal Health, Tokyo, Japan) on day 0 intramuscularly, and 25 mg $PGF_{2\alpha}$ (Tromethamine dinoprost: Pronalgon® F, Pfizer Animal Health, Tokyo, Japan) was injected intramuscularly on day 7. The observation of ovaries, artificial insemination and evaluation

procedure were the same in Experiment 2.

Plasma P_4 concentrations were measured by the Enzyme-Linked Fluorescent Assay (ELFA) method (VIDAS® Progesterone, Japan bioMérieux, Tokyo, Japan) using an automated immunoassay analyzer (miniVIDAS®, Japan bioMérieux, Tokyo, Japan) for the blood samples from Experiment 2 - 4. The sensitivity of the assay was 0.1 *ng/ml*, and the intra- and interassay coefficients of variation was less than 10%.

All statistical analysis was performed using the JMP™ ver. 5.1.1J software (SAS Institute Japan Inc., Tokyo, Japan). The data for testing of frequency was analyzed by Fisher's exact probability test in the case of comparison of the frequency in two groups. The likelihood by P_4 patterns and of correlation between existence of wall structure and plasma P_4 concentrations in Experiment 2 were assessed by the likelihood ratio test from the test probability. Parity, BCS and days interval between calving and CIDR insertion, between CIDR removal and the day of estrus

and between CIDR removal and the day of first AI were assessed by the Bartlett's test for equality of variances and were then analyzed by the unpaired *t*-test. The numbers of samples in patterns Ⅱ and Ⅲ in Experiment 2 were too small to analyze statistically. Comparison of the changes in follicle diameter was assessed by the paired *t*-test.

3.3. Results

Retention rate of CIDRs in the experimental cows was 100% in these studies.

Experiment 1: From the results of measuring plasma concentrations of P_4 on day 0, 11 cows were < 1.0 *n*g/m*l* and 17 cows were ≥ 1.0 *n*g/m*l* (Table 3.1). In the group with P_4 < 1.0 *n*g/m*l*, CLs were formed in 87.5% in the 7 days insertion of a CIDR and in 100% in the 14 days insertion of a CIDR group. In the group with P_4 ≥ 1.0 *n*g/m*l*, CLs were formed in 77.8% in the 7 days

insertion of a CIDR and in 50.0% in the 14 days insertion of a CIDR group. As the results of overall, CLs were formed in 75.0% of cows, and 42.9% of cows were conceived within 60 days after CIDR removal. There are no significant differences in the all parameters between 7 days and 14 days insertion groups.

Experiment 2: The likelihood of each pattern of plasma P_4 concentrations on day -7 and day 0 and the results for reproductive performance with the CIDR and $PGF_{2\alpha}$ treatment in Experiment 2 are shown in Table 3.2.

There was a significant difference in the incidence of each pattern of plasma P_4 concentrations ($P<0.01$). However, there was no significant difference in any parameters between patterns I and Ⅳ ($P>0.05$). A CL formed on day 21 in 92.9% of the cows, the days interval between CIDR removal and conception was 24.0 ± 6.5 days and 89.3% (overall) of the cows were pregnant within 60 days.

The changes in the diameters of 33 cysts in the 23 cows in patterns Ⅰ and Ⅳ on day -7, day 0 and day 7 in Experiment 1 are shown in Table 3.3 and the images by ultrasound of two cystic follicles of the same cow at both sides of the ovaries are shown in Figures 3.3 and 3.4.

The sizes of cysts in patterns Ⅰ and Ⅳ, single cysts ≥25 mm in diameter and multiple cysts ≥18 mm in diameter did not change from day -7 to day 0 ($P>0.05$); however, their diameters decreased significantly during CIDR insertion from day 0 to day 7 ($P<0.05$), but there was no clear decrease in single cysts ≥25 mm in pattern Ⅰ.

The wall structures around the anechoic areas of 50 cystic follicles (2 follicles/cow: 25 cows) were carefully examined by ultrasonography in Experiment 2 (Table 3.4). In 40 of the follicles (20 cows), no clear wall structure ≥2 mm or fibrin network inside the anechoic area were not observed; however, the plasma P_4 concentrations were ≥1.0 *n*g/m*l* in 14 of the 40

follicles (35.0%) even though no CLs could be observed at both right and left ovaries. In the other 10 follicles (5 cows), wall structures ≥2 mm were observed; however, the plasma P_4 concentrations were <1.0 *n*g/m*l* in 2 of the 10 follicles (20.0%). Based on the likelihood ratio test, the existence of a ≥2 mm wall structure around the follicle indicated that the plasma P_4 concentrations of the follicle tended to be higher than 1.0 *n*g/m*l* (P=0.056); however, follicles without wall structure or those whose walls were <2 mm thick tended to produce low P_4 concentrations (P=0.056, Figure 3.5).

Experiment 3: In 27 of the 55 cows (49.1%), the plasma P_4 concentrations were <1.0 *n*g/m*l* on day 0, and in 28 of the 55 cows (50.9%), they were ≥1.0 *n*g/m*l* on day 0 (Table 3.5). There were no significant differences in any parameters for each of the plasma P_4 patterns in Experiment 3 (P>0.05), and there were also no significant differences in any overall parameters compared with Experiment 2 (P>0.05).

When the consolidated data on reproductive

parameters of patterns Ⅰ and Ⅲ in Experiment 2 were compared with the data of the cows with plasma P_4 concentrations <1.0 *n*g/m*l* on day 0 in Experiment 3, there were no significant differences in any parameters ($P>0.05$, Table 3.6). In addition, the consolidated data of patterns Ⅱ and Ⅳ in Experiment 2 were compared with the data with plasma P_4 concentrations ≥1.0 *n*g/m*l* on day 0 in Experiment 3 and there were no significant differences between both groups in any parameters ($P>0.05$). On the other hand, comparing consolidated data of patterns Ⅰ and Ⅱ in Experiment 2 with the data of the cows with plasma P_4 concentrations <1.0 *n*g/m*l* in Experiment 3, there were no significant differences between the groups ($P>0.05$) but cows in patterns Ⅲ and Ⅳ in Experiment 2 were compared with cows with plasma P_4 concentrations ≥1.0 *n*g/m*l* in Experiment 3, there were more cows inseminated within 60 days after CIDR removal and cows pregnant within 60 days after CIDR removal in Experiment 2 with cows in Experiment 3 ($P<0.05$, Table 3.7). There were no

significant differences in the other parameters.

Experiment 4: During day 0 to day 7, no ovulation of any cystic follicles was observed in both CIDR group and GnRH group; however, ovulation of a coexistent follicle was observed 30% in GnRH group (Table 3.8). In contradiction to this, no ovulation of a coexistent follicle was observed in CIDR group. The CL formation on day 21 was 100% in CIDR group and 70% in GnRH group but there was no significant difference ($P>0.05$). Days interval between $PGF_{2\alpha}$ administration and conception was 24.7 ± 10.4 days (mean ± SEM) in CIDR group and 33.6 ± 17.4 days in GnRH group and the conception rates within 60 days from $PGF_{2\alpha}$ administration were 69.2% vs 60.0%, respectively; however, these were not significant ($P>0.05$).

3.4. Discussion

We investigated COD in Japanese Black cows

that were more than 40 days postpartum and that farmers complained as being in anestrous after calving, evaluated whether or not 7-day insertion of a CIDR combined with $PGF_{2\alpha}$ injection at CIDR removal could be a practical treatment against COD and evaluated whether or not this protocol could contribute to benefit of farmers.

Recent data collected using ultrasonography indicates that follicles typically ovulate at 17 mm in diameter, and thus, follicles that persist at that diameter or greater may be considered to be "cystic" [42]. Ginther *et al.* [38] reported that normal ovulatory follicles in dairy cattle reach an average diameter of 16 ± 0.4 mm or 13.9 ± 0.4 mm at ovulation (two vs. three waves of follicle growth, respectively). Crane *et al.* [20] also used this criterion to diagnose cystic ovaries in their study in the case of existence of multiple follicles. In the present study, we used 25 mm as the cut-off diameter in the case of single cysts and 18 mm in the case of multiple cysts.

In Experiment 1, the efficacy of 7-day exposure of P_4 from a CIDR could be recognized against COD preliminarily and clinically, and it was not different from the reproductive efficacy of 14-day exposure of P_4. Todoroki, *et al.* [103] reported the excellent efficacy against COD in Japanese Black donor cows with single or multiple insertions of CIDRs; however, 7-day insertion of a CIDR could be sufficient to treat COD clinically in commercial Japanese Black cows in our present study.

In Experiment 2, since the cysts were examined for 1 week before CIDR insertion in experimental cows in which anestrous continued until initiation of observation, differentiation of COD was not performed absolutely in accordance with the guidelines [107] in Japan. Measurement of plasma P_4 concentrations on day -7 and day 0 showed that 13 of 28 cows (46.4%) had concentrations <1.0 *ng/ml* (pattern Ⅰ) and 10 of 28 cows (35.7%) had concentrations ≥1.0 *ng/ml* (pattern Ⅳ) at both time points. The cows exhibiting pattern Ⅰ

might have had an FC or might have been experiencing non-cyclic estrus, and those exhibiting pattern Ⅳ might have had an LC or might have been exhibiting cyclic estrus. This result might suggest that FCs tend to be more common than LCs, as reported previously [12, 36, 65].

According to ultrasonographic observation of the cysts in Experiment 2, 20% of the cysts had a non-functional luteal structure, even in the case of the presence of a wall ≥2 mm thick around the cavity (plasma P_4 concentrations: <1.0 *n*g/m*l*); on the other hand, 35% of the cysts had luteal function, even in the case that no wall was observed or that it was <2 mm thick (plasma P_4 concentrations: ≥1.0 *n*g/m*l*). In addition to this, the obtained appearance ratio, 35% in this study was not different from 22% as the probability of appearance ratio of the follicles with no clear wall structures or <2 mm of thickness but the plasma P_4 concentrations were ≥1.0 *n*g/m*l*. That is, although some reasons were considerable, which were the

artificial error of observation of walls, or of CLs, or of sensitivity of the ultrasound equipment including low resolution image, but at least 20% of the follicles with <2 mm of wall structure around the cavity or no clear wall found out by ultrasonography may produce P_4 in this study. On the other hand, although there were not sufficient numbers of samples of the follicle group with $\geq$2 mm of wall structures, the study should be continued to collect more samples.

This indicates that the accuracy of diagnosis of cysts based on ultrasonography and plasma P_4 concentrations was 80% for cysts with luteal function and 65% for cysts without luteal function, respectively. Thus, it was not easy to differentiate COD by observation for existence of a wall around of the cavity, even by using ultrasound from a morphological standpoint.

On the other hand, Douthwaite and Dobson [28] described that the wall thickness of cysts is positively correlated with the plasma P_4 concentrations ($r = 0.52$).

We also found that the P_4 concentrations in cows with cysts with a wall structure $\geq$2 mm thick were significantly higher than for cysts without a wall or with a wall less than 2 mm thick ($P<0.05$) and that for cysts that did not have an observable wall or that had a wall less than 2 mm thick, the P_4 concentrations tended to be lower, but this result was not significant ($P>0.05$). However, there are some reports indicating that the P_4 concentrations might vary and contradict ultrasound images when trying to classify cysts [30], and this contradiction may be due to the stage of the cyst and age of the luteal tissue present [12]. Jeffcoate and Ayliffe [44] described that the plasma P_4 concentrations in some cows with either an FC or LC are similar on the day of treatment and that, therefore, differentiating the types of cyst would be of little value. On the other hand, in practice, measurement of the plasma P_4 concentrations is not practical under general field conditions or in veterinary activities due to the time required until receipt of the results from the laboratory,

and therefore, the information itself is posterior evidence that may not represent the condition at the respective moment. Thus, although it is no wonder that the plasma P_4 concentrations must be assayed in order to diagnose COD more accurately in addition to ultrasonographic examination and rectal palpation, selecting the correct treatment strategy by diagnosis using plasma P_4 concentrations and/or ultrasonography in addition to rectal palpation is not easy for clinicians as common veterinary practice in the field.

The plasma P_4 concentrations of 5 in the 28 cows (17.9%) exhibiting patterns Ⅱ and Ⅲ in Experiment 1 changed from day -7 to day 0. Based on ultrasonographic findings, the cysts did not significantly decrease in size during the 7 days ($P>0.05$). The ovaries of 2 in 28 cows (7.1%) exhibiting pattern Ⅱ became partly luteinized, and the plasma P_4 concentrations of 3 in 28 cows (10.7%) exhibiting pattern Ⅲ decreased from above 1 *n*g/m*l* to below that level during the 7-day examination. Nakao *et al.* [65]

described the possibility of emergence of a new follicle that produces estrogen in this case; however, this may be a delicate matter of timing because the size of the newly emerged follicles of these cows in this study were equal to or less than 10 mm in diameter on day 0. According to Yoshioka *et al.* [111], the P_4 levels in blood could change beyond a concentration of 1 *n*g/m*l* when an FC turned over. Although the observed cysts of patterns Ⅱ and Ⅲ might have been at this delicate moment, it was impossible to categorize these cows as clearly having an FC or LC and others properly on day 0 even after observation for 7 days; this was also the case for the cysts of patterns Ⅰ and Ⅳ. Furthermore, it was also impossible to select a proper treatment strategy under the field conditions.

During 7 days of CIDR insertion, the average size of cysts in patterns Ⅰ and Ⅳ decreased significantly ($P<0.05$) or tended to be small in single cyst $\geq$25 mm in diameter in pattern Ⅰ. It has been shown that P_4 administration regresses cysts by suppressing pulsatile

LH [11] and that the cysts decrease in size.

The likelihoods of formation of a CL on day 21 were extremely high in patterns I (100%), Ⅳ (90.0%), overall in Experiment 2 (92.9%) and overall in Experiment 3 (83.6%). These ratios are relatively higher than those reported in studies of dairy cows using GnRH, GnRH analogue or hCG for FCs and a Progesterone Releasing Intravaginal Device for ovarian cysts [66, 68, 112]. These results indicated that a high percentage of cows with COD ovulated within 14 days after CIDR removal. However, the cysts did not ovulate, but newly emerged follicles ovulated. The conception rates within 60 days after CIDR removal were equal to or more than 50.0%, except for that of pattern Ⅱ. The overall conception rates in Experiments 2 and 3 were 71.4% and 54.5%, respectively. Thus, even if the cysts remained in the ovaries after this treatment, the cattle could conceive by AI.

The data in Experiment 2 could be divided into two patterns based on the plasma P_4 concentrations on

day -7 and day 0, cows with <1.0 *n*g/m*l* and cows with ≥1.0 *n*g/m*l*, and these categorized patterns could be recognized as the same situation in each cow with plasma P_4 concentrations of <1.0 *n*g/m*l* and ≥1.0 *n*g/m*l* on day 0 in Experiment 3. Therefore, statistical analyses were performed to compare the consolidated data for cows exhibiting patterns Ⅰ and Ⅲ in Experiment 2 with the data for cows with plasma P_4 concentrations <1.0 *n*g/m*l* in Experiment 3. And also to compare the consolidated data for cows exhibiting patterns Ⅱ and Ⅳ in Experiment 2 with the data for cows with plasma P_4 concentrations ≥1.0 *n*g/m*l* in Experiment 3 (Table 3.6). And the consolidated data for cows with patterns Ⅰ and Ⅱ in Experiment 2 were compared with the data for cows with plasma P_4 concentrations <1.0 *n*g/m*l* in Experiment 3. The consolidated data for cows with patterns Ⅲ and Ⅳ in Experiment 2 were compared with the data for cows with plasma P_4 concentrations ≥1.0 *n*g/m*l* in Experiment 3 (Table 3.7). As a result, the ratio of cows inseminated

within 60 days after CIDR removal and cows pregnant within 60 days after CIDR removal were significantly higher for consolidated patterns Ⅲ and Ⅳ in Experiment 2 than for cows with plasma P_4 concentrations of $\geq$1.0 *ng/ml* in Experiment 3 ($P<0.05$). These results indicated that a longer observation period might bring better reproductive results. However, further investigation is needed because there were no significant differences in other parameters and in comparisons between the consolidated data of patterns Ⅰ and Ⅱ in Experimental 2 and the cows with <1.0 *ng/ml* in Experiment 3. Also the investigation is needed between patterns Ⅰ and Ⅲ in Experiment 2 and the cows with <1.0 *ng/ml* on day 0 in Experiment 3, and between patterns Ⅱ and Ⅳ in Experiment 2 and the cows with $\geq$1.0 *ng/ml* on day 0 in Experiment 3 ($P>0.05$).

On the other hand, the average days interval between CIDR removal and both estrous detection and conception were not significantly different between

Experiments 2 and 3 (days interval to estrus of 16.5 ± 4.4 vs 11.2 ± 2.8, respectively, $P>0.05$; days interval to conception of 24.0 ± 6.5 vs 24.4 ± 5.3, respectively, $P>0.05$). All experimental cows had been in anestrus until treatment after calving. Therefore, these results indicated that the timing, not only of estrous detection, but also of conception was accelerated by at least 7 days in the cows utilized in Experiment 3 compared with Experiment 2. The total production cost of a Japanese Black calf per dam per day was calculated as 1,377 yen in the fiscal 2005 [75]. Therefore, the opportunity cost obtainable by farmers from this 7-day acceleration is equivalent to 9,639 yen. This suggests that an early decision to start treatment with CIDR combined with $PGF_{2\alpha}$ against COD could bring an economic benefit to farmers/producers.

When comparing the reproductive efficacy of the CIDR and $PGF_{2\alpha}$ combination regimen with one of major current treatment procedure, GnRH administration followed by $PGF_{2\alpha}$, for treatment of cows with COD

whose plasma P_4 concentrations were <1.0 *ng/ml* both on day -7 and day 0, any significant differences in the evaluated parameters were not recognized in the Experiment 4. Therefore, a CIDR insertion for 7 days followed by $PGF_{2\alpha}$ administration could be replaceable regimen of GnRH protocol; however, the number of samples was small and cows with other plasma P_4 patterns were not compared yet; therefore, this evaluation study should be continued. Furthermore, in the present study, the 7-day CIDR insertion and $PGF_{2\alpha}$ administration at CIDR removal had sufficient efficacy; however, it has recently been reported that P_4 exposure for 3 days, but not 1 day, appears to be sufficient to reinitiate estradiol responsiveness of the hypothalamus [39]. Therefore, further advantage may come up with this CIDR insertion and $PGF_{2\alpha}$ administration regimen in the near future.

In conclusion, 7-day insertion of a CIDR combined with $PGF_{2\alpha}$ administration at CIDR removal could be valuable for treatment against COD. This

usage would minimize the risk of incorrect treatment, and early initiation of treatment can provide an economic benefit to farmers.

3.5. Acknowledgements

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3.6. Tables and Figures

Table 3.1. Overall reproductive results of experimental cows with COD in Experiment 1.

Plasma P_4 concentrations on Day 0 (*n* g/m*l*)	< 1.0		≥ 1.0		Overall
CIDR insertion	7 days	14 days	7 days	14 days	
Parameters					
N (% of total)	8 (28.6)	3 (10.7)	9 (32.1)	8 (28.6)	28 (100)
Parity (Mean ± SEM)	5.0 ± 1.3	5.3 ± 1.9	6.7 ± 1.1	6.9 ± 1.0	6.0 ± 0.7
BCS (Mean ± SEM)	2.66 ± 0.15	2.83 ± 0.14	3.08 ± 0.13	3.41 ± 0.18	3.03 ± 0.10
Days interval between calving and CIDR insertion (Mean ± SEM)	74.6 ± 6.5	161.7 ± 39.3	82.8 ± 5.7	121.0 ± 45.9	102.3 ± 15.6
Formation of a CL at 14 days after CIDR removal (%)	7 (87.5)	3 (100)	7 (77.8)	4 (50.0)	21 (75.0)
Cows inseminated within 60 days after CIDR removal (%)	7 (87.5)	2 (66.7)	5 (55.5)	6 (75.0)	20 (71.4)
Cows pregnant within 60 days after CIDR removal (%)	4 (50.0)	1 (33.3)	2 (22.2)	5 (62.5)	12 (42.9)

Day 0: the day of CIDR insertion
The cows included in this data received up to 3 AIs.
1) There are no significant differences in any parameters between 7 days and 14 days of CIDR insertion in the same group of P_4 concentrations.

Table 3.2. Descriptive reproductive parameters of cows with COD characterized by hormonal condition on day -7 and day 0 in Experiment 2.

Pattern	I	II	III	IV	Overall
Plasma P_4 concentrations (*n* g/ml)					
Day -7	< 1.0	< 1.0	≥ 1.0	≥ 1.0	
Day 0	< 1.0	≥ 1.0	< 1.0	≥ 1.0	
Parameters					
N/N (% of total)	13 (46.4)	2 (7.1)	3 (10.7)	10 (35.7)	28(100)
Parity (Mean ± SEM)	6.9 ± 1.0	7.0	5.3 ± 1.5	5.2 ± 0.6	6.1 ± 0.6
BCS (Mean ± SEM)	3.10 ± 0.11	3.13	3.42 ± 0.08	2.95 ± 0.11	3.08 ± 0.07
Days interval between calving and CIDR insertion (Mean ± SEM)	167.2 ± 32.2	97.0	138.3 ± 64.8	103.6 ± 23.4	136.4 ± 18.8
Formation of a CL on d 21 (%)	13 (100)	1 (50.0)	3 (100)	9 (90.0)	26 (92.9)
Days interval between CIDR removal and estrus (Mean ± SEM)	3.1 ± 0.4	4.5	4.3 ± 1.9	2.8 ± 0.3	3.2 ± 0.3
N	13	2	3	10	28
Days interval between CIDR removal and first AI (Mean ± SEM)	16.0 ± 6.5	67.5	11.3 ± 8.8	8.1 ± 3.6	16.5 ± 4.4
N	13	2	3	10	28
Days interval between CIDR removal and conception (Mean ± SEM)	24.7 ± 10.4	67.5	40.5 ±38.5	8.4 ± 4.0	24.0 ± 6.5
N	11	2	3	9	25
Cows inseminated within 60 days after CIDR removal (%)	12 (92.3)	0 (0)	3 (100)	10 (100)	25 (89.3)
Cows pregnant at the first AI (%)	8 (61.5)	2 (100)	1 (33.3)	7 (70.0)	18 (64.3)
Cows pregnant within 60 days after CIDR removal (%)	9 (69.2)	0 (0)	2 (66.7)	9 (90.0)	20 (71.4)

Day -7: initial day of visit. Day 0: the day of CIDR insertion. Day 7: the day of CIDR removal.
The cows included in this data received up to 3 AIs.
1) There is significant difference in the incidence of each plasma P_4 pattern ($P<0.01$).

Table 3.3. The changes in the diameters of cystic structures on day -7, day 0 and day 7 in Experiment 2.

	Number of follicles	Number of cows	Diameter (mm, mean ± SEM)		
			Day -7	Day 0	Day 7
Pattern I					
Single ≥ 25 mm	6	5	30.0 ± 1.7	30.5 ± 1.5	27.7 ± 2.3
Multiple ≥ 18 mm	15	8	19.8 ± 0.6	18.8 ± 0.4 ^{A}	13.7 ± 2.0 ^{B}
Subtotal	21	13	22.6 ± 1.3	22.3 ± 1.3 ^{A}	17.7 ± 2.2 ^{B}
Pattern IV					
Single ≥ 25 mm	4	4	25.7 ± 4.3	31.3 ± 0.9 ^{A}	25.0 ± 1.2 ^{B}
Multiple ≥ 18 mm	8	6	21.7 ± 2.5	20.0 ± 0.6 ^{a}	14.8 ± 2.1 ^{b}
Subtotal	12	10	21.1 ± 1.6	23.8 ± 1.7 ^{A}	17.8 ± 2.2 ^{B}

Day -7: initial day of visit. Day 0: the day of CIDR insertion. Day 7: the day of CIDR

1) Different superscripts in the same line indicate a significant difference; $P<0.01$ for large characters, and $P<0.05$ for small characters.
2) The categorized sizes of the cysts are based on the sizes on day 0.
3) The CIDR was inserted from day 0 to day 7.

Table 3.4. The relationship between observed walls around cavities of cystic follicles and plasma P_4 concentrations.

	Number of follicles (%)	
Thickness of wall structure	≥2 mm	<2 mm
Plasma P_4 concentrations		
≥ 1.0 *n*g/m*l*	8 (80.0)	14 (35.0)
< 1.0 *n*g/m*l*	2 (20.0)	26 (65.0)
Total	10 (100)	40 (100)

The probability of appearance of the follicles with <2 mm of wall structure in each group of plasma P_4 concentrations were the same as the probability of appearance at 22% in ≥1.0 ng/ml group and 78% in <1.0 ng/ml of plasma P_4 concentrations by the likelihood ratio test from the test probability. Therefore, at least 20% of the follicles with <2 mm of wall structure around the cavity or no clear wall found out by ultrasonography may produce P_4 in this study.

Table 3.5. Overall reproductive results of experimental cows with COD in Experiment 3.

Plasma P_4 concentrations on day 0 (*n*g/m*l*)	< 1.0	≥ 1.0	Overall
Parameters			
N (% of total)	27 (49.1)	28 (50.9)	55 (100)
Parity (Mean ± SEM)	6.0 ± 0.7	6.2 ± 0.5	6.1 ± 0.4
BCS (Mean ± SEM)	2.97 ± 0.08	3.13 ± 0.08	3.05 ± 0.06
Days interval between calving and CIDR insertion (Mean ± SEM)	138.3 ± 19.4	103.1 ± 16.1	122.6 ± 13.0
Formation of a CL on d 21 (%)	26 (96.3)	20 (71.4)	46 (83.6)
Days interval between CIDR removal and estrus (Mean ± SEM)	3.3 ± 0.4	3.2 ± 0.3	3.2 ± 0.2
N	26	20	46
Days interval between CIDR removal and first AI (Mean ± SEM)	10.7 ± 3.7	11.9 ± 4.5	11.2 ± 2.8
N	26	20	46
Days interval between CIDR removal and conception (Mean ± SEM)	25.0 ± 5.8	18.7 ± 5.8	24.4 ± 5.3
N	19	18	37
Cows inseminated within 60 days after CIDR removal (%)	24 (88.9)	19 (67.9)	43 (78.2)
Cows pregnant at the first AI (%)	10 (37.0)	14 (50.0)	24 (43.6)
Cows pregnant within 60 days after CIDR removal (%)	16 (59.3)	14 (50.0)	30 (54.5)

Day 0: the day of CIDR insertion. Day 7: the day of CIDR removal.
The cows included in this data received up to 3 AIs.
1) There are no significant differences in any parameters by plasma P_4 concentrations on day 0.

Table 3.6. Descriptive reproductive parameters of cows with COD characterized by consolidated data in Experiments 2 and 3.

Experiment	Experiment 2		Experiment 3	
Pattern	I +III	II +IV		
Plasma P_4 concentrations (*n*g/m*l*) Day 0	< 1.0	≥ 1.0	< 1.0	≥ 1.0
Parameters				
N	16	12	27	28
Parity (Mean ± SEM)	6.6 ± 0.8	5.5 ± 0.7	6.0 ± 0.7	6.2 ± 0.5
BCS (Mean ± SEM)	3.16 ± 0.09	2.98 ± 0.09	2.97 ± 0.08	3.13 ± 0.08
Days interval between calving and CIDR insertion (Mean ± SEM)	161.8 ± 28.1	102.5 ± 20.0	138.3 ± 19.4	103.1 ± 16.1
Formation of a CL on d 21 (%)	16 (100)	10 (83.3)	26 (96.3)	20 (71.4)
Days interval between CIDR removal and estrus (Mean ± SEM)	3.3 ± 0.5	3.1 ± 0.3	3.3 ± 0.4	3.2 ± 0.3
N	16	12	26	20
Days interval between CIDR removal and first AI (Mean ± SEM)	15.1 ± 5.5	18.3 ± 7.4	10.7 ± 3.7	11.9 ± 4.5
N	16	12	26	20
Days interval between CIDR removal and conception (Mean ± SEM)	27.3 ± 10.0	20.0 ± 8.4	25.0 ± 5.8	18.7 ± 5.8
N	12	10	19	18
Cows inseminated within 60 days after CIDR removal (%)	15 (93.8)	10 (83.30)	24 (88.9)	19 (67.9)
Cows pregnant at the first AI (%)	9 (56.3)	9 (75.0)	10 (37.0)	14 (50.0)
Cows pregnant within 60 days after CIDR removal (%)	11 (68.8)	9 (75.0)	16 (59.3)	14 (50.0)

Day -7: initial day of visit. Day 0: the day of CIDR insertion. Day 7: the day of CIDR removal.
The cows included in this data received up to 3 AIs.

Table 3.7. Descriptive reproductive parameters of cows with COD characterized by consolidated data in Experiments 2 and 3.

Experiment	Experiment 2		Experiment 3	
Pattern	I + II	III+IV		
Plasma P_4 concentrations (*n*g/m*l*)				
Day -7	< 1.0	≥ 1.0	-	-
Day 0	-	-	< 1.0	≥ 1.0
Parameters				
N	15	13	27	28
Parity (Mean ± SEM)	6.9 ± 0.9	5.2 ± 0.6	6.0 ± 0.7	6.2 ± 0.5
BCS (Mean ± SEM)	3.10 ± 0.09	3.06 ± 0.10	2.97 ± 0.08	3.13 ± 0.08
Days interval between calving and CIDR insertion (Mean ± SEM)	157.9 ± 27.8	111.6 ± 22.2	138.3 ± 19.4	103.1 ± 16.1
Formation of a CL on d 21 (%)	14 (93.3)	12 (92.3)	26 (96.3)	20 (71.4)
Days interval between CIDR removal and estrus (Mean ± SEM)	3.3 ± 0.4	3.2 ± 0.5	3.3 ± 0.4	3.2 ± 0.3
N	15	13	26	20
Days interval between CIDR removal and first AI (Mean ± SEM)	22.9 ± 7.3	9.1 ± 3.5	10.7 ± 3.7	11.9 ± 4.5
N	15	13	26	20
Days interval between CIDR removal and conception (Mean ± SEM)	31.8 ± 9.8	14.6 ± 7.7	25.0 ± 5.8	18.7 ± 5.8
N	12	10	19	18
Cows inseminated within 60 days after CIDR removal (%)	12 (80.0)	13 (100.0) [a]	24 (88.9)	19 (67.9) [b]
Cows pregnant at the first AI (%)	10 (66.7)	8 (61.5)	10 (37.0)	14 (50.0)
Cows pregnant within 60 days after CIDR removal (%)	9 (60.0)	11 (84.6) [c]	16 (59.3)	14 (50.0) [d]

Day -7: initial day of visit. Day 0: the day of CIDR insertion. Day 7: the day of CIDR removal.
The cows included in this data received up to 3 AIs.

Table 3.8. Comparative study of CIDR vs GnRH in reproductive parameters in cows with COD and with < 1.0 *n*g/m*l* plasma P_4 concentrations both on day -7 and day 0 in Experiment 4.

Treatment	CIDR	GnRH
Plasma P_4 concentrations (*n*g/m*l*)		
Day -7	< 1.0	< 1.0
Day 0	< 1.0	< 1.0
Parameters		
N	13	10
Parity (Mean ± SEM)	6.9 ± 1.0	6.0 ± 0.9
BCS (Mean ± SEM)	3.10 ± 0.11	3.30 ± 0.15
Days interval between calving and d 0 (Mean ± SEM)	167.2 ± 32.2	164.3 ± 45.7
Ovulation of a cystic follicle during d 0 to d 7 (%)	0 (0)	0 (0)
Ovulation of a coexistent follicle during d 0 to d 7 (%)	0 (0)	3 (30.0)
Formation of a CL on d 21 (%)	13 (100)	7 (70.0)
Days interval between d 7 and estrus (Mean ± SEM)	3.1 ± 0.4	18.8 ± 8.3
N	13	9
Days interval between d 7 and first AI (Mean ± SEM)	16.0 ± 6.5	18.8 ± 8.3
N	13	9
Days interval between d 7 and conception (Mean ± SEM)	24.7 ± 10.4	33.6 ± 17.4
N	11	7
Cows inseminated within 60 days from d 7 (%)	12 (92.3)	8 (80.0)
Cows pregnant at the first AI (%)	8 (61.5)	4 (40.0)
Cows pregnant within 60 days from d 7 (%)	9 (69.2)	6 (60.0)

Day -7: initial day of visit. Day 0: the day of CIDR insertion or GnRH administration.
Day 7: the day of CIDR removal and/or $PGF_{2\alpha}$ administration.
The cows included in this data received up to 3 AIs.
1) There are no significant differences in any parameters.

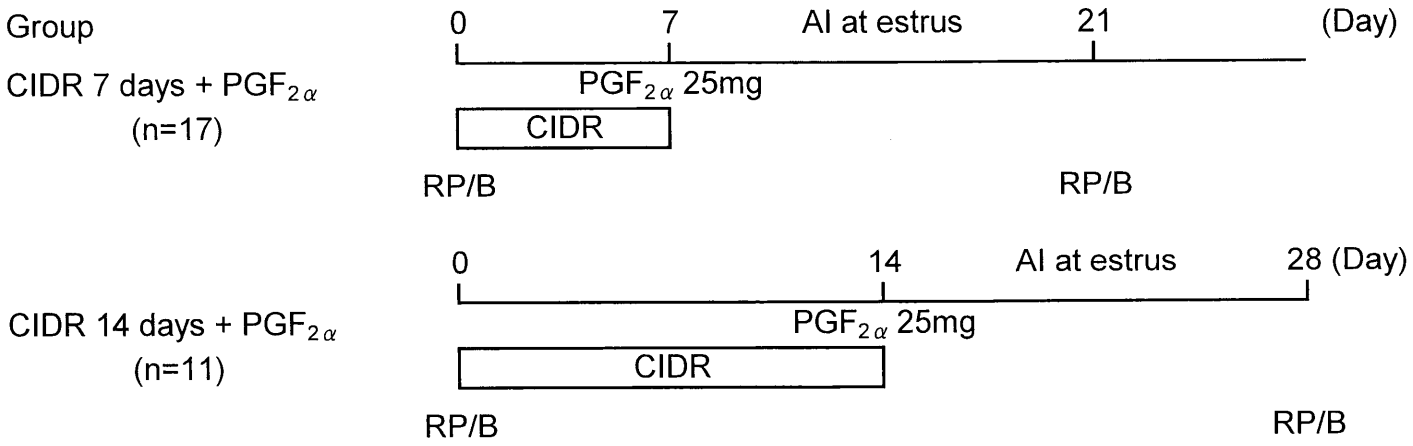


Figure 3.1. Schematic diagram of treatment protocols in Experiment 1.
$PGF_{2\alpha}$: tromethamine dinoprost, RP/B: Rectal Palpation and/or Ultrasound and blood sampling

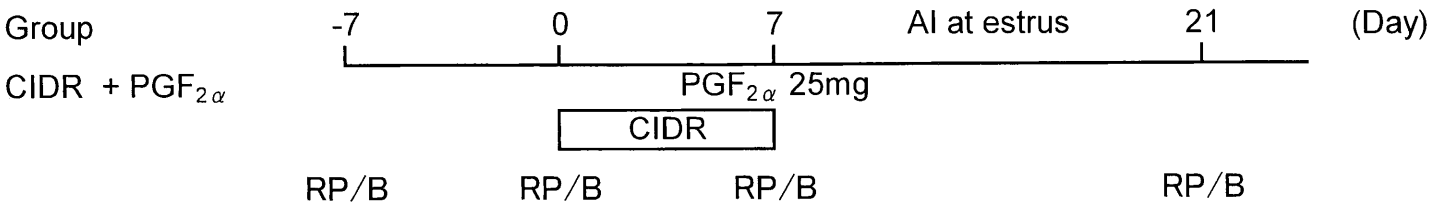
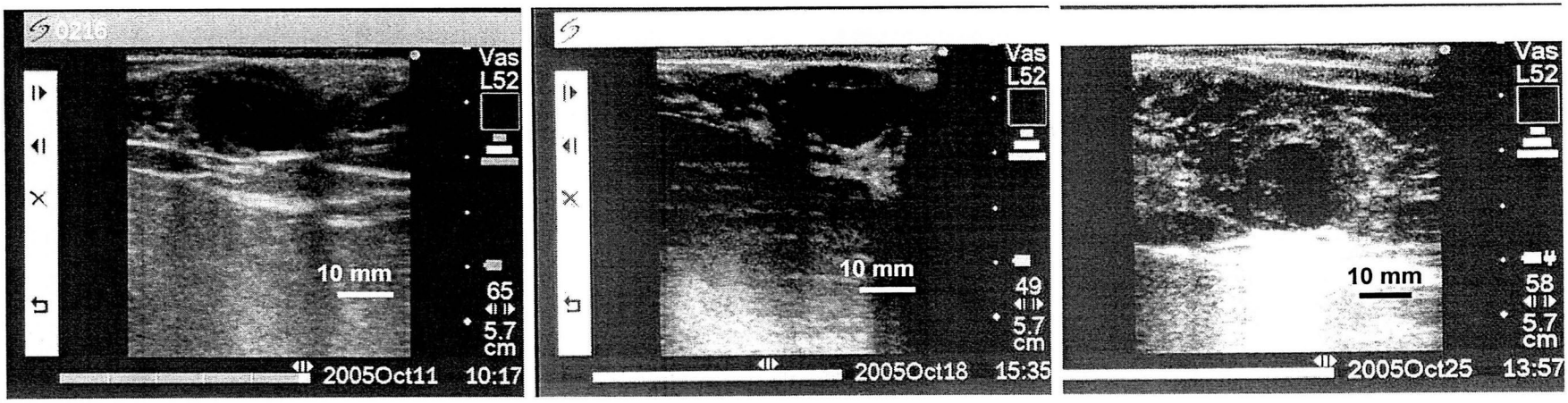
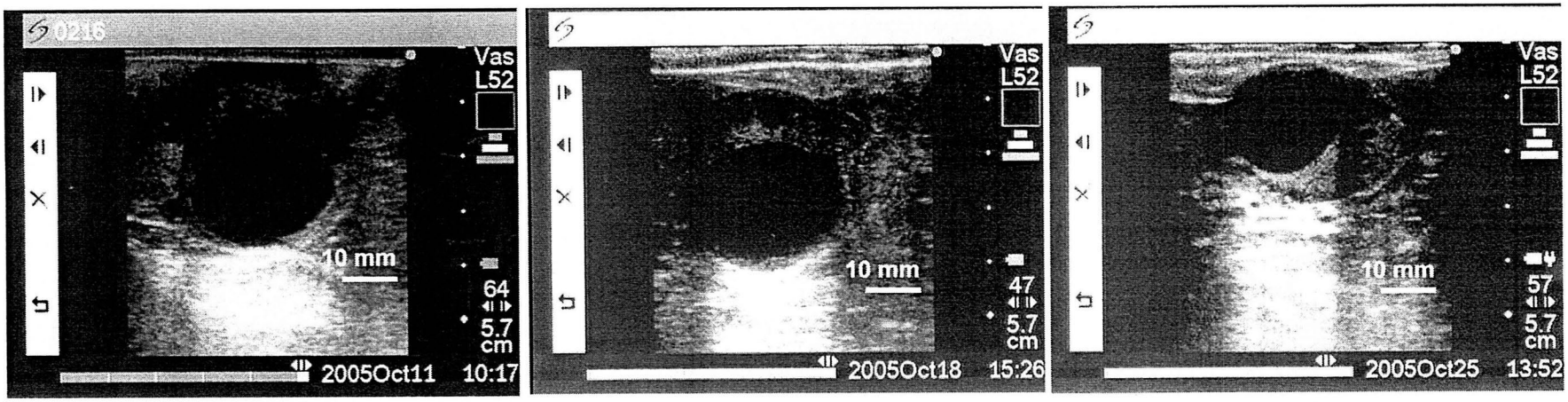


Figure 3.2. Schematic diagram of treatment protocols in Experiment 2.
$PGF_{2\alpha}$: tromethamine dinoprost, RP/B: Rectal Palpation and/or Ultrasound and blood sampling



(A) (B) (C)

Figure 3.3. A case of morphological changes of the cystic follicle on the left ovary in the CIDR treated cow in Experiment 2. (A): day -7, (B): day 0, A CIDR was inserted, (C): day 7, CIDR was removed. Plasma P_4 concentrations of the cow were 2.24 *ng/ml*, 0.93 *ng/ml* and 4.37 *ng/ml*, respectively.
(Cow name: Fujiko no 3, Parity: 3)



(A) (B) (C)

Figure 3.4. A case of morphological changes of the cystic follicle on the right ovary in the CIDR treated cow in Experiment 2. (A): day -7, (B): day 0, A CIDR was inserted, (C): day 7, CIDR was removed. Plasma P_4 concentrations of the cow were 2.24 *n*g/m*l*, 0.93 *n*g/m*l* and 4.37 *n*g/m*l*, respectively.
(Cow name: Fujiko no 3, Parity: 3)

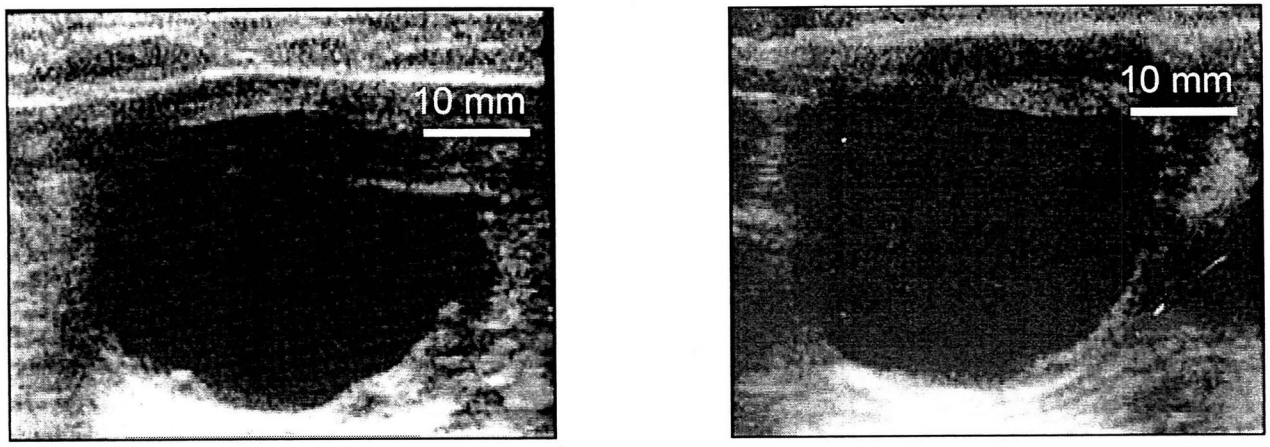


Figure 3.5. (*Left*) A case of the cystic follicle with 2 mm of wall structure (Right ovary). No CLs were found out at any side of the ovary. Plasma P_4 concentration at this moment was 1.32 *n*g/m*l* (Cow name: Michiyo, Parity: 8). (*Right*) A case of the cystic follicle with no wall structure (Left ovary). No CLs were found out at any ovary; however, plasma P_4 concentration at this moment was 1.14 *n*g/m*l* (Cow name: Hibari, Parity: 2).

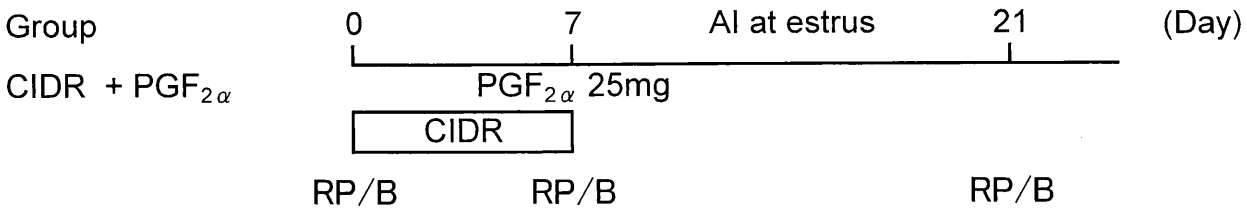


Figure 3.6. Treatment protocol in Experiment 3.
$PGF_{2\alpha}$: tromethamine dinoprost, RP/B: Rectal Palpation and/or Ultrasound and blood sampling

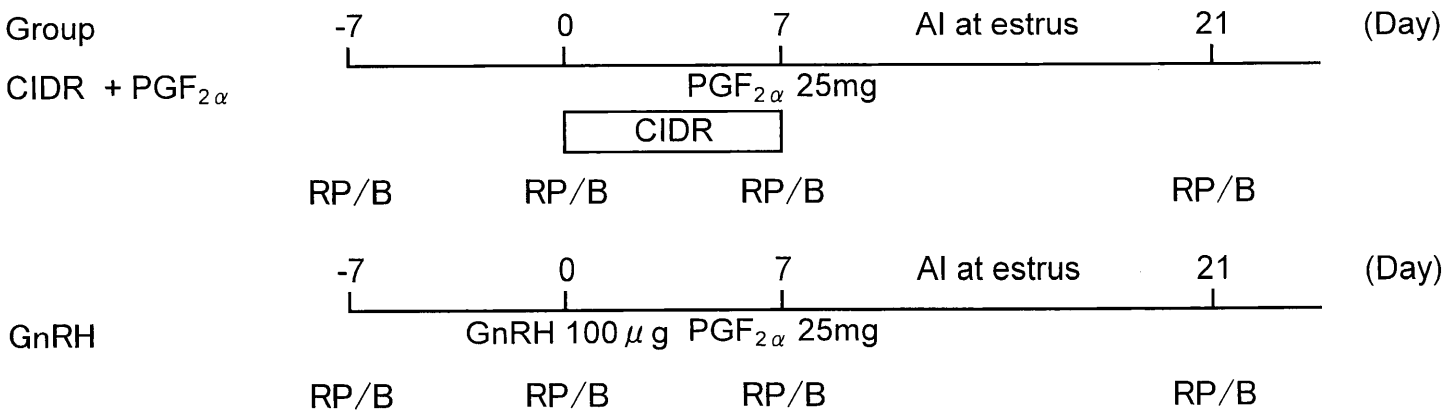


Figure 3.7. Schematic diagram of treatment protocols in Experiment 4.
GnRH: fertirelin acetate, $PGF_{2\alpha}$: tromethamine dinoprost, RP/B: Rectal Palpation and/or Ultrasound and blood sampling

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