A CIDR-Based Timed Embryo Transfer Protocol Increases the Pregnancy Rate of Lactating Repeat Breeder Dairy Cows

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Abstract. This study evaluated the pregnancy rates following either a controlled internal drug release (CIDR)-based timed artificial insemination (TAI) or an embryo transfer (TET) protocol compared with that following a single PGF2α injection and AI after estrus (AIE) in lactating repeat breeder dairy cows. Fifty-three lactating dairy cows diagnosed as repeat breeders were used in this study and were randomly assigned to the following three treatments. (1) Cows, at random stages of the estrous cycle, received a CIDR device and 2 mg estradiol benzoate (EB; Day 0), a 25 mg PGF2α injection at the time of CIDR removal on Day 7 and a 1 mg EB injection on Day 8. The cows then received TAI 30 h (Day 9) after the second EB injection using dairy semen (TAI group, n=13). (2) Cows, at random stages of the estrous cycle, received the same hormonal treatments as in the TAI group. The cows then received TET on Day 16 using frozen-thawed blastocysts or morula embryos collected from Korean native cattle donors (TET group, n=13). (3) Cows, at the luteal phase, received a 25 mg injection of PGF2α and AIE using dairy semen (control group, n=27). The ovaries of the cows in the TET group were examined by transrectal ultrasonography to determine ovulation of the preovulatory follicles, and blood samples were collected for serum progesterone (P4) analysis. The pregnancy rate was significantly higher in the TET group (53.8%) than in the control (18.5%) or TAI (7.7%) groups (P<0.05). The ultrasonographic observations demonstrated that all the cows in the TET group ovulated the preovulatory follicles and concomitantly formed new corpora lutea. Accordingly, the mean serum P4 concentration remained constant between Day 0 and Day 7 of the luteal phase, decreased dramatically on Day 8 (P<0.01) and subsequently increased by Day 16 (P<0.01). These data suggest that the CIDR-based TET protocol can be used to effectively increase the pregnancy rate in lactating repeat breeder dairy cows.

Key words: Controlled internal drug release (CIDR), Lactating repeat breeder dairy cow, Pregnancy rate, Timed artificial insemination (TAI), Timed embryo transfer (TET) (J. Reprod. Dev. 53: 1313–1318, 2007)
errors, endocrine dysfunction, failures of ovulation or fertilization and early embryonic mortality [2–10]. Hormonal treatments, such as gonadotropin-releasing hormone (GnRH) or human chorionic gonadotropin (hCG), have been used to increase the rate of pregnancy for repeat breeder cows [10–14], although success has been limited. Furthermore, Kasimanickam et al. [15] used the Ovsynch protocol to treat repeat breeder dairy cows and produced an overall conception rate of 21%. In their study, no differences in conception rate were detected between cows receiving timed artificial insemination (TAI) and AI after estrus (AIE). Recently, Kim et al. [16] demonstrated that a controlled internal drug release (CIDR)-based TAI protocol is an effective technique to increase the pregnancy rate of non-lactating repeat breeder dairy cows that have a highly extended postpartum interval determined through fine regulation of folliculogenesis and synchronous ovulation. Interestingly, embryo transfer (ET) can increase the probability of maintenance of pregnancy in lactating dairy cows by minimizing the negative effects of high milk production and heat stress on both the quality of the oocyte and early development of the embryo [17]. Similarly, this ET technique has been introduced as an efficient tool to improve fertility for repeat breeder dairy cattle [18, 19]. It has also been reported that a progesterone (P4)-based timed ET (TET) protocol enables more recipients to be selected without a decrease in pregnancy rate compared with synchronization of estrus using PGF2α [20]. Thus, we proposed that the CIDR-based TET protocol would be an effective protocol to improve fertility in repeat breeder cows. Furthermore, a comparison of the pregnancy rates of lactating repeat breeder dairy cows that received either TAI or TET has not yet been conducted. In this study, we evaluated the pregnancy rate following either a CIDR-based TAI or TET protocol compared with that following a single PGF2α injection and AIE in lactating repeat breeder dairy cows.

**Materials and Methods**

*Experimental animals and study design*

This study was performed on five Holstein dairy farms (Chungbuk Province, Korea). The lactating Holstein-Friesian cows were maintained in freestall facilities, fed a total mixed ratio and milked twice daily. The average milk yield was 9,782 kg per year per cow. Fifty-three lactating dairy cows were selected as repeat breeders for the study. Our definition of a repeat breeder cow was similar to the definitions used in previous studies [2, 21–23]; the cows had no gross abnormalities of the reproductive tract, which was determined by rectal palpation and transvaginal ultrasonography (Sonoace 600 with a 7.5 MHz linear array transducer; Medison, Seoul, Korea), and had normal estrous cycles, but failed to conceive after repeated inseminations (2 or more times). All experiments were performed with the approval of the Animal Ethics Committee at the College of Veterinary Medicine, Chungbuk National University (Cheongju, Chungbuk, Korea).

The 53 lactating repeat breeder dairy cows were randomly assigned to three treatments as follows. (1) Cows, at random stages of the estrous cycle, received a CIDR device containing 1.9 g of P4 (CIDR™, InterAg, Hamilton, New Zealand) and 2 mg estradiol benzoate (EB) (SY Esrone; Samyang, Seoul, Korea; Day 0), an injection of 25 mg PGF2α (Lutalyse; Pharmacia & Upjohn, Puurs, Belgium) at the time of CIDR removal on Day 7 and 1 mg EB injection on Day 8. The cows then received TAI 30 h (Day 9) after the second EB injection using dairy semen of known fertility (TAI group, n=13). (2) Cows, at random stages of the estrous cycle, received the same hormonal treatments as in the TAI protocol. The cows then received TET on Day 16 using frozen-thawed blastocysts or morula embryos collected from Korean native cattle donors (TET group, n=13). A detailed description of the embryo production, embryo freezing/thawing and ET methods is provided below. (3) Cows in the luteal phase (8–13 days after estrus) received a 25 mg injection of PGF2α and AIE using dairy semen of known fertility (control group, n=27). All hormone injections were administered intramuscularly (im). AI was performed by a skilled artificial inseminator, and ET was performed by an experienced veterinarian, respectively.

Diagnosis of pregnancy was determined 60 days after AI or ET using both ultrasonography and rectal palpation. Pregnancy rate was defined as the percentage of treated cows that conceived from TAI, TET and AIE for the TAI, TET and control groups, respectively.

The characteristics of the selected repeat breeders were as follows. The mean (± SEM) numbers of lac-
tations for the TAI, TET and control groups were 1.8 ± 0.2, 2.8 ± 0.5 and 2.6 ± 0.3, respectively. The mean body condition scores (point scale from 1 to 5; Edmonson et al. [24]) were 4.2 ± 0.1, 4.1 ± 0.1 and 4.2 ± 0.1, respectively. The postpartum intervals (days) were 341.1 ± 26.9, 231.9 ± 9.1 and 274.7 ± 16.8, respectively. The numbers of previous inseminations were 3.6 ± 0.6, 2.9 ± 0.5 and 3.4 ± 0.2, respectively.

Embryo production, freezing/thawing and transfer

Two Korean native cows that were between Days 8 and 12 of their estrous cycles were superovulated with 28 mg of porcine follicle stimulating hormone (FSH) (Antrin-R10®; Kawasaki Mitaka Pharmaceutical, Tokyo, Japan) in twice daily im injections, with a gradual decrease over 4 days (5, 5, 4, 4, 3, 3, 2 and 2 mg). Along with the 5th and 6th injections of FSH, 25 mg and 15 mg PGF2α were administered, respectively. The cows received 200 µg gonadorelin (GnRH) (Fertagyl®; Intervet, Boxmeer, Netherlands) 12 h after the final (8th) injection of FSH. The cows were artificially inseminated 24 and 36 h after the final injection of FSH using commercial semen from a Korean native bull. The embryos were recovered 7 days after the first insemination by flushing with Dulbecco’s phosphate buffered saline (D-PBS, Gibco) supplemented with 0.1% polyvinyl alcohol (PVA, Sigma-Aldrich).

The recovered embryos were evaluated according to the International Embryo Transfer Society Manual [25] for the stage of development and quality. The morulae and blastocysts rated as 1 or 2 in quality were equilibrated in 1.8 M ethylene glycol in D-PBS supplemented with 0.5% bovine serum albumin (IFP9620; Research Institute for the Functional Peptides, Yamagata, Japan) for 10 min and were loaded individually into 0.25 ml straws (IMV, L’Aigle, France). The straws were placed into a chamber of a programmable freezer (CL863; Cryo-Logic, Victoria, Australia) that was precooled to −7 C. After 3 min, the straws were seeded, maintained for another 7 min and then cooled a rate of 0.3 C/min to −35 C before being immersed in liquid nitrogen. The straws were thawed for 10 sec in air followed by immersion for 20 sec in a 37 C water bath.

The frozen-thawed embryos were directly transferred into the uterine horn ipsilateral to the ovary containing the CL on Day 16.

Ovarian ultrasound scanning and the progesterone assay

The ovaries of the cows in the TET group were examined by transrectal ultrasonography before insertion of the CIDR device and the EB injection (Day 0), before the injection of PGF2α and removal of the device (Day 7) and 24 h (Day 8) and 216 h (Day 16) after the PGF2α injection in order to observe the changes in ovarian structures (follicles and CL). All visible follicles (antral diameter ≥4 mm) and CLs were measured for each cow. The ovaries of the cows were assessed via ultrasonography to determine the presence of preovulatory follicles on Days 7 and 8. Subsequent ovulation and concomitant formation of CLs were diagnosed when a preovulatory follicle disappeared and was confirmed by the appearance of a new CL on Day 16. Before ET, the cross-sectional diameters of CLs were measured.

Blood samples were collected from the tail vein on Days 0, 7, 8 and 16 for analysis of serum P4 concentrations for the cows in the TET group. After 2 h at 5 C, the samples were centrifuged at 2,500 × g for 10 min, and the sera were collected, immediately frozen and thereafter kept at −20 C until the assay. P4 concentrations were determined by fluorimunoassay (1234 Delfia Fluorometer; Wallac, Turku, Finland). The sensitivity of the assay was 0.25 ng/ml. The intra- and interassay coefficients of variation for the serum P4 analyses were 8.7, 8.5, 6.7, 8.5, 11.5 and 9.6% and 4.0, 5.4, 6.6, 6.9, 10.5 and 8.1% for the standard concentrations of 0, 0.31, 1.26, 3.14, 12.6 and 37.7 ng/ml, respectively.

Statistical analyses

The pregnancy rates of the control, TAI and TET groups were compared using Fisher’s exact test (SAS Version 8.1 [26]). The changes in serum P4 concentrations during the treatments for the cows in the TET group were analyzed using ANOVA (SAS Version 8.1). Mean values were compared using Duncan’s multiple comparison test. A probability level of P<0.05 was considered significant.

Results

The pregnancy rate was significantly higher (P<0.05) in the TET group (53.8%) than in the control (18.5%) and TAI (7.7%) groups (Table 1). The ultrasonographic observations demonstrated that
all repeat breeder cows in the TET group ovulated preovulatory follicles and concomitantly formed new CLs (mean ± SEM diameter of CLs: 21.1 ± 1.1 mm). The mean serum P4 concentration on Day 0 (6.0 ± 0.8 ng/ml) was maintained at a level that was comparable to Day 7 of the luteal phase (9.2 ± 1.6 ng/ml) in the repeat breeder cows of the TET group (Fig. 1). In this group, the mean serum P4 concentrations then dramatically decreased on Day 8 (1.4 ± 0.3 ng/ml, P<0.01) and subsequently increased on Day 16 (5.5 ± 0.5 ng/ml, P<0.01; Fig. 1).

### Discussion

This study evaluated the pregnancy rate following either a CIDR-based TAI or a TET protocol compared with that after a single PGF_2α injection and AIE in lactating repeat breeder dairy cows. Our data showed that the pregnancy rate was significantly higher in the TET group compared with either the control or TAI groups. In addition, the ultrasonographical observations and serum P4 analyses during the TET procedure demonstrated that all the repeat breeder cows ovulated the preovulatory follicles and concomitantly formed new CLs under the proper endocrine regulation.

Our results showing that the pregnancy rate after TET was greater than either that after AIE (control) or TAI indicates that induction of a timed ovulation and concomitant CL formation alone was not enough to increase the pregnancy rate; additional provision of a viable embryo may be needed to increase the fertility of lactating repeat breeder dairy cows. Interestingly, a previous study showed that the pregnancy rates following surgical ET of normal and repeat breeding dairy cows did not differ, indicating that the maternal environment of most repeat breeders was satisfactory for maintaining pregnancy [27]. Likewise, ET probably overcomes some of the problems related to oocyte maturation, fertilization and early embryo development and embryo passage into the uterus in repeat breeder cows. In an experiment similar to ours [17], the pregnancy rate was higher in ET lactating dairy recipients (45.8%) than in AI lactating dairy cows (33.0%) on day 46 after estrus, which is consistent with our results. In addition, the pregnancy rate following TET in the present study was similar to that (48.9%) following ET in repeat breeder dairy cattle using frozen-thawed embryos collected from Japanese black cows [19]. Moreover, Dochi et al. [18] demonstrated that ET using frozen-thawed in

### Table 1. Comparison of the pregnancy rates after a CIDR-based timed AI (TAI) or ET (TET) and after AI at estrus (AIE; control) in lactating repeat breeder dairy cows

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. of cows treated</th>
<th>No. of cows with AIE, TAI or TET</th>
<th>No. of cows pregnant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control¹</td>
<td>27</td>
<td>24</td>
<td>5 (18.5) a</td>
</tr>
<tr>
<td>TAI²</td>
<td>13</td>
<td>13</td>
<td>1 (7.7) a</td>
</tr>
<tr>
<td>TET³</td>
<td>13</td>
<td>13</td>
<td>7 (53.8) b</td>
</tr>
</tbody>
</table>

a,b Values with different superscripts within the same column are significantly different (P<0.05).

Control¹: cows received a 25 mg injection of PGF_2α during the luteal phase (8–13 days after estrus) and AIE. TAI²: cows received TAI 30 h (Day 9) after the second EB injection in the CIDR-based TAI protocol. TET³: cows received TET using frozen-thawed blastocysts or morula embryos on Day 16 in the CIDR-based TET protocol.

![Fig. 1. Serum progesterone concentrations (mean ± SEM) before CIDR insertion and EB injection (Day 0), before CIDR removal and PGF_2α injection (Day 7), before the second EB injection (Day 8) and before ET (Day 16) in the cows of the timed ET group. a,b: Values with different letters are significantly different between days within the same group (P<0.01).](image-url)
vitro produced blastocysts with or without an additional AI could increase the pregnancy rate in repeat breeder Holstein cattle. On the other hand, the pregnancy rate following TAI in the present study was lower than that (27.7%) following TAI using non-lactating repeat breeder dairy cows in a previous study [16]. The lower rate in this study may have resulted from various factors related to lactation status, postpartum interval, nutrition or herd management. Taken together, these results indicate that the ET technique using frozen-thawed in vivo or in vitro embryos may increase the pregnancy rate in repeat breeder dairy cattle.

Our ultrasonographic observations confirmed that all the cows in the TET group ovulated the preovulatory follicles and concomitantly formed new CLs; therefore, all the cows in this group received ET. In this study, the mean diameter of the CL on the day of ET (Day 16) was similar to the results of Garcia (28), in which the mean diameters of the CLs of pregnant and non-pregnant cows measured on the day of ET (7 days after standing estrus) were 20.0 and 20.6 mm, respectively. In addition, the serum P4 concentrations measured in this study showed that the endocrine system was correctly regulated during the TET procedure. Therefore, these results indicate that the CIDR-based TET protocol has an advantage that enables more cows per treated animals to be used as recipients and guarantees timely ovulation and concomitant CL formation. Other studies [29–31] have also shown that the P4-based TET scheme provides acceptable pregnancy rates (42.4–63.4%) in beef recipients.

In conclusion, the pregnancy rate following the CIDR-based TET protocol was higher than that following the CIDR-based TAI protocol. This suggests that provision of a viable embryo into the uterus in conjunction with timed ovulation and concomitant CL formation may be needed to increase the pregnancy rate in lactating repeat breeder dairy cows.

Acknowledgements

The authors thank Dr. Daehyun Chung (Department of Statistics, Chungbuk National University, Cheongju, Korea), for statistical analysis of the data.

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