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Optimization of *In Vivo* Embryo Production and Pregnancy following Embryo Transfer in Hanwoo Cattle

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ABSTRACT

Embryos formed *in vivo* were collected from 171 donors housed in *Chung Cheong Buk-Do Institute of Livestock and Veterinary Research* of the Chungbuk community during the years 2009～2012. We evaluated annual embryo collection, effect of follicle stimulating hormone (FSH), controlled internal drug release (CIDR) and prostaglandin (PG) administration to the donor for superovulation and controlling the estrus cycle, seasonal effects of embryo collection and compared the number of embryos recovered as per the collection days and pregnancy rate. In all, 1,243 embryos were collected from 118 donors with an average of 7.31 ± 5.35 embryos per donor, out of which 69.4% were transferable. Dosages of FSH required for inducing superovulation in various donors were compared. Average number of embryos collected from donors administered with 30 AU of FSH (7.13 ± 5.74 per donor) was not significantly different from that of donors who were given an injection of 24 AU of FSH (7.53 ± 4.91 per donor). However, the percentage of transferable embryos in the 30AU FSH-administered group (63.2 %, 449 of 711) was higher than that in the 24AU FSH-administered group (77.8%, 414 of 532). In the group of donors under a natural estrus cycle, the FSH dose administered did not influence the number of transferable embryos produced (7.49 ± 6.25 per donor for 30 AU of FSH vs 7.49 ± 4.92 per donor for 24 AU of FSH). However, in donors administered with CIDR and PG for controlling the estrus cycle, the FSH dose affected the average number of transferable embryos collected (4.25 ± 2.87 per donor for 30 AU of FSH vs 8.50 ± 6.36 per donor for 24 AU of FSH). We collected embryos from donors 6, 7 or 8 days after artificial insemination (AI). Results showed that the percentage of transferable embryos among those collected 8 days after AI was significantly higher than that among embryos collected 6 or 7 days after AI. Seasonal variations did not affect number of recovered embryos and pregnancy rates in natural estrus cycle and CIDR treatment groups (48.28% and 42.55%) but higher than pregnancy rate of frozen embryos (19.63%). These results indicated that administration of FSH beyond a threshold dose (at least 24 AU) has no beneficial effect on the production embryos and that collection of embryos 7～8 days after AI is optimal for embryo recovery. CIDR treatment induced superovulation in short term and had no influence on the natural estrus cycle. Finally, although good-quality embryos were transferred, freezing significantly reduced the pregnancy rates after transfer.

(Key words: *in vivo* embryo, follicle stimulating hormone (FSH), controlled internal drug release (CIDR), prostaglandin (PG), Hanwoo)

INTRODUCTION

Embryo transfer (ET) is a step in the process of assisted reproduction, in which embryos are transferred into the uterus of a female recipient with the intent to establish a pregnancy. This technique (which is often used in connection with *in vitro* fertilization [IVF]), can be applied in humans and animals, and the procedure has helped to lower the number of infertility cases across the nation and led to increased livestock production. One of the advantages of embryo transfer techniques in animals is that, it allows top-quality female livestock to have a greater influence on the genetic makeup of a herd or flock in much the same way as the artificial insemination has allowed greater use of superior sires. et also allows the continued train-

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ing and use of animals in competitions while producing offspring. The general epidemiological aspects of embryo transfer indicate that the transfer of embryos provides the opportunity to introduce desirable genetic material into populations of livestock, while greatly reducing the risk for transmission of infectious diseases.

In Korea, ET has been widely studied and industrially applied since the 80's. In early 2000, Hanwoo was produced on a large scale by using dairy cattle as recipients to conserve of economic loss because of quota system of milk. However, because most of the Hanwoo embryos were obtained from slaughterhouses, their genetic backgrounds were unknown and this had a negative impact on breeding (Kim *et al.*, 2006). This is in contrast to the *in vivo* produced embryos selectively collected from genetically well-identified donors. Therefore, ET using *in vivo* produced embryos has the potential to increase the number of genetically superior cattle within a relatively short period (Casida *et al.*, 1943; Christensen, 1991; Smith, 1984). However, since the production of transferable embryos *in vivo* is relatively more difficult and laborious than that *in vitro*, efficient treatment for inducing superovulation has become an important basic procedure.

It has been widely reported that treatments to induce superovulation often leads to low efficiency of conception, high costs of production and embryonic lethality during early developmental stages. Various direct factors, including nutrition, hormone sensitivity (Shea *et al*., 1984), type of hormone used (Quaresma *et al*., 2003; Kim *et al*., 1997; Staigmiller *et al*., 1992), ratio of hormones (Willmot *et al*., 1990), timing of administration (Goulding *et al*., 1990) and the amount (Pawlyshn *et al*., 1986; Donaldson, 1984) of hormone administered, affects the efficiency of superovulation. Indirect factors, including body condition score, breed, season (Sreenan *et al*., 1983) and timing of embryo collection (Bulsson *et al*., 1977), also affect the efficiency of superovulation. Therefore, standardization of procedures/conditions used for inducing superovulation and the collection of *in vivo*-produced embryos are required to improve the efficiency of conception, lower the cost of production and to minimize embryonic lethality. Recently, progesterone-releasing units called controlled internal drug release (CIDR) inserts have been widely used to induce superovulation. CIDR has the advantage that it is not affected by natural estrus cycle and induces superovulation with an efficiency that is comparable to that of directly administered hormones (Andrade *et al*., 2003). Therefore, optimization of CIDR inserts may lead to efficient superovulation.

The objective of this study was to optimize the procedures used for the *in vivo* production of transferable embryos Additionally, we evaluated the efficiency rate of the live offspring produced from transferred embryos.

MATERIALS AND METHODS

1. Animals

We recruited 171 Hanwoo donors from *Chung Cheong Buk-Do Institute of Livestock and Veterinary Research* and Chungbuk community. All donors are registered and genetic superiority guaranteed by *Korea Animal Improvement Association*. A total of 241 recipients with good corpus luteum and normal uterus, living under normal nutritional conditions, were selected.

2. Superovulation and Artificial Insemination (AI)

For the recipients under natural estrus cycle, 24 or 30 AU of FSH (Antorin, Kawasaki, Japan) was administered once every 12 hr for 3 days to 9 days after heat. Two days after the injection of FSH, PGF2α (Lutalyse, Pfizer, Belgium) was administered. gonadotrophin releasing hormone (GnRH) (Fertagyl, MSD, Belgium) was additionally administered 48 hr after $PGF_{2\alpha}$ injection. AI was performed 2 times after GnRH administration with a 12 hr interval between each AI procedures (Fig. 1 A). To analyze the effect of prostaglandin (PG), following the administration of 25 mg of $PGF_{2\alpha}$, 30 AU of FSH was injected after 11 days of natural estrus cycle, followed by $PGF_{2\alpha}$ again 2 days after FSH injection. GnRH (0.2 mg) was administered 48 hr after injecting $PGF_{2\alpha}$. Following this, AI was carried out using frozen semen, 2 times with a 12 hr interval (Fig. 1 B). Four days after the insertion of Progesterone Releasing Intravaginal Device (CIDR-PLUS, InterAG, New Zealand) into the vagina of cows using a CIDR injector, estrus cycle was induced by the administration of FSH (30 AU) for 3 days with an interval of 12 hr between each FSH injection. $PGF_{2\alpha}$ was administered 2 days after FSH injection. Three days later, CIDR-PLUS was removed. AI was carried out using frozen semen, 2 times with a 12 hr interval between the procedures, after the injection of GnRH (0.2 mg; Fig. 1 C).

3. Collection and Transfer of Embryos Produced *In Vivo*

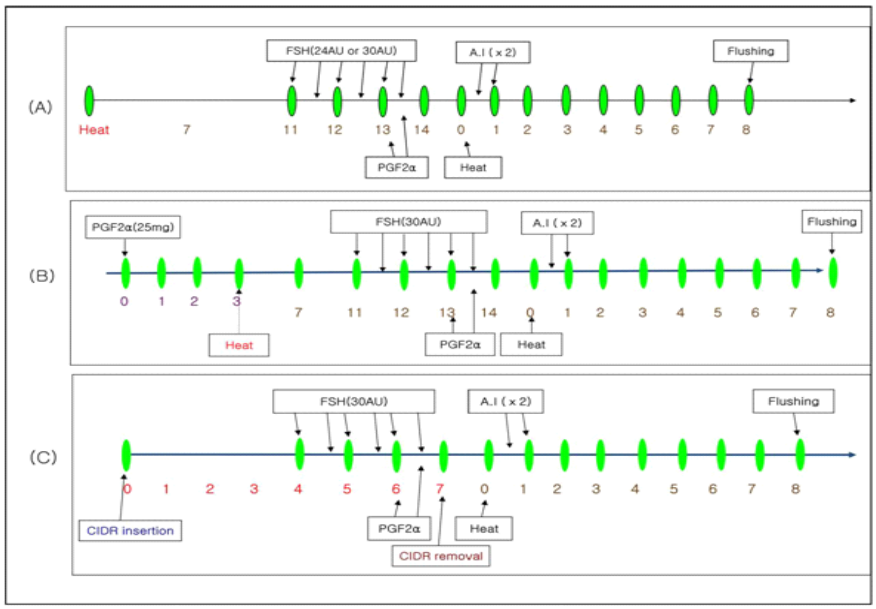

Fig. 1. Hormone treatment routine adapted for the superovulation of donors.
(A) Effects of the dosage of FSH administered for the superovulation of donors on *in vivo* embryo production (Experiment 1).
(B) Effects of prostaglandin (PG) administration on *in vivo* embryo production (Experiment 2).
(C) Effects of controlled internal drug release (CIDR) inserts on *in vivo* embryo production (Experiment 3).

Seven days after AI, embryos developed *in vivo* were collected using a Foley Catheter (Fujihira Industry Co., Japan). Typically, local anesthesia was induced by administering 2% Lidocaine (Jeil Pharm. Co. Ltd., Korea) (5～7 ml) between 1st and 2nd lumbar and embryos were flushed using a Foley Catheter. Collected embryos were evaluated and classified according to the developmental stage as morula, compact morula, early blastocyst, blastocyst, expanded blastocyst or hatching blastocyst. Evaluated embryos were transferred into uterus of recipients with a corpus luteum.

4. Cryopreservation and Thawing of Embryos

Embryos were frozen according to the procedures described in the user guide provided by the manufacturer of the liquid nitrogen freezer (CL8000; CryoLogic, Australia). Briefly, embryos were equilibrated for 7 min in bovine embryo freezing medium (IFP Co., Japan, Cat#: IFP9620) containing 1.8 M ethylene glycol and loaded into 0.25 mL straw. Following this, temperature was lowered from room temperature to −6℃ at a rate of cooling of 1 ℃/min. After 2 min, seed each straw by grasping the straw with a forceps dipped in liquid nitrogen (LN_2) at the air part and stay for 10 min in −6℃. Additional chilling was achieved by cooling at a rate of 0.3℃/min to −32 ℃. Subsequently, the embryos were stored in liquid nitrogen tank. Frozen embryos were thawed by exposing for 10 sec, followed by warming for 30 sec in a water bath maintained at 25℃.

5. Analysis of Pregnancy and Estimation of Body Weight of the Newborn Calf

Three months after embryo transfer, a rectal exam was performed to evaluate pregnancy. Body weight of the newborn calf was also estimated.

6. Statistical Analysis

Data were statistically analyzed using the generalized linear model of the Statistical Analysis System (SPSS 17.0), ANOVA and the chi-square test. Significance was determined using a Tukey's multiple range. $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

1. Annual *In Vivo* Production of Embryos

The number of embryos produced *in vivo* during the period of 2009～2012 from *Chung Cheong Buk-Do Institute of Livestock and Veterinary Research* and 171 donor of Chungbuk area are shown in Table 1. In, the year 2010, 288 embryos, produced *in vivo*, were collected with the highest recovery rate (81.8

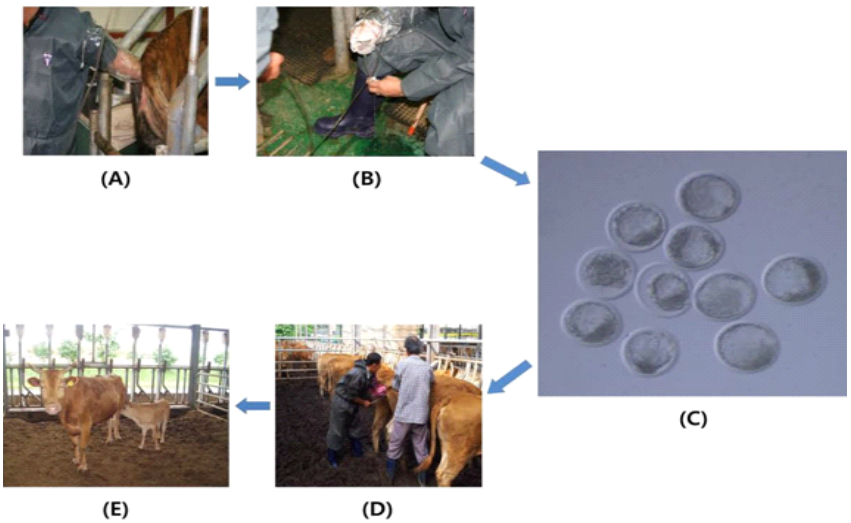

Fig. 2. Procedures for the *in vivo* production and transfer of embryos.
(A) Rectal (corpus luteum) palpation, (B) Flushing, (C) Transferable embryos, (D) Embryo transfer, (E) Offspring produced by embryo transfer.

%, 288/352). The recovery rate was lowest in 2012 (59.4%, 207/348). In total, 1,243 embryos were collected from 118 donors during the 4 year period with an average of 10.53 ± 7.31 embryos collected per donor. The total recovery rate of transferable embryos (69.4%) was substantially lower than that found in an earlier report (83.3%) (Kim *et al*., 2004). However, the average number of transferable embryos per donor (7.31 ± 5.35) was higher in our study than in that reported by others (6.5 ± 5.4 for CIDR-treated animals) (Son *et al*., 2006). Although these differences could not be readily explained, the involvement of factors such as feeding habits, nutrients, types/dose of hormones cannot be ruled out. Parity of donors did not influence the recovery of embryos (data not shown).

2. Effect of the Dose of FSH Administered on the Production of Embryos

We evaluated the effect of the dose of FSH administered on the induction of superovulation in donors (Table 2). Depending on the dosage of FSH administered (30 or 24 AU), donors were divided into two groups. A total of 63 donors administered with 30 AU of FSH produced 711 embryos, out of which 63.2% (449 of 711) were transferable. From the 55 donors administered with 24 AU of FSH, 532 embryos were collected and 77.8% (414 of 532) of the embryos were transferable. Average number of embryos collected per donor was higher 30AU FSH group (11.29 ± 8.56) than in 24AU FSH group. However, the average number of transferable embryos

Table 1. Annual *in vivo* production of embryos

Year	No. of donors	No. of embryos (mean ± S.D)		Transferable / total (%)
	Flushed/treated (%)	Total	Transferable	
2009	24/36 (66.7)	192 (8.00 ± 4.35)	136 (5.67 ± 3.42)	70.8
2010	33/50 (66.0)	352 (10.67 ± 5.89)	288 (8.73 ± 5.34)	81.8
2011	33/46 (84.6)	351 (10.64 ± 6.16)	232 (7.03 ± 4.21)	66.0
2012	28/39 (71.8)	348 (12.43 ± 10.88)	207 (7.39 ± 7.32)	59.4
Total	118/171 (69.0)	1,243 (10.53 ± 7.31)	863 (7.31 ± 5.35)	69.4

Table 2. Comparison of the dosage of FSH with the corresponding number of embryos produced *in vivo*

FSH dose	Flushed / treated (%)	No. of embryos (mean ± S.D)		
		Total	Transferable	Transferable of total (%)
24 AU	55/82 (67.1)	532 (9.67 ± 5.50)	414 (7.53 ± 4.91)	77.8
30 AU	63/89 (70.8)	711 (11.29 ± 8.56)	449 (7.13 ± 5.74)	63.2

collected per donor from 30AU FSH and 24AU FSH groups were not significantly different (30AU FSH: 7.13 ± 5.74, 24AU FSH: 7.53 ± 4.91). Donaldson (1984) reported that administration of FSH exceeding 50 mg reduced both the total number of embryos collected and the number of transferable embryos. However, Lauria *et al*. (1983) reported that administration of more than 46.5 mg of FSH increased the total number of embryos. Together with these reports, our findings clearly show that excessive administration of FSH may not have beneficial effects on the production of embryos *in vivo*.

3. Effect of CIDR and PG on the Production of Embryos

Table 3 shows efficiency of *in vivo* embryo production under natural estrus cycle, following CIDR treatment and PG administration, or after the administration of PG. Although the number of embryos collected from natural estrus group was lower than that from 24AU FSH group, the number of transferable embryos collected showed a reverse trend. Out of 29 embryos collected from 3 donors administered with CIDR and PG, 17 were transferable (58.6%) in 24AU FSH group. However, when we administered 30 AU of FSH to donors treated with CIDR and PG, the number embryos collected was 4.75 ± 3.10 per donor and the number of transferable embryos was 17 (89.5%). In PG group, 105 embryos were collected from 13 donors. The average number of embryos collected per donor was 10.50 ± 6.36 and the number of transferable embryos was 65 (61.9%). Taken together, donors group treated with CIDR and PG in combination with 24 AU of FSH showed highest rate of embryo recovery (14.50 ± 2.12) and produced more transferable embryos per donor (8.50 ± 6.36). Our results are similar to that reported by Gouveia *et al*. (2002) who collected 10.5～12.6 embryos per donor (7.4～9.6 transferable embryos per donor) from CIDR and Folltropin-V treated animals. Several reports have shown that the efficiency of *in vivo* embryos production in CIDR-treated donors were not significantly different from that of donors under natural estrus cycle (Lafri *et al*., 2002: Andrade *et al*., 2003). Therefore, these results suggest that CIDR treatment is beneficial for inducing superovulation in short term and does not affect the natural estrus cycle.

4. Efficiency of Embryo Recovery when the Embryos were collected at Different Time Points following Artificial Insemination.

Table 4 shows the efficiency of embryo recovery at various time points after artificial insemination. We collected embryos

Table 3. Comparison of natural estrus cycle with CIDR + PG and PG treatments employed for producing embryos

Treatment	FSH dose	Flushed/treated (%)	No. of embryos (mean ± S.D)		
			Total	Transferable	Transferable/total (%)
Nature	24 AU	53/79 (67.1)	503 (9.49 ± 5.51)	397 (7.49 ± 4.92)	78.9
	30 AU	49/69 (71.0)	587 (11.98 ± 9.07)	367 (7.49 ± 6.25)	62.5
CIDR+PG	24 AU	2/3 (66.7)	29 (14.50 ± 2.12)	17 (8.50 ± 6.36)	58.6
	30 AU	4/7 (57.1)	19 (4.75 ± 3.10)	17 (4.25 ± 2.87)	89.5
PG	30 AU	10/13 (76.9)	105 (10.50 ± 6.36)	65 (6.50 ± 3.41)	61.9

Table 4. Effects of *in vivo* embryo production according to flushing days after artificial insemination

Flushing day	No. of donors	No. of embryos	Degenerated	No. of embryos (%)					
				M[1)]	CM[2)]	EB[3)]	B[4)]	EX[5)]	Sub total
6	6	83	34 (41.0)	31 (37.4)	18 (21.7)				49 (59.0)
7	49	548	155 (28.3)	98 (17.9)	178 (32.5)	58 (10.6)	57 (10.4)	2 (0.4)	393 (71.7)
8	63	612	191 (31.2)	21 (3.4)	118 (19.3)	92 (15.0)	182 (29.7)	8 (1.3)	421 (68.8)

[1)] morula, [2)] compact morula, [3)] early blastocyst, [4)] blastocyst, [5)] expanded blastocyst.

6, 7, or 8 days after artificial insemination. On the sixth day after AI, 83 embryos were collected from 6 donors, out of which 59.0% (49 of 83) were transferable. On the 7th day after AI, 548 embryos were collected from 49 donors, out of which 71.7% (393 of 548) were transferable. On the 8th day after AI, 612 embryos were collected from 63 donors and 68.8% (421 of 612) of these embryos were transferable. These results indicated that although more embryos and higher number of morula stage embryos were collected on the 7th day after insemination than on the 8th day, there were no significant differences in total embryo recovery and the quality of embryos collected on 7th and 8th days after AI. Our results also indicated that recovery rate embryos collected after 7～8 days after AI was significantly higher than that collected 6 days after AI. Therefore, embryo collection performed 7～8 days after AI leads to optimal for recovery.

5. Seasonal Effects on Embryo Production

As shown in Table 5, there were are no significant seasonal variations in embryo recovery. Average number of embryos collected per donor in spring (10.09 ± 5.39), summer (11.00 ± 6.51), fall (10.20 ± 11.06) and winter (10.14 ± 4.95) were similar. Various groups have reported differing results regarding the seasonal effects on embryos collection. Some reports suggested that seasons affected the collection of embryo number (Greve *et al*., 1979; Hasler *et al*., 1983; Sreenan, 1983; Almeida, 1987), while others suggested that there was no seasonal variation in embryo collection (Crister *et al*., 1980; Massey and Oden, 1984; Darrow *et al*., 1982). It is likely that these differences stemmed from regional differences, as well as differences in farm management and nutrition.

6. Pregnancy Rate

We compared the ability of freshly collected and cryopreserved embryos to contribute to pregnancy. There were no significant differences in the pregnancy rates between natural estrus (48.28%) and CIDR-treated (42.55%) groups. Compared to fresh embryos, transfer of cryopreserved embryos led to sig-

Table 5. Seasonal differences on *in vivo* embryo production

Seasons	No. of donors	No. of recovered (mean ± S.D)			
		Oocytes unfertilized	Transferable embryos	Embryo degenerated	Sub total
Spring (Mar-May)	32	29 (2.90 ± 3.63)	236 (7.38 ± 4.52)	58 (1.81 ± 2.49)	323 (10.09 ± 5.39)
Summer (Jun-Aug)	54	110 (4.78 ± 4.66)	382 (7.07 ± 4.43)	102 (1.92 ± 2.70)	594 (11.00 ± 6.51)
Autumn (Sep-Nov)	25	21 (2.10 ± 2.28)	195 (7.80 ± 7.99)	39 (1.56 ± 2.69)	255 (10.20 ± 11.06)
Winter (Dec-Feb)	7	2 (2.00 ± 0.00)	50 (7.14 ± 4.74)	19 (2.71 ± 1.80)	71 (10.14 ± 4.95)

Table 6. Pregnancy following transfer of fresh or cryopreserved embryos

Embryo	Synchronization	No. of recipients	No. of recipients delivery	Delivery rate(%)
Fresh	Nature	87	42	48.28[b]
	CIDR+PG	47	20	42.55[b]
	Subtotal	134	62	46.27[b]
Frozen-thawed	Nature	107	21	19.63[a]

[ab] Values with different susperscripts within the same column significantly differed ($p<0.01$).

nificantly lower (19.63%) pregnancy rates. Heyman (1985) reported that freezing procedure damages 10～40% of the cells in embryos. There were no significant differences in body weights of calves between groups (data not shown).

In conclusion, our results suggest that administration of FSH exceeding a threshold dosage (at least 24 AU) has no beneficial impact on the production embryos and embryo *in vivo*. Embryo collection 7～8 days after AI is optimal for embryo recovery. We did not observe any seasonal differences in the number of embryos collected. We showed that CIDR treatment induces superovulation with no influence of natural estrus cycle. Finally, transfer of cryopreserved embryos led to reduced pregnancy rates.

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