Effect of Estrus Synchronization Protocols and Gonadotropin Releasing Hormone Treatments on the Pregnancy and Fetal Loss Rate after Transfer of Korean Native Cattle Embryos to Holstein Recipients

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ABSTRACT

This study examined pregnancy and fetal loss rates according to different estrus synchronization protocols and injection of gonadotropin releasing hormone (GnRH) after transfer of Korean Native Cattle embryos to Holstein recipients. In Experiment 1, recipients received no treatment (Control, $n = 119$); two injections of prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$) 11 days apart (PGF group, $n = 120$); GnRH (day 0)-$PGF_{2\alpha}$ (day 7)-GnRH (day 9) (Ovsynch group, $n = 120$); and CIDR (day 0)-$PGF_{2\alpha}$ and CIDR removal (day 7)-GnRH (day 9) (CIDR group, $n = 110$). In Experiment 2, the control group was received no treatment of GnRH. The treatment groups were received GnRH at embryo transfer (ET) (day 0), 7 days later, 14 days later, ET and 7 days later, 7 and 14 days later, or ET, 7 and 14 days later. Recipients were assigned to treatment randomly and received two *in vitro* produced blastocysts. Pregnancy was diagnosed at day 60 by palpation per rectum. Fetal loss to term was determined by palpation every 90 days thereafter. In Experiment 1, the pregnancy rate in the CIDR group (59.1%) were higher than in the Control group (42.0%) ($p<0.01$); fetal loss rates were similar for all groups (12.0 to 18.5%). In Experiment 2, the pregnancy rate in Day 0+7+14 group was higher (60.2%) than the control (40.2%) ($p<0.01$) and resulted in a lower fetal loss ($p<0.05$) than the control (4.6 vs. 11.4%). There were no significant difference between other treatment and the control ($p>0.05$). These results show that pregnancy rates of bovine embryos can be enhanced by CIDR insertion or GnRH 3× treatment. Additionally, fetal loss may be reduced with GnRH treatment after ET.

(Key words : embryo transfer, estrus synchronization, GnRH, CIDR, pregnancy and fetal loss rates)

INTRODUCTION

In vitro production of Korean Native Cattle (KNC) embryos and transfer to Holstein recipients has been promoted for proliferation of KNC and increased meat production. A commercial company using this technique was established, and the production of *in vitro* embryos and use of embryo transfer (ET) has increased every year since 1993 (Hwang *et al.*, 1993). There has been much progress with *in vitro* embryo production, but low pregnancy rates and high fetal losses have limited the success of such procedures (Schmidt *et al.*, 1996). The pregnancy rate of 20 studies over a decade was only 30±10% with *in vitro* embryos (Peterson and Lee, 2003), considerably lower than artificial insemination (AI) (Pursley *et al.*, 1995) or using *in vivo*- produced embryos (Schmidt *et al.*, 1996). Furthermore, fetal loss in recipient that received *in vitro*- produced embryos was 22 to 47% (Hasler *et al.*, 1995; Park *et al.*, 2005), much greater than that of AI or transfer of *in vivo* produced embryos (Santos *et al.*, 2004).

The success of ET depends on factors associated with the embryo, recipient, and interaction between them (Spell *et al.*, 2001). Factors related to the embryo that determine success include whether or not the embryo was frozen, the quality of the embryo, and the developmental stage (Hasler, 2001). The recipient factors that determine success include the corpus luteum (CL) diameter, uterine infection, nutrition, hormonal balance, and environment (Broadbent *et al.*, 1991; Lukaszewska and Hansel, 1980; Putney *et al.*, 1989; Spell *et al.*, 2001). Synchrony of the stage of the estrous cycle between donor and recipient is particularly important (Misra *et al.*, 1999). There are

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many estrous/ovulation synchronization protocols, including $PGF_{2\alpha}$, controlled intravaginal drug release (CIDR containing 1.38 to 1.9g progesterone), progesterone-release intravaginal device, and the Ovsynch and Presynch protocols (Martinez *et al.*, 2002; Pursley *et al.*, 1995; Stevenson *et al.*, 1987; Yavas and Wallon, 2000). There is no recommended synchronization protocol for transfer of *in vitro*-produced KNC embryos to Holstein cattle, and no reports on fetal losses with different estrous synchronization protocols.

In addition, hormonal imbalances, such as low concentrations of plasma progesterone, pose major challenges to improvement of pregnancy rates and reduction of fetal losses (Bulman and Lamming, 1978; Wilmut *et al.*, 1985). In addition, progesterone plays a major role in stimulating the production of several endometrial proteins, growth factors and the secretion of interferon-τ (Garrett *et al.*, 1988; Mann and Lamming, 2001). Exogenous progesterone, human chorionic gonadotropin (hCG), gonadotropin releasing hormone (GnRH), and its agonist have been used to regulate CL function and to increase progesterone concentration (Macmillan *et al.*, 1986; Sianangama and Rajamahendran, 1992; van Cleeff *et al.*, 1996). However, exogenous progesterone has side effects like suppressing LH and inducing follicular turnover (Bo *et al.*, 1995). GnRH and hCG are equally effective in inducing accessory CL formation (Schmitt *et al.*, 1996). However, repeated use of hCG can induce antibody formation that neutralizes the hCG molecule and dramatically reduces binding to its receptors (Sundby and Torjesen, 1978). Therefore, we chose GnRH as the drug of choice to improve pregnancy rates in this study.

Thus this study was performed to investigate the effects of four different estrous synchronization protocols (Experiment 1) and different GnRH treatments after ET (Experiment 2) on pregnancy and fetal loss rates after transfer of *in vitro*-produced KNC embryos to Holstein recipients.

MATERIALS AND METHODS

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

1. *In Vitro* Maturation

The ovaries of KNC were obtained at a slaughterhouse and transported within 5 h at 25 to 28℃ to the laboratory in 0.9% saline supplemented with 25 μg/ml gentamicin. Cumulus oocyte complexes (COCs) were obtained by aspiration from 2 to 6 mm follicles. Only COCs with compact cumulus layers and evenly granulated ooplasm were selected. The COCs were washed three times in tyrode's albumin lactate pyruvate (TALP) medium (Bavister *et al.*, 1983) supplemented with 25 mM HEPES and 3 mg/ml bovine serum albumin (BSA). Groups of 15 COCs were placed in 50 μL drops of TCM199 (Gibco, USA) supplemented with 0.2 mg/ml pyruvate, 10% fetal bovine serum (FBS), 1 μg/ml follicle stimulating hormone, 10 μg/ml luteinizing hormone (LH), and 1 μg/ml Estradiol-17β under mineral oil for 20 h at 39℃ with an atmosphere of 5% CO_2 in air and maximum humidity.

2. *In Vitro* Fertilization

Frozen Korean Native bull semen was thawed for 1 min in 37℃ water and placed on the top of a discontinuous Percoll density gradient composed of 2 ml 45% Percoll over 2 ml 90% Percoll. The sample was centrifuged for 20 min at 700 × g at room temperature. The spermatozoa collected at the bottom of the fraction were washed in sperm-TALP, which consisted of TALP medium supplemented with 3 mg/ml BSA (fraction V), for 10 min at 350 × g. Spermatozoa were counted in a hemocytometer and diluted in an appropriate volume with sperm-TALP to give a concentration of 25×10^6 spermatozoa/ ml.

After maturation, the COCs were washed three times in fer-TALP, which consisted of TALP medium supplemented with 6 mg/ml BSA and 2 μg/ml heparin. Groups of 15 COCs were placed in 48 μl drops of fer-TALP. A 2 μl aliquot of prepared spermatozoa suspension was added to each fertilization drop to obtain a final concentration of 1×10^6 spermatozoa/ml. Dishes were incubated for 20 h at 39℃ with 5% CO_2 in the air and maximum humidity.

3. *In Vitro* Culture

After fertilization, the presumptive zygotes were stripped of cumulus cells and washed three times before they were transferred to 20 μl drops of Charles Rosenkrans 1 amino acid (CR1aa) medium (Rosenkrans *et al.*, 1993) with 3 mg/ml BSA. On day 3 of culture, the culture medium was exchanged to CR1aa medium supplemented with 10% FBS. All cultures were carried out under mineral oil at 39℃ with 5% CO_2 in air and maximum humidity. On Day 7 of IVC, the middle blastocysts of excellent or good quality (Linder and Wright, 1983) were used for ET.

4. Embryo Transfer

This study was conducted on 21 farms located in Kyongbuk

province, Korea. Holstein heifers (n = 1,519) between 12 and 14 months of age and weighing in over 270 kg were used as recipients.

In Experiment 1, heifers (n = 469) at one dairy farm were assigned randomly to control and three synchronization treatment groups. Recipients in the control group (n = 119) displayed signs of estrous naturally. For recipients in the treatment groups, injection was initiated at random stages of estrous cycle (day 0). The PGF group (n = 120) was synchronized with two injections of 25 mg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, lutalyse, Pharmacia, Belgium) on day 0 and 11. The Ovsynch group (n = 120) was synchronized with 0.01 mg GnRH (Receptal, Intervet, Holland) on day 0, followed by $PGF_{2\alpha}$ on day 7 and GnRH on day 9. The CIDR group (n = 110) was received 1.9g progesterone (CIDR-plus, InterAg, New Zealand) insert on day 0. The recipients was removed a progesterone insert and an injection of $PGF_{2\alpha}$ on day 7. On day 9, the recipients received an injection of GnRH.

In Experiment 2, a total of 1,050 heifers raised at twenty dairy farms were used. From each farm, the heifers were assigned randomly to control or one of six treatment groups. Each group in Experiment 2 contained 150 recipients. The estrous of recipients in Experiment 2 were synchronized with two injection of $PGF_{2\alpha}$ on 11 day apart. The control group was not injected with GnRH. The day of ET (Day 0), day 7 (Day 7), and day 14 (Day 14) groups were each given a single injection of GnRH on those days. Day 0+7 and Day 7+14 groups were given single injections of GnRH on days 0 and 7, and on days 7 and 14. Day 0+7+14 group was given single injections of GnRH on days 0, 7, and 14.

Heifers with observed signs of estrous were selected as recipients. On day 7 after estrous detection, the selected recipients were palpated per rectum for the presence of a CL. Only heifers with CL were used as ET recipients. Two blastocysts were transferred non-surgically to the central portion of the uterine horn ipsilateral to the ovary with a CL. The same veterinarian performed all transfers.

5. Pregnancy Diagnosis

Pregnancy was diagnosed 60 day after ET by palpation per rectum. Fetal loss was calculated as the same method in pregnancy diagnosis from 60 day to term of gestation every 90 days.

6. Statistical Analysis

Data on the pregnancy and fetal loss rates of Experiment 1 were analyzed by the χ^2-test. Data for the pregnancy and fetal loss rates of Experiment 2 were analyzed by the General Linear Models (GLM) procedure with the Statistical Analysis System (SAS; Cary, NC, USA) after arcsine transformation of the percent rates of pregnancy and fetal loss. Treatment means were compared by t-tests. P-values < 0.05 were considered to be significant.

RESULTS

Experiment 1: Effect of Estrus Synchronization Protocols

The pregnancy rate for Control group was 42.0% (Table 1), and PGF and Ovsynch group had statistically similar rates. However, the pregnancy rate of CIDR group was 59.1%. This was significantly greater than that of Control group (p<0.01). The fetal loss rates ranged from 12.0 to 18.5% and were not different among Control and treatment groups.

Experiment 2: Effect of GnRH

The pregnancy rate of the controls was 40.2% (Table 2), and treatment protocols for all single and double GnRH protocols had no significant effect on pregnancy rates, which ranged from 40.2 to 47.7%. However, Day 0+7+14, treated with GnRH injections on Days 0, 7, and 14 had a pregnancy rate of 60.2%, which was significantly higher than that of Control group (p< 0.01). The fetal loss rate in Day 0+7+14 was 4.6%, which was significantly lower than that of Control group (p<0.05).

DISCUSSION

Several protocols have been developed to improve the herd

Table 1. Effects of estrus synchronization protocols on the rates of pregnancy and fetal loss in Holstein heifers following the transfer of Korean Native Cattle embryos produced *in vitro*

Group	N	Pregnancy rate (%)	*P*	Fetal loss rate (%)	*P*
Control	119	42.0	–	12.0	–
PGF	120	45.8	0.08	15.2	0.59
Ovsynch	120	50.8	0.06	13.1	0.52
CIDR	110	59.1	0.01	18.5	0.31

P values for PGF, Ovsynch, and CIDR-PGF-GnRH treatment groups were based upon comparison with the control.

Table 2. Effects of post-ET GnRH treatments on the rates of pregnancy and fetal loss in Holstein heifers following the transfer of Korean Native Cattle embryos produced *in vitro*

Group	N	Pregnancy rate (%)	*P*	Fetal loss Rate (%)	*P*
Control	150	40.2±3.1	–	11.4±3.6	–
Day 0	150	46.6±3.1	0.14	7.7±3.3	0.22
Day 7	150	40.2±2.9	0.40	13.9±4.0	0.32
Day 14	150	45.9±2.9	0.15	4.6±2.8	0.06
Day 0+7	150	47.7±3.0	0.08	6.6±3.1	0.16
Day 7+14	150	44.1±3.9	0.27	7.0±3.1	0.18
Day 0+7+14	150	60.2±2.9	0.01	4.6±2.0	0.05

P values for Day 0, Day 7, Day 14, Day 0+7, Day 7+14 and Day 0+7+14 treatment groups were based upon comparison with the control.

pregnancy rates by synchronizing estrus and improving embryo survival. Bovine estrus can be synchronized with different drugs such as progesterone, estradiol, progesterone-estradiol combinations, $PGF_{2\alpha}$, GnRH, and GnRH agonist (Martinez *et al.*, 2002; Pursley *et al.*, 1995; Stevenson *et al.*, 1987; Yavas and Wallon, 2000). The pregnancy rate of recipients with natural estrous is similar to that of $PGF_{2\alpha}$-treated animals, but synchronization with progesteron insert has the highest pregnancy rate after AI or ET (Bényei *et al.*, 2005; Wilmut *et al.*, 1985). In fresh and frozen ETs, the pregnancy rates of recipients with natural or with $PGF_{2\alpha}$-induced estrous is 24.0 to 41.0% (Bényei *et al.*, 2005; Dochi *et al.*, 1998), and progesterone insert-induced estrous ranged from 41.7% to 62.5% (Bényei *et al.*, 2005). In this study, the pregnancy rate in CIDR group, 59.1%, was significantly higher than that of the control group. The combination of progesterone insertion with GnRH injection increases estrous behavior and synchronizes ovulation (Gouveia *et al.*, 2002; Takaaki *et al.*, 2004). These results suggest that progesterone insertion can be used to precisely time the ovulation so that it is synchronized with the developmental state of the *in vitro* produced embryo. This will contribute to the success of ET.

Hormonal balance, including appropriate concentrations of plasma progesterone, is a major factor for the maintenance of pregnancy (Wilmut *et al.*, 1985). The increase of plasma progesterone concentration during the early luteal phase post-AI or post-ET has beneficial effects on pregnancy (Bulman and Lamming, 1978). Exogenous progesterone, hCG and GnRH are useful in inducing higher concentrations of progesterone by inducing accessory CL (Schmitt *et al.*, 1996; Sianangama and Rajamahendran *et al.*, 1998; van Cleeff *et al.*, 1996). The administration of GnRH on day 5 or 6 of the estrous cycle induces the acute LH release, the ovulation of dominant follicles, and formation of accessory CL that results in higher progesterone concentrations (Rajamahendran *et al.*, 1998). The pregnancy rate increases and early embryo loss decreases in accordance with the increase in plasma progesterone concentration (Nishigai *et al.*, 1998). In this study (Table 2), the single injection with GnRH on day 0, 7 and 14 or double injection with GnRH on days 7 and 14 after ET did not increase pregnancy rates. However, treatments on days 0, 7, and 14 significantly increased them. MacMillan *et al.* (1986) also reported that the pregnancy rate improved when GnRH treatment was given between days 11 and 13 but not between days 7 and 10 after AI. While some have reported pregnancy rates of multiple treatments with hCG did not differ from single treatments (Christie *et al.*, 1979), but this study found that three administrations of GnRH significantly increased the pregnancy rate compared to single or double treatments. From the results in this and previous studies (Macmillan *et al.*, 1986; Sheldon and Dobson, 1993), the timing of the GnRH treatment post-ET or post-AI may influence the pregnancy rate.

To reduce economic losses in ET, it is necessary to focus not only on enhancing pregnancy rates, but also minimizing embryonic and fetal losses in cattle. *In vitro*-produced blastocysts have higher fetal loss rates (Hasler *et al.*, 1995) and in some cases, increased rates of abnormalities, such as hydroallantois of recipients (van Wagtendonk *et al.*, 1998). Fetal loss in recipients that received *in vitro* embryos was 22 % to 47 % (Hasler *et al.*, 1995; Park *et al.*, 2005), much higher than 11% to 13% with AI and *in vivo* embryos (Santos *et al.*, 2004). Several factors affect pregnancy losses in cattle, such as the quality of the embryo itself, inadequate uterine environment, and infections (Santos *et al.*, 2004). The early death of embryos occurs at a high frequency from 9 to 17 days after ET, and low concentrations of plasma progesterone are associated with early embryonic loss (Bulman and Lamming, 1978; Lukaszewska and Hansel, 1980). Enright *et al.* (2000) also found that most of the embryonic losses occur before day 100 of gestation. Pregnancy loss rates do not differ among cows with AI following different estrous-inducing protocols (López-Gatius *et al.*, 2002). In our study, there were no differences in fetal loss

between recipients synchronized with three different protocols or naturally occurring estrus. However, a series of single injections with GnRH on days 0, 7, and 14 significantly reduced fetal loss rates. The reduction in fetal loss may be due to GnRH induction of a new accessory CL, resulting in a higher plasma progesterone concentration (Rajamahendran *et al.*, 1998). The higher progesterone levels stimulate the secretion of interferon-τ (Mann and Lamming, 2001). It also inhibits the secretion of $PGF_{2\alpha}$ and the luteolytic cascade around day 16 to 18 of the estrous cycle.

In conclusion, the pregnancy rates of *in vitro* produced bovine embryos can be enhanced by an estrous synchronization protocol using progesterone insert on day 0, $PGF_{2\alpha}$ on day 7, and GnRH on day 9. The serial injection with GnRH on days 0, 7, and 14 after ET are also effective in increasing pregnancy rates and reducing fetal loss.

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