Effects of Number of Embryos Transferred, the State of Uterus and Ovary on Pregnancy Rates, and Artificial Induction of Twins with Hanwoo IVF Embryos

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ABSTRACT

The purpose of this experiment is to compare the pregnancy rate (PR) according to the state of the ovaries and uterus, according to the number of embryos transferred from cows and heifers and to investigate the method of artificial twin induction with Hanwoo *in vitro* fertilized (IVF) embryos by embryo transfer (ET). Looking at the PR according to the condition of the ovaries and uterus, the result was not influenced by the condition of the ovaries, but was significantly influenced by the state of the uterus. The PR according to the number of embryos transferred from cows was 36.8%, 53.0%, 50.5% for 1, 2, and 3 embryos respectively, and although there was a higher frequency of twin calves with 3 embryos than with 2, the calving rate was the highest with 2 embryos. In case of heifers, the transfer of 1 embryo showed the best pregnancy and calving rate, and although the PR was similar with 2 embryos (67.7 versus 66.4), in case of 2 embryos transferred there was high frequency of embryonic loss(6.1%) occurred when a cow was diagnosed at 28 and 53 d after ET, total loss (21.3%; sum of fetal death, abortion and stillbirth after pregnant diagnosis at 60 day).

(Key words : PR, IVF, ET, Twins)

INTRODUCTION

In spite of the potential of ET itself, there is a tendency to avoid this method due to elements such as the low PR, high cost of embryo production, and malformation. However, the ability to select and reproduce embryos with superior genetic qualities through this process is unrivaled, and it can be used as a method to resolve the low PR in dairy cattle and beef cattle. In addition, the artificial induction of twins is a technique attracting attention as it increases the breeder's income, and adds the individuals with outstanding genetic traits.

This research was conducted with the purpose of exploring ways to increase the results of ET in the actual use of Hanwoo (Korean native cattle) IVF embryos with Holstein cows (multiparae), heifers (nulliparae) and Hanwoo cows. That is, to discover a suitable number of embryos that positively influences the pregnancy rate of ET, a method of artificial induction of twins. Accordingly, the transfer of fresh embryos is an important tool to increase the probability of pregnancy of lactating Holstein cows because it can bypass the negative effects of milk production and low P4 on the early embryo (Demetrio *et al*., 2007). Several studies reported that the association between plasma progesterone concentration and PR in cattle (Binell *et al*., 2001; Thatcher *et al*., 2001; Thatcher *et al*., 2002). Higher P4 concentrations have been associated with incrased embryo development and the capacity of the conceptus to secrete interferon-τ (Mann *et al*., 1999) and higher PR (Baruselli *et al*., 2001; Fuentes S and Fuente J, 1997; Marques *et al*., 2003; Santos *et al*., 2000). Other studies, however, did not demonstrate the effect of increased P4 concentrations on PR (Hasler *et al*., 1980; Nogueira *et al*., 2002; Spell *et al*., 2001; Tríbulo *et al*., 1997). In addition, there is the added purpose of comparing the PR according to the state of the uterus and ovaries(i.e. corpus luteum; CL), in order to reveal how the state of these affects the pregnancy rate.

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MATERIALS AND METHOD

In Vitro Embryo Production

After selecting COCs with clear cytoplasm and well attached cumulus cells from the follicular fluid that has extracted egg follicles from slaughter house ovaries, these were put in the maturation medium, a mixture of EGF 30 ng/ml with 5% FBS in Medium 199 (Gibco, 123-40) and allowed to incubate for 20~22 hours at 5% CO_2, 38.5℃, a maximum humidity in air. The maturation-incubated COCs were washed 3 times with m-BO (Yoon *et al*., 2009) and put in the fertilizing medium.. The fertilizing medium used F-TALP, and it was made up of 114 mM NaCl, 3.2 mM KCl, 25.0 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 10.0 mM lactate (sodium salt), 2.0 mM $CaCL_2$, 0.5 mM $MgCl_2$, 0.2 mM pyruvate, 5.0 mM glucose, 6.0 mg FAF-BSA (fatty acid free BSA), 20.0 μM penicillamine, 10 μM hypotaulin, 1.0 μM epinephrine, 20 μg/ml heparin, 1 μl/ml gentamycin in purified water. The frozen semen was thawed at 35℃ water for 30s and the semen was separated through centrifugation at 800 rpm in m-BO 2 times for 8 minutes each time, and then put in the fertilizing medium to a concentration of 2×10^6/ml. After a fertilization time of 18~20 hours, presumptive zygotes were washed 3 times in 5% FBS, then they were cultivated for 26~28 hours with 50% of the maturation medium replaced with the same maturation medium for cultivation into a zygote. After this cultivation, only the zygotes that are beyond the 4 cell stage were selected and cultivated in the maturation medium changed to 0.5% BSA only in Medium199 until it developed into the blastocyst stage (cultured for 6~8 d after IVF) which can then be transferred. The culture medium was replaced by 50% with the same medium every 48 hours, and only the embryos that developed to blastocyst stage were selected and used for transfer.

Artificial Estrus Induction

As the need arises, estrus was artificially induced in heifers and cows by 2 does of $PGF_2\alpha$ (Lutalyse, Upjohn, Belgium) i.m. with 11 days apart, and 2 days after 2^{nd} $PGF_2\alpha$ i.m., recipients were treated with gonadotrophin releasing hormone (GnRH) i.m. for inducing ovulation.

Embryo Transfer

ETs carried out non-surgically and embryos were placed deep in the uterine horn ipsilateral to CL (contralateral to CL for artificial induction of twins in Hanwoo cow with AI) 7 days after estrus (9 days after secondary $PGF_2\alpha$ injection). All transfers were conducted by the same practician. The transfer procedure took 3~10 minutes, and recipient cows were given 250 mg of FM (Flunixin Meglumin; Fluximin, Bomac New Zealand) i.m. just before the transfer.

Pregnancy Diagnosis

Pregnancy was diagnosed at Day 28 and Day 50 after ET by transrectal ultrasonography (Tringa Linear, Esaote Piemedical). Pregnancy was also determined on the basis (Kastelic *et al*., 1991) of the uterine cavity and the presence of uterine luminal fluid, an embryonic vesicle, fetus and heart beat, and palpations of the middle uterine artery.

Comparison of PR according to the State of Uterus and Ovaries

Recipient cows selected were dairy cows from farms which were observed to be experiencing estrus was a natural or induced artificially. The uterus were classified into A, B, and C, according to tone, degree of contraction to original size and site in pelvic cavity or abdomen, and ovaries were classified into A and B according to the integrity(size, protrusion and tone) of CL, and the texture felt by hand or sonography to compare the PR. When performing rectal palpation, recipients with a ovary as cystic ovarian follicles have been rejected. Transfers were conducted 7 days after estrus, and 2 embryos were transferred into each subject deep in the uterine horn where the CL is located.

Comparison of PR according to the Number of Transferred Embryos

Dairy cows which were observed estrus (natural or induced) were used for the comparison of the conception rate in cows according to the number of transferred embryos. The PR was measured in 321 cows with state of the uterus of A and above, and in cows with state of ovaries above A or B. In addition, a comparison was conducted of the pregnancy rate, embryonic loss, abortion and twin calving rate, in order to determine the most suitable number of embryos. Pregnancy diagnosis was conducted twice at 35 days and at 60 days in pregnant to compare the embryonic loss.

Comparison of the pregnancy rate in heifers according to the number of embryos was made for dairy heifers as the recipients, which were over 13 months old, with 2.5~3.5 BCS, and observed estrus normally.

Artificial Induction of Twining in Hanwoo Cows

With the goal of inducing artificial twin impregnation in Hanwoo cows, a comparison was conducted between the pregnancy rate and rate of twin calves production by comparing the method of transferring 2 IVF embryos 7 days after Artificial Insemination (AI) into the uterine horn contralateral to CL, and of transferring an 2 embryos or 3 embryos into deep uterine horn ipsilateral to CL without AI.

Statistics

The significance test in *t*-test was conducted to analyze the data incase of need as below.

To compare PR following the states of ovary and uterus, 321 cows with state of the uterus of A and in cows with state of ovaries above A or B were tested for pregnancy rate by 3 repetition.

95 Hanwoo cows (multiparae) were used to compare PR following ET method for twins between two groups by 3 repetition.

RESULTS AND DISCUSSION

The Effects following the States of Ovary and Uterus

ET was performed on 506 Holstein cows (multiparae) that were lactating 6～12 month after calving, with reproductive organs with no clinical diseases (endometritis, vaginitis, and follicular cysts). According to the results of this experiment in Table 1, ovary A on uterus A, ovary B on uterus A, ovary A on uterus B, ovary B on uterus B, ovary A on uterus C, ovary B on uterus C, showed a 58.6%, 55.2%, 16.4%, 16.4%, 7.6%, 10.5 % of PR respectively.

This shows that although there is a close correlation between the state of the uterus and PR, ovaries have a relatively lower correlation. After ovulation, CL forms in the ovary and newly formed CL and the plasma progesterone (P4), which is produced in the CL, has absolute influence on implantation and maintaining impregnation. It was said that the PR of ET increases according to the diameter and number of CL, which is in the same context as the theory that this increase in number and size of CL results in an increased concentration level of P4 in the blood which has a direct effect on maintaining conception (Massip *et al*., 1995). Baruselli *et al*. (2010) reported that PR were positively correlated with the diameter of the CL and the number of CL at ET when repeat-breeder Holstein cows were used as recipients, and de Feu *et al*. (2008) have reported that the differences in periovulatory steroid concentrations and size of ovarian structures do not explain the differences in embryo quality But, Spell *et al*. (2001) published that there were no significant differences in mean CL diameter, luteal volume or plasma progesterone concentration among recipients that did or did not become pregnant after embryo transfer, and reported that suitability of a potential embryo transfer recipient is determined by observed estrus and a palpable CL, regardless of size or quality. Morever, Bényei *et al*.(2006) reported that internal factors (age and size of CL, embryo development and quality score, synchronization methods, age of recipient, quality of transfer and reuse of recipients) is not a major influence on the results of pregnancy from embryo transfer but external factors (synchronization methods, month, embryo origin and farm effects), and Misra *et al*. (1999) reported that efforts to increase pregnancy rate following embryo transfer in buffalo should include prevention of luteolysis during the first week of transfer and a reduction in the incidence of embryonic mortality. And other reports (Remsen and Roussel, 1982; Hasler *et al*., 1987; Donaldson and Ward, 1987; Coleman *et al*., 1987) fai-

Table 1. Comparison of PR according to conditions of uterus and CL

*Grade of uterus	**Grade of CL	No. of cows	No. of pregnants	Pregnancy rate
A	A	196	115	58.6%[a]
	B	125	69	55.2%[a]
B	A	73	12	16.4%
	B	67	11	16.4%
C	A	26	2	7.6%
	B	19	2	10.5%

*; Grade of uterus.

A; Have a normal size, site(just past site of cavity) and softness by contraction to origin.

B; Larger, some stiffen, more dropped in abdominal cavity than normal.

C; Larger than B, lack of elasticity, much dropped to abdominal cavity and could palpate should be pulled.

**; Grade of ovary.

A; Normal size and have CL with protrusion over 1 mm of height.

B; Much bigger or smaller size to normal ovary and have CL not palpable but not cystoma.

[a] Values in same column is not different (p>0.5).

Table 2. PR and rate of birth to twins following number of embryos transferred

No. of transferred embryos	No. of recipients*	No. of pregnant(%)	No. of birth to twins (%)	No. of birth to triplets (%)
1	38	14(36.8)	0	0
2	162	86(53.0)	13(15.1)	0
3	87	44(50.5)	28(63.6)	3(6.8)

*; Cows that have clinically healthy reproductive organs and have shown normal estrus.

led to indicate any correlation between CL size and pregnancy rate of recipients, they used palpation per rectum to determine CL size(Spell *et al*., 2001). The reports written above showed two faces that the integrity of CL or plasma P4 concentration is or is not the most factor for implantation, conception, embryonic survival and mataining of pregnancy.

In this experiment as well, in the ET procedure, it is not the state of the recipient cow's ovaries but the state of the uterus which directly affects the PR if there is observations of curtain estrus and presence of palpable CL regardless of size. Accordingly, it could be supposed that the most influential factor is the uterine environment.

Impact of the Number of Transferred Embryos

We investigated the effects that the number of transferred embryos had on the pregnancy and twin calving rate in the transfer of Hanwoo IVF embryos with the aim of getting high-priced Hanwoo calves from normal dairy cows (multiparas). Sakaguchi *et al.* (2002) reported that the survival rate of embryos, including indigenous ones, was not affected by the number of embryos transferred, and a significantly higher twinning rate (68 % of pregnant recipients at 80 days after transfer) was achieved when two IVP embryos were transferred, as compared with the rate when one IVP embryo was transferred (24%). Results in this study are shown in Table 2.

As the results, the PR when 1, 2, and 3 embryos were transferred to Holstein cows (multiparae) was 36.8%, 53.0%, and 50.5% respectively. It was highest with the transfer of 2 and 3 embryos, and twin calving rate was highest with 3 embryos at 63.6%. There was not much difference in the embryonic loss rate and abortion rate, and there was no difference in the calving rate. It was similar with result of Franco *et al*. (2006) that transferring of two embryos into the uterine horn ipsilateral to the CL might be use for increasing pregnancy rates in dairy cow recipients than transferring of one embryo. Morever, Bertolini *et al*. (2002) reported that a tendency for higher calving rate was in the recipients that received two embryos in the uterine horn ipsilateral to the CL as compared to recipients that received one embryo, it was also similar with results of this experiment. When Hanwoo IVF embryos are transferred to Holstein cows with the purpose of increasing profit, transferring 3 embryos showed the best results in all aspects: PR, calving rate, and twin calving rate. Although loss of reproductive functions (Freemartin) which occurs in the case of male and female twins in the production of twin calves is a minus, it is still expected to considerably contribute to increase the profits of farmers.

A comparison was also conducted between the transfer of 1 embryo and 2 embryos in Holstein heifers (nulliparae).

As shown in Table 3, there was no significant difference in the PR between transferring 1 embryo and transferring 2 embryos. This results are similar with Del Campo *et al.* (1983) that considerable loss occurred greater than day 30 resulting in a low survival rate by day 110 (27%) when an embryo was present in each horn, and then the presence of two embryos would de-

Table 3. Effects of one or two embryos transferred to heifers*

No. of embryos transfered	No. of heifers	No. of pregnant at 35 days(%)	No. of pregnant at 60 days(%)	No. of heifers showing loss**(%)	Rate of calving (calvings/pregnants at 60 days)	Rate of twining (twinings/pregnants at 60 days)
1	186	131 (70.4)	126 (67.7)	3 (2.38)	97.6% (123/126)	
2	197	143 (72.5)	131 (66.4)	28 (21.3)	78.6% (103/131)	6.1% (8/131)

*; Nulliparous Holstein heifers.
loss**; fetal death, abortion, stillbirth after pregnancy diagnosis at 60 day.

Table 4. Twins effects following transfer method

Method	No. of cows	No. of pregnant(%)	No. of twins for pregnants(%)
*2 embryos with AI	52	43(82.6)	27(62.7)[a]
**2 embryos without AI	46	29(63.0)	11(37.9)
**3 embryos without AI	43	27(62.7)	17(62.9)[a]

*; Embryos were transferred at contralateral uterus of CL after A.I.
**; Embryos were only transferred at ipsilateral uterus of CL
[a] Values in same column is not different (p>0.5)

crease the survival probability of both embryos later in gestation. However, loss (embryonic loss, abortion, and dystocia) rate for 2 transferred embryos was 21.3%, higher than with 1 embryo with 2.38%. There is a most likely explanation for the increased loss of pregnancy. The loss is presumed that nulliparae have not sufficient uterine capacity than multiparae, and there is uterine crowding that afford embryos to be under unfavorable circumstances. There is similar results obtained in another studys (Franco *et al*., 2006; Anderson *et al*., 1979). As the final outcome, the best way to produce the most number of healthy calves is to transfer 1 Hanwoo IVF embryo to each Holstein heifer (nulliparous), and there are no benefits from transferring 2 embryos.

Artificial Production of Twin Calves in Hanwoo Cows

Artificial production of twin calves was also induced in Hanwoo cows to increase productivity.

As shown in Table 4, when combining AI and ET in Hanwoo (transferring 2 IVF embryos into the uterine horn where corpus luteum is not present 7 days after AI) the PR and twin calving rate are 82.6% and 62.7%, showing better results than with ET alone. With ET alone, transferring 2 embryos showed a lower PR than transferring 3 embryos, but a better calving rate. Penny *et al*. (1995) have reported that single IVF embryos transferred non-surgically to the uterine horn contralateral to the CL of 43 previously inseminated cows resulted in a calving rate of 72% with a twinning rate of 38.7%, and in 45 cows, two IVF embryos were transferred non-surgically to one uterine horn resulted in a calving rate of 51.1% with a twinning rate of 39.1%. And there is similar result for PR of ET with AI that transferring of two IVP embryos to previously inseminated recipients induced a significantly higher twinning rate during early pregnancy than that of one IVP embryo transfers (Sakaguchi *et al*., 2002). This concurs with the results of the present study showing that transferring 2 or more embryos have negative effects on embryonic death rate or maintaining impregnation in heifers, but in cows transferring 2 or 3 embryos the PR is better than when transferring just 1 embryo for increasing the number of cattle by increasing PR and inducing production of twin calves. And transferring of two embryos with AI show the best results in PR than transferring without AI. That result is similar with the result of Dochi *et al*. (2008) that transfer of frozen-thawed IVF embryos during the same cycle in which AI was done improved PR. Consequently, the results obtained in this study persuade transferring two embryos with AI for production twinning in multiparous Hanwoo cows, which in turn is more effective in increasing farmer's income.

In the actual use of Hanwoo IVF embryo transfer, to sum up the results of this experiment, the PR is not influenced by ovaries while it is significantly influenced by the state of the uterus. According to the number of embryos transferred, the twin calving rate was high in cows which had 3 embryos transferred, but the calving rate was best with the transfer of 2 embryos. In heifers, the transferring of 1 embryo shows the best pregnancy and calving rate, but while the PR was similar with 2 embryos, there was a higher loss. Thus in the case of heifer dairy cattle, I think it is best to transfer only 1 top quality embryo when transferring Hanwoo embryos.

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