Pregnancy Rate of *In Vitro* Produced Korean Cattle Embryos according to Transport Time Course

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ABSTRACT

This study was to investigate pregnancy rate of IVM/IVF/IVC Korean cattle (registered in government) embryos according to transport time course. For the production of embryos, oocytes recovered from slaughtered excellent grade cow and highly motile frozen-thawed bull semen (purchased from LIMC, KPN#497) was used. *In vitro* produced embryos were cultured in CR1aa medium for 8 days and some of them were frozen. The rate of average cleavage (>2-cell) was 83.0% (308/371) and blastocyst rate at day 8 was 34.7% (107/308). Among *in vitro* produced blastocyst embryos at day 8, most healthy embryos were freshly transferred on production day and some frozen embryos were direct transferred on appropriate day. These embryos were produced in a laboratory, embryo transfer (ET) was planned in 10 areas of the remote island (Jeju) from the laboratory by airplane. Thus, we examined the pregnancy rate in recipient cow according to embryo of transport time course before ET. From embryo transferred 44 recipient cows, overall pregnancy was 40.9% (18/44), these 18 cows were all calved [single, 94% (17/18); twin, 6% (1/18)] and total embryo implantation rate was 26% (19/66). Comparing transport time in the base of 6 hr, pregnancy rate in ET group required less 4 hr (60%, 9/15) was significantly higher than that required more 6 hr (26.3%, 5/19). In direct ET of freezing embryos, the pregnancy rate was 40% (4/10). However, it was difficult to find the meaning of temperature, pH and corpus luteum quality of recipients on comparison of pregnancy rate. When the cell death level of embryos according to storage time in thermos (straw container) before ET was measured by TUNEL staining, apoptotic index was increased with storage time-dependent. These results demonstrated that long distance transfer of IVM/IVF/IVC embryos is possible and the time of embryo transport is very important for the pregnancy rate on field trial.

(Key words : Korean cattle, Embryo transfer, Transport time, Pregnancy, Apoptosis)

INTRODUCTION

Ever since Edwards reported the *in vitro* culture of bovine embryos in 1965 (Edwards, 1965), many researchers reported the IVM and IVF of bovine oocytes. Following the success of bovine oocytes fertilized *in vitro,* calf production was reported (Hanada *et al.,* 1986). Until now, many people are using the embryo transfer to improve the effect of breeding stock. For industrial utilization of embryo transfer, embryos must be churned out at extremely low cost and improve the pregnancy rate before everything else.

The effects of a large number of factors on the pregnancy rates of cattle embryos were examined over a period of years at several different locations. The quality and developmental stage of embryos are important factors in determining the success of embryo transfer. Kubish *et al.* (2004) showed that transfer of expanded blastocysts resulted in a significant higher pregnancy rate than transfer of embryos of early or hatched stage. Intracellular pH regulates a variety of cellular processes such as enzyme activity, cell division, differentiation and protein synthesis (Boron., 1986). Ocon *et al.* (2003) observed that low pH reduced development to the blastocyst stage and low pH is involved in the early embryonic death of mouse eggs (Leclerc *et al.,* 1994). Bovine embryos are susceptible to thermal stress

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during developmental stages. Exposure of ovary to low temperature during transport may significantly decrease *in vitro* development (Cho *et al.*, 2006). Also, exposure of preimplantation embryos to elevated temperature causes disruption of continued development (Rivera *et al.*, 2001). An important factor in the success of embryo transfer is the degree of estrous cycle synchrony between donor and recipient animal. Whight *et al.* (1981) reported a reduction of pregnancy rates if synchrony deviated by only 12 hr. Beside, the effects of transfer number of embryo (Numbae *et al.*, 2000), recipient factors (Kim *et al.*, 2005a), technical aspects in practitioner (Kim *et al.*, 2005b) and environmental factors (Halser, 1987) were studied for improve the pregnancy rate of embryo transfer. After all many research, the efficiency industrial utilization of embryo transfer and the pregnancy rate of cows were still low. Especially, there is no report whether long distance transportation before ET will be affected on pregnancy rate of IVM/IVF/IVC fresh embryos.

This study carried out to investigate the pregnancy rate of Korean cattle (registered in government) embryos according to transport time course before ET.

MATERIALS AND METHODS

Production of Korean Cattle IVM/ IVF/ IVC Blastocysts

Chemicals were obtained from Sigma Chemical Company (St. Louis, MO) and media from GIBCO (Grand Island, NY), unless otherwise stated.

The culture procedures employed in the production of preimplantation embryos from follicular oocytes of Korean cattle were as outlined by Park *et al.* (1998). Briefly, ovaries of excellent grade cow were obtained from a slaughterhouse and cumulus-oocyte complexes (COCs) were aspirated from visible follicles (2~6 mm in diameter). The COCs were then washed with HEPES-buffered Tyrode's medium and cultured in maturation medium composed of TCM199 + 10% fetal bovine serum (FBS) supplemented with 0.2 mM sodium-pyruvate, 1 ug/ml follicle-stimulating hormone, 1 ug/ml estradiol-17β, and 25 ug/ml gentamycin sulfate at 39℃, 5% CO_2 incubator. After incubation for 22~24 hr in IVM medium, the COCs were inseminated using highly motile sperm recovered from frozen-thawed bull semen (purchased from LIMC, KPN#497) separated on a discontinuous percoll column. Fertilization was assessed as cleavage rate (≥2-cell) after 44±2 hr co-incubation with the sperm. For *in vitro* culture, cleaved embryos were cultured in CR1aa (Rosenkrans *et al.*, 1993) medium supplemented with 3 mg/ml fatty acid-free BSA and then transferred into 10% FBS added CR1aa medium at day 4 after IVF. For the study, day 8 blastocysts produced *in vitro* after IVF were mainly used.

Vitrification

Vitrification procedures were indicated in previous study (Kim *et al.*, 1998). Solutions used for vitrification were prepared in modified Dulbecco's phosphate buffered saline (D-PBS) supplemented with 10% FBS. Vitrification solutions were mixtures of glycerol (G) and ethylene glycol (EG). Selected blastocysts for experiments were first exposed to 10% (v/v) G for 5 min, then were transferred to 10% G plus 20% EG (v/v) for 5 min, and finally were transferred to 25% G plus 25 % EG (v/v) (G25EG25). And then they were transferred into vitrification solution section of 0.25 ml French mini straw (IMV, L'Aigle, France) and was heat-sealed within 30 to 45 sec. Before being plunged into liquid nitrogen (LN_2), straw was placed horizontally on styrofoam box which included LN_2 and exposed by the cold nitrogen vapor for 3 min. Average embryo numbers loaded in each straw were three.

Thawing procedures were performed as described previously (Lee *et al.*, 2000). Thawing of straw was carried out in air for 10 sec and then in water bath of 25℃ for 20 sec. Total needed time for one-step dilution is about 2 min. After the contents of straw were mixed into single column, straw was placed vertically plug-end down and plug-end up in 25℃ water bath for 30 sec each.

Recipients

Recipients were used for the nonsurgical embryo transfer after natural estrus or synchronization by single intramuscular injection of 25 mg lutalyse of derivatives $PGF_2\alpha$. The recipients used in the present study were both parous cows and nulliparous heifers. The corpus luteum (CL) was examined by palpation per rectum for morphological quality (good, fair and poor) and no abnormal structures on the ovary or uterus immediately before one-step dilution and transfer of the embryo.

Embryo Transfer, Pregnancy Diagnosis and Calving

For fresh ET, good-quality embryos (expanded and expanding blastocysts) were removed from culture on day 8, placed in the ET medium, CR1aa supplemented with Flavonoid and HEPES that our team developed, and packaged individually in straws (IMV, L'Aigle, France). Straws were sealed with Parafilm, placed in thermos set at 39℃ and carried to the Jeju island by airplane.

One-step diluted straws were transferred directly into recipient cows by placing the straw into an embryo transfer gun. This embryo transfer into uterus of re-

cipients was carried out within 10 min after dilution. Pregnancies were confirmed at first when recipient cows did not return to the subsequent estrus cycle, and later by manual palpation per rectum on day 45, 90. Ten days before the expected beginning of the calving season, recipients were checked for parturition signs every 2 hr. All newborns had nasal passages cleared, were helped to breath, had navels dipped into iodine solution and were encouraged to suckle within 30 min of birth.

TUNEL Assay

Blastocysts were washed three times in PBS (pH 7.4) containing 1 mg/ml polyvinylpyrolidone (PBS/PVP) and then fixed in 4% paraformaldehyde in PBS for 1 hr at room temperature (RT). After fixation, embryos were washed in PBS/PVP and permeabilized by incubation in 0.5% Triton X-100 for 1 hr at RT. Embryos were then washed twice in PBS/PVP and incubated with fluorescein-conjugated dUTP and the terminal deoxynucleotidyl transferase enzyme (Roche, Mannheim, Germany) in the dark for 1 hr at 37℃. After counterstaining with a solution of 40 mg/ml propidium iodide (PI) plus 50 mg/ml RNase A for 1 hr at 37℃ to label nuclei, embryos were washed in PBS/PVP, mounted with slight cover slip compression and examined by fluorescence microscopy (Olympus, Tokyo, Japan).

Statistical Analyses

The apoptotic index was evaluated by analyses of variance using the general linear model (PROC-GLM) in the SAS software program. Differences of $p<0.05$ were considered significant.

RESULTS

For the production of embryos, bovine oocytes completely surrounded by at least 3 layers of cumulus cells were used. Highly motile frozen-thawed bull semen and previously matured cumulus oocytes were cultured for 24 hr in 50 ul drop fertilization media under mineral oil.

The cleavage rate of IVM-IVF oocytes was 83% (308/371). Development rates to the morular and blastocyst among cleaved embryos were 39% (120 /308) on day 6, 34.7% (107/308) on day 8, respectively (Table 1).

Fig. 1. Fraternal twin calves born out after embryo transfer (right, December 27, 2007) into recipient mother (left).

In 10 areas of Jeju island most healthy embryos were freshly transferred on production day and some embryos were frozen and direct transferred into 44 recipient cows on appropriate day. Total embryo implantation rate was 26% (19/66). Overall pregnancy was 40.9% (18/44). In 18 of pregnant cows, aborted cow was not and 18 cows were calved [single, 94% (17/18); twin, 6% (1/18)], 19 calves were lived (Fig. 1 and Table 2).

Table 2. Pregnancy and calving rates according to transfer of *in vitro* produced blastocysts

Items	No.	Percent(%)
No. of transferred cows	44	
No. of transferred embryos	66	-
No. of pregnancy cows	18/44	41
No. of abortion cows	-	-
No. of calving cows	18	100
single	17/18	94
twin	1/18	6
No. of implanted embryos	19/66	29
No. of dead calves	0/19	
No. of living calves	19/19	100

Table 1. *In vitro* development of follicular oocytes of Korean cattle

No. (%) of examined oocytes	No. (%) of IVF	No. (%) of embryos developed to		
		≥8 cell on day 4	≥ morular on day 6	≥ blastocyst on day 6
371	308 (83.0)	233 (75.6)	120 (39.0)	107 (34.7)

Table 3. Comparison of pregnancy rates according to transport time course of fresh embryos or direct transfer of one-step thawed freezing embryos

	Factor	No. of recipients transferred	No. (%) of pregnancy recipients	No. of living calves
Overall		44	10 (40.9)	19
Fresh	Long time(≥6 hr)*	19	5 (26.3)	5
	Short time(≤4 hr)	15	9 (60.0)	10
Freezing**		10	4 (40.4)	4

* Transport time before embryo transfer from lab to recipient cow.
** Direct transfer of one-step thawed freezing embryo.

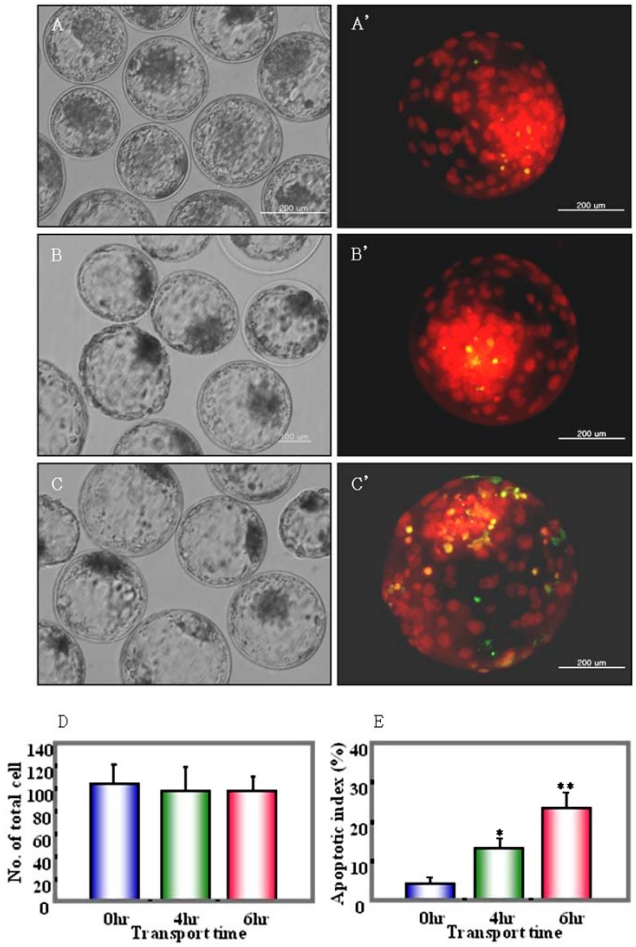

Fig. 2. Fluorescence microscopy images and apoptotic index of nuclei of bovine day 8 blastocysts according to transport time course using TUNEL staining. PI was used to stain chromatin. Embryos kept in 0.25CC straw 0 hr (Control, A-A′), 4 hr group (B-B′) and 6 hr group (C-C′). Green, fragmented DNA; red, chromatin. Total number of cells (D) and apoptotic index (E) counted from TUNEL stained blastocysts in each treatment groups. Stars indicate statistically significant differences among treatment groups (p<0.05).

In this study, we compared to pregnancy rate by transport time in the base of 6 hr before ET. In fresh embryos, pregnancy rate in ET group required less 4 hr (60%, 9/15) was significantly higher than that required more 6 hr (26.3%, 5/19). Pregnancy rate of ET group required more 6 hr was decreased than that of direct ET group of freezing embryos (40%, 4/10, Table 3).

When the cell death level of embryos according to storage time in thermos (straw container) before ET was measured by TUNEL staining, apoptosis was increased with storage time-dependent (Fig. 2).

DISCUSSION

Embryo transfers (ET) have been performed in cattle for over 30 years. To improve the pregnancy rate, the many studies went off. However despite continued refinement of culture conditions, pregnancy rates have generally remained below 50% (Peterson *et al.*, 2003).

In this work, the pregnancy rate of cows receiving IVM/IVF/IVC Korean cattle was analyzed according to transport time course before ET. The main findings were that (1) in fresh embryos, pregnancy rate in ET group required less 4 hr was significantly higher than that required more 6 hr and freezing embryos; (2) Pregnancy rate of ET group required more 6 hr was decreased than that of direct ET group of freezing embryos; (3) Apoptosis was increased with storage time-dependent.

The factors affecting the pregnancy rate in ET were to be classified into embryo factors, recipient factors, technical aspects and environmental factors.

In aspect of embryo factors, Hasler *et al.* (2001) and Kubisch *et al.* (2004) demonstrated that the pregnancy rate is influenced by embryo grade and stage. Specially, Hasler *et al.* (2001) reported that for fresh embryos pregnancy rates were 68.3% and frozen-thawed embryo pregnancy rate were 56.1%. In our analysis, stage of using embryos was expanded blastocyst, pregnancy rate of fresh embryos in ET group required less 4 hr substantially increased than freezing embryos (60

% and 40%). Consequently, our data showed the similar trend with result of Hasler (2001). Its primary reason for just a little bit low pregnancy rate (not significance) was thought that unlike Halser's *in vivo*-derived embryos we used *in vitro* produced embryos. Also, it has been reported that freezing and thawing process decreases embryo viability, which is attributed to physical and chemical damage induced during the cryopreservation process (Baguisi *et al.*, 2000). On the other hand, pregnancy rate of fresh embryos in ET group required more 6 hr was low above all things (26.3%).

Recipient factors include recipient age, breed, CL quality and so on. Numabe *et al.* (2000) reported that it makes no difference on pregnancy rate whether recipient parity is or not. Hwang *et al.* (2004) reported high pregnancy rate in cows but Hasler *et al.* (2001) reported high pregnancy rate in heifers. Consequently, it might be a difference according to a researcher (individual variation). Also, according to quality of CL, Niemann *et al.* (1985) reported that pregnancy rate for recipient having CL of C grade was high. On the other hand, Hwang *et al.* (2004) reported that pregnancy rate of recipient having A grade of CL was high. In our analysis, the majority of the recipients had a CL score of "good" or "fair". Similarly, the CL (Coleman *et al.*, 1987), recipient parity (Numabe *et al.*, 2000) and recipient age did not have a significant effect on the pregnancy rate as was found.

Sediel *et al.* (1980) reported that pregnancy rate showed from 26% to 67% according to experience of technician. Kim *et al.* (2005b) reported that pregnancy rate was higher in group having experience of ET more than 500 times than less than 500 times. In our studies, it thought that there was no technical problem on the pregnancy rate, because we get help from 2 technician (experienced of ET more than 700 times).

Temperature (Rivera *et al.*, 2001) and pH (Leclerc *et al.*, 1994; Ocon *et al.*, 2003) may modify function of early embryo. In our analysis, we found no affect of the temperature and pH during the transportation of long distance of embryos. That's why low temperatures or sudden temperature and pH changes have not observed even if the transportation time of embryos required more 6 hr. In viewpoint of pH stability, everyone knows that HEPES added within ET medium that our team developed has no changed pH during the transportation time of embryos required more 6 hr. However, it was difficult to find the regional meaning on comparison of pregnancy rate because there was not sufficient ET trial in 10 areas of Jeju island.

On the other hand, high concentrations of reactive oxygen species (ROS) in the microenvironment surrounding the pre-implantation embryo *in vitro* may disturb the balance between the formation of ROS and antioxidants, leading to oxidative stress, which is generally thought to be harmful for embryonic development (Watson *et al.*, 1994; Blondin *et al.*, 1997). Flavonoids are polyphenolic compounds that behave as an antioxidant against the free radicals (Serafini *et al.*, 1998). In recently, ROS scavenger such as superoxide dismutase and polyphenol compounds has beneficial effect on embryo development *in vitro*. In our preliminary study, addition of flavonoid in ET medium brings beneficial effects on *in vitro* survival of embryos (Data not shown).

Hwang *et al.* (1988) examined the factor affecting on pregnancy rate in non-surgical transfer of bovine embryos and reported that pregnancy rate of embryo transfer within 5~15 min was high than more than 15 min. Also, Kim *et al.* (2005a) reported that transfer time of blastocyst did not have a significant effect on the pregnancy rate. But, in these two results, time had been measured except transportation time of embryo from raise the embryo impregnator at to uninstalled time of embryo impregnator soon after ET. As mentioned earlier, in our studies, working time in ET had been measured including 4~6 hr of transportation times of embryo.

To investigate the cell apoptosis on transportation time of long distance of embryo, when the cell death level of embryos according to storage time in thermos (straw container) before ET was measured by TUNEL staining, there was no difference in the number of total cells among all groups (102±18, 98±21, 98±17). But, percent of apoptotic cells in ET groups required less 4 hr and more 6 hr (13±2.3% and 23±4%) was significantly higher than that in the control group (5±1.7%) (p>0.05). Apoptosis was increased with storage time-dependent. It thought that pregnancy rate was low as a result apoptotic cell increased.

Taken together, these results demonstrated that long distance transfer in our Korean cattle IVM/IVF/IVC-ET system is possible and the time of embryo transportation is very important for the pregnancy rate on field trial.

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