Effects of IVM and IVF Duration on *In Vitro* Development and Cell Numbers of Embryos in Korean Native Cattle

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ABSTRACT

The present study was performed to investigate the effects of *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) duration on the development of Korean Native Cattle embryos. The time of blastocyst formation and the quality of blastocysts based on cell numbers were examined. The cleavage rate increased with the length of IVF duration in the groups of 18-hr IVM, but was constant in the groups of 24-hr IVM. The development rate to the 8-cell stage was significantly higher in the IVM 18: IVF 20 group than in the IVM 24: IVF 24 group. The development rate to the blastocyst stage was highest in the IVM 18: IVF 20 group, significantly different from that of the IVM 18: IVF 16, IVM 24: IVF 20 and IVM 24: IVF 24 group. The time of blastocysts formation tended to be shorter when IVM and IVF duration were decreased. The number of inner cell mass, trophoblast and the total cells were significantly higher in the IVM 18: IVF 16 group than in the IVM 24: IVF 24 group ($P<0.05$). These results demonstrated that the IVM and IVF duration should be adequate for the efficient production of bovine embryos, and it might particularly be essential to determine the proper combination of IVM and IVF duration.

(Key words : *In vitro* Maturation, *In vitro* Fertilization, Embryo development, Cell numbers, KNC)

INTRODUCTION

There have been many researches on the production of bovine blastocysts *in vitro*, since the birth of the first IVF calf (Brackett, 1982). However, the overall development of oocytes to the blastocyst stage has remained 10 to 30%, and the reproducibility of the results was low (Dominko and First, 1997). The reasons are considered to be the inadequate conditions of *in vitro* (Ward et al., 2002). Therefore, adequate conditions for IVM and IVF must be determined to improve the production of transferable bovine blastocyst *in vitro* (Nagai, 2001).

In vitro maturation of bovine oocytes is affected by several factors such as transport time and temperature from the slaughterhouse to the laboratory (Yang et al., 1990), follicle size (Blondin and Sirard, 1995), developmental stage of oocyte (Hagemann et al., 1999), oocyte diameter (Hyttel et al., 1997), composition of medium (Lonergan et al., 1997), hormones (Zuelke and Brackett, 1990), serum (Avery et al., 1999) and duration (Dominko and First, 1997; Prokofiev et al., 1992; Semple et al., 1993; Ward et al., 2002). The speed at which the oocyte undergoes maturation has implications for subsequent development (van der Westerlaken et al., 1994). Inappropriate timing of maturation could lead to the formation of abnormal chromatin (Dominko and First, 1997), the possibility of oocyte aging (Hunter and Greve, 1997) and impaired development (Marston and Chang, 1964).

The successful IVF of bovine oocytes is affected by sperm capacitating (Fukui et al, 1990; Kochhar and King, 1998), incubation periods (Fukui et al., 1990; Dode et al, 2002), bull and its lot (Parrish et al., 1986), heparin concentration (Fukui et al., 1990) and reactive oxygen species (ROS, Kim et al., 1999). With regard to the relationship between IVF duration and embryo development, the fertilization rate rose with the increase of IVF duration (Rehman et al., 1994), but the development rate to the blastocyst stage was decreased with the increase of IVF duration (Sumantri et al., 1997). In human IVF, the shorter co-incubation between oocytes and spermatozoa resulted in higher fertilization rates, and achieved better quality embryos and higher pregnancy than those with the use of longer overnight gamete co-incubation (Dirnfeld et al., 1999; Gianaroli et al., 1996).

In addition to the proportion of oocytes developing to the blastocyst stage, the quality of these blastocysts is important in determining success of embryo transfer. Blastocyst quality has been assessed by the morphology of the blastocyst (Linder and Wright, 1983), hatching rate

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(Hernandez-Ledezma et al., 1996), blastocyst cell number (Du et al., 1996; Papaioannou and Ebert, 1988) and cryotolerance (Men et al., 2002).

The purposes of this study were 1) to investigate the effects of the combination of IVM and IVF durations on subsequent embryo development and the day of blastocyst formation of Korean Native Cow (KNC) oocytes, 2) to examine the embryo quality as measured by the mean cell number of blastocysts.

MATERIALS AND METHODS

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

Oocyte Collection and *In Vitro* Maturation

The ovaries of KNC were obtained at a slaughterhouse and transported within 5 hr to the laboratory at 25 to 28 °C in 0.9% saline supplemented with 25 μg/mL gentamicin. Cumulus oocyte complexes (COCs) were obtained by aspiration from 2 to 6 mm follicles with an 18-gauge needle attached to a 10-mL disposable syringe. Only COCs with compact cumulus layers and evenly granulated ooplasm were selected for treatments.

The COCs were washed three times in HEPES-Tyrode's albumin lactate pyruvate (HEPES-TALP) medium composed of TALP medium supplemented with 25 mM HEPES and 3 mg/mL BSA. Groups of 15 COCs were placed in 50 μL drops of TCM199 (Gibco, Cat, no. 12340-030, Grand Island, NY, USA) supplemented with 0.2 mg/mL Na-pyruvate, 10% fetal bovine serum (FBS), 1 μg/mL FSH, 10 μg/mL LH, and 1 μg/mL Estradiol-17β under mineral oil at 38.5 °C under 5% CO_2 in air with maximum humidity.

Sperm Preparation and *In Vitro* Fertilization

A single frozen semen straw of a Korean Native bull (KNB) was thawed for 1 min in 37 °C water and then placed on top of a discontinuous Percoll density gradient composed of 2 mL 45% Percoll over 2 mL 90% Percoll in a 15 mL centrifuge tube (Corning, NY, USA). The sample was centrifuged for 20 min at 700 ×g at room temperature. The spermatozoa collected at the bottom of the fraction were washed for 10 min at 350 × g in sperm-TALP medium, which consisted of TALP medium supplemented with 3 mg/mL BSA (fraction V). Spermatozoa were counted in a hemacytometer and diluted with fer-TALP medium to give a concentration of 25 × 10^6 spermatozoa/mL. Fer-TALP medium consisted of TALP medium supplemented with 6 mg/mL BSA and 10 μg/mL heparin.

After maturation, the COCs were washed three times in fer-TALP medium. 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 16, 20 and 24 hr at 38.5 °C with 5% CO_2 in air and maximum humidity.

Preparation of Bovine Oviduct Epithelial Cells and *In Vitro* Culture

Bovine oviduct epithelial cells (BOEC) were obtained weekly from the genital tracts of slaughtered KNCs and transported to the laboratory within 5 hr at 4 °C and dissected free from the ovaries and other tissue. The oviduct was blotted on sterile gauze to remove excess fluid and blood. The oviduct was place into a culture dish with TCM199, and grasped with a forceps at the isthmic end and scraped gently toward the infundibulum with a rounded end of test tube. The mass of epithelial cells was transferred into a 15 mL centrifuge tube and washed three times in TCM199 by centrifugation (10 min, 200 × g). After centrifugation, the pellet was vigorously extruded at least 5 times through a 21-gauge needle on a 3 mL syringe and was rewashed in 3 mL TCM199 containing 10% FBS. After the final wash, 500 μL of the epithelial cell suspension was added to each well of 4-well dish (NUNC, Roskilde, Denmark) and cultured at 38.5 °C under 5% CO_2 in air with maximum humidity. By 48 hr, the BOEC formed vesicles which were washed three times in CR1aa medium supplemented with 10% FBS. Groups of 20 vesicles were transferred to 50 μL drops of CR1aa medium supplemented with 10% FBS under mineral oil.

After fertilization, the presumptive zygotes were stripped of cumulus cells by pipetting and were washed three times before they were transferred to 50 μL drops of CR1aa medium with 3 mg/mL BSA and incubated for 2 d. Then the culture medium was changed to CR1aa medium supplemented with 10% FBS and co-cultured with BOEC. All cultures were carried out under mineral oil at 38.5 °C with 5% CO_2 in the air and maximum humidity.

Differential Staining of Blastocysts

The zona of blastocysts was removed through treatment with 0.5% pronase and washed 5 times in HEPES-TALP medium. Zona-free blastocysts were incubated in a 1:5 dilution of rabbit anti-bovine whole serum in HEPES-TALP medium for 1 hr. After washing 5 times in HEPES-TALP medium, blastocysts were reincubated in a 1:10 dilution of a guinea pig complement in HEPES-TALP medium supplemented with 4 μg/mL propidium iodide (PI) and 4 μg/mL bisbenzimide for 1 hr. Presumptive stained blastocysts were mounted on a slide glass and the cell numbers were examined under a fluorescence microscope (Olympus, Tokyo, Japan). Bisbenzimide-stained inner cell mass (ICM) nuclei appeared blue

and trophectoderm (TE) nuclei, which were stained with both bisbenzimide and PI, gave red or pink fluorescence.

Statistical Analysis

Data on the embryo development and the time of blastocyst formation were analyzed by the x^2-test. The cell number was arcsine transformed and analyzed by the General Linear Models Procedure with the Statistical Analysis System (SAS; Cary, USA). Treatment means were compared with the Duncan's multiple range test; P values < 0.05 were considered to be significantly different.

RESULTS

Effects of IVM and IVF Duration on Embryo Development

In general terms of IVM 18-hr groups, as the duration of co-incubation increased, the cleavage rate decreased, and it was significantly higher in IVF 24-hr group than in IVF 16-hr group (Table 1). However, the co-incubation time in IVM 24-hr group had no significant effect on the cleavage rates. The development rate to the 8-cell stage was higher in the IVM 18:IVF20-hr and IVM 18: IVF 24-hr group compared to the IVM 24: IVF 24-hr group ($P < 0.05$). Additionally, the development rate to the blastocyst stage was significantly greater in the IVM 18: IVF 20-hr group than in the IVM 24: IVF 20-hr and IVM 24: IVF 24-hr groups.

Effect of IVM and IVF Duration on Blastocyst Formation

In IVM 18-hr group, there was trend for the rate of blastocyst formation on Day 7 and Day 9 to increase with increased duration of IVF, but on Day 8, it was decreased with increased duration of IVF. In IVM 24-hr group, on Day 7, the rates of blastocyst formation was higher in the IVF 20-hr than the IVF 24-hr group ($P< 0.05$). On Day 8, however, the rate of blastocysts formation was significantly higher in the IVF 24-hr than IVF 20-hr group ($P<0.05$). On Day 9, there was no different among the treatment groups.

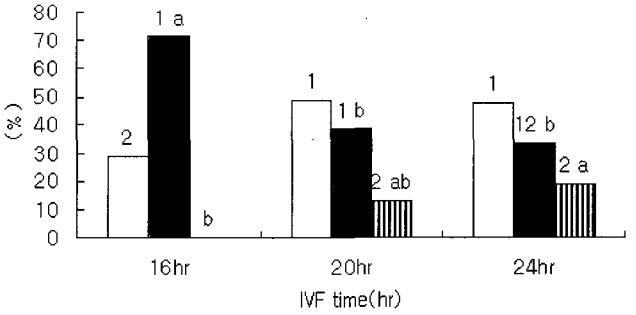

Fig. 1. Effect of the IVM (18 hr) and IVF(16, 20, 24 hr) durations on blastocyst formation from day 7(□), day 8(■) and day 9(▥). [ab]Values in the same column with different superscripts are significantly different ($P<0.05$). [123]Values in the same row with different superscripts are significantly different ($P<0.05$).

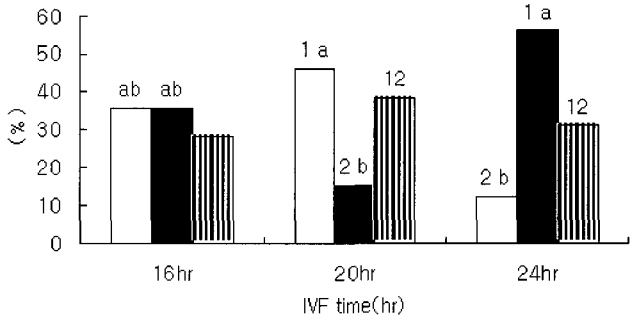

Fig. 2. Effect of the IVM (24 hr) and IVF (16, 20, 24 hr) duration on blastocyst formation from day 7(□), day 8(■) and day 9(▥). [ab]Values in the same column with different superscripts are significantly different ($P<0.05$). [123]Values in the same row with different superscripts are significantly different ($P<0.05$).

Table 1. Effects of the combination of IVM and IVF duration on *in-vitro* development of Korean Native Cow embryos

IVM (hr)	IVF (hr)	N	No. (%) of embryos developed to		
			≥2-cell	8-cell	Blastocyst
18	16	165	85(51.8±6.5)[a]	48(29.0±3.7)[ab]	28(17.3±2.1)[a]
	20	230	156(66.5±9.3)[ab]	88(37.3±5.3)[b]	72(32.0±5.4)[b]
	24	215	152(70.5±0.6)[b]	76(35.3±4.0)[b]	53(24.0±5.8)[ab]
24	16	129	85(65.3±4.4)[ab]	45(33.0±4.1)[ab]	30(21.5±3.1)[ab]
	20	235	160(68.3±3.5)[ab]	53(23.3±6.3)[ab]	33(13.8±3.8)[a]
	24	193	121(60.0±3.6)[ab]	36(18.3±4.2)[a]	24(13.3±4.1)[a]

[ab]Values in the same column with different superscripts are significantly different ($P<0.05$).

Table 2. Effect of the combinations of IVM and IVF duration on ICM, TB and total cell number of Korean Native Cow blastocysts

Maturation time(hr)	Fertilization time(hr)	No. of examined oocytes	No. of cells		
			Total	ICM[1]	TB[2]
18	16	15	138.3± 9.8[a]	38.8±4.5[a]	99.5± 6.5[a]
	20	20	129.3± 6.3[ab]	31.7±2.3[a]	97.6± 5.9[a]
	24	17	83.9± 4.7[c]	26.2±3.3[ab]	57.7± 3.6[bc]
24	16	12	72.4±11.5[cd]	15.1±3.1[b]	57.3±10.1[bc]
	20	24	109.3± 5.1[b]	36.0±3.5[a]	73.4± 3.0[b]
	24	14	59.6± 3.0[d]	12.9±1.0[b]	46.7± 3.7[c]

[1]ICM: Inner cell mass.
[2]TB: Trophoblast.
[ab]Values in the same columns with different superscripts are significantly different ($p<0.05$).

Effect of IVM and IVF Duration on Cell Number

In IVM 18-hr groups, the mean total cell number in blastocysts was decrease with increased IVF duration, and it was significantly higher in the IVF 16-hr group than in the IVF 24-hr group ($P<0.05$). The mean total cell number in IVM 24-hr groups was significantly higher in the IVF 20-hr group than in the other treatment groups ($P<0.05$). There were not differ in the mean number of ICM cells among IVM 18-hr treatment groups, but in IVM 24-hr groups, it was significantly higher in the blastocysts produced IVF 20 hr compared to the IVF 16 or 24 hr. The mean number of TE cells in the IVM 18: IVF 16-hr and IVM18: IVF 20-hr groups were significantly higher than other treatment groups ($P <$ 0.05).

DISCUSSION

The present study was performed to examine the effects of IVM and IVF duration on the development of Korean Native Cow embryos. Additionally, we examined the time of blastocysts formation and the quality of these blastocysts in terms of cell number.

Ward et al. (2002) and Monaghan et al. (1993) were highest at the 24 hr of IVM, and in the research of Prokofiev et al. (1992) there was no difference in the embryo development rate according to IVM duration. However, Semple et al. (1993) and Dominko and First (1997) reported that the fertilization rate and development to blastocyst were higher at 14 or 16 hr than that at 24 hr of IVM, which is similar to the result of this experiment (Table 1). The reasons of this are unclear, but the increase of IVM duration may cause the aging of the oocyte, which in turn has a detrimental effect on the embryo development. The IVM of oocytes is long (over 17 hours), chromatin decondensation and the formation of sperm aster in the fertilized zygote are delayed (Long et al., 1994).

In the relationship between IVF duration and subsequent embryo development, the cleavage rate rose with the increase of IVF duration (Rehman et al., 1994; the present study Table 2, the groups of IVM18), but the development rate of to the blastocyst stage went down with the increase of IVF duration (Sumantri et al., 1997; the present experiment Table 2, the groups of IVM24). This is probably because the tendency of polyspermy increases with the increase of IVF duration as reported by Chian et al. (1991). The chromatin decondensation and the formation of sperm aster were delayed with the increase of IVM and IVF duration of bovine oocytes (Long et al., 1994), we can assume the possibility of the aging of eggs. Moreover it is believed that toxic substances produced from cumulus cells are absorbed into the eggs and lower the embryo development rate (Hunter and Greve, 1997). Moreover, in the present experiment (Table 2), the fertilization rate and the blastocysts development rate were significantly low when IVM and IVF duration were decreased (IVM18: IVF16). In addition, inferring from the relationship between the number of cells and the pregnancy rate (Park et al. 2003), we expect that, as these blastocysts have a large number of cells, they may have a high pregnancy rate.

The ultimate test of embryo quality is a pregnancy and production of a live offspring. However, due to the impracticality of transferring every embryo, the methods for assessing embryo quality include morphology of the blastocyst (Linder and Wright, 1983), hatching rate (Hernandez-Ledezma et al., 1996), blastocyst cell number (Du et al., 1996; Papaioannou and Ebert, 1988) and cryotolerance (Men et al., 2002). Blastocyst cell number is an especially good indicator of the embryo quality and health (Papaioannou and Ebert, 1988) and is closely

related to the viability of embryos (Willadsen and Polge, 1981). There are many factors such as amino acid (Park et al. 2004), maturation condition (Park et al., 2003), and culture conditions (Du et al., 1996) affect the cell number of blastocyst. In addition, Du et al. (1996) reported that the blastocysts produced *in vivo* have a larger number and proportion of ICM cells than those produced *in vitro*. Our results showed that in terms of the number of cells, the quality of blastocysts produced in IVM 18: IVF 16-hr was highest. Park et al. (2003) reported that the blastocysts have a larger cell number represent a higher pregnancy rate. Therefore, these blastocysts may have a high pregnancy rate than "long" IVM and IVF duration.

In human, the short exposure of the oocyte to spermatozoa may have a favourable effect on implantation rates by improving embryo quality (Dirnfeld et al., 1999; Gianaroli 1996). The overnight exposure of oocytes to large numbers of spermatozoa led to inferior embryo quality, which may have been due to suboptimal culture conditions, overloaded by the excessive amounts of reactive oxygen species (ROS) and other products of metabolites. From this study, in bovine IVF, the shortening of oocyte-spermatozoa co-incubation may improve embryo quality by preventing the negative effect caused by oxygen free radicals produced by spermatozoa during prolonged co-incubation.

In conclusion, both IVM and IVF durations can affect on development of bovine embryos, and subsequently influence the blastocyst quality. Particularly, the combination of IVM and IVF duration must be considered preciously.

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