J. Emb. Trans. (2009) Vol. 24, No. 4, pp. 281～287

Production and Transfer of *In Vitro* fertilized Hanwoo Embryos with Serum-free Media

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ABSTRACT

As a simple and economical method for *in vitro* produced embryos, we have used BSA instead of serum for the production and embryo transfer of Hanwoo *in vitro* fertilized (IVF) embryos and obtained the following results:

1) When using serum (FBS; fetal bovine serum) or BSA-containing culture media as the initial culture media for immature oocytes, it is regarded as inappropriate to add only BSA to the culture solutions from maturation of the immature oocytes to development stage culture, but serum still needs be added though there is no significant difference in the concentration, with a change from 5% to 10%.
2) The results of culturing IVF embryos after development (4 cell stage) in the Medium199 solutions containing BSA instead of serum (FBS) showed that 0.3% BSA concentration is not optimal and 0.5% or higher BSA concentration has no significant difference among 0.5%, 0.7%, 1% and 2% ($p > 0.05$).
3) The post-freezing survival ratio after development in 5% FBS-Medium199 showed that 1% BSA concentration of the culture solution is the most suitable in the BSA concentrations of 0.3% (51%), 0.5% (67%), 0.7% (69%), 1% (77%) and 2% (75%).
4) The pregnancy rates of the transplanted fresh(not frozen) blastocyst had no significant concentration dependency ($p > 0.5$), and the average pregnancy rate was 63.8%. 14% of overweight calves were found among the calves given birth to by the transfer of IVF blastocysts cultured in the serum-added culture solution, but none was found in the experimental groups in which BSA was added instead of serum.

(Key words : FBS, BSA, *in vitro* fertilization, embryo transfer)

INTRODUCTION

Embryo transfer (ET) derived from *in vivo* and *in vitro* has great effect on animal improvement and superagencies reproduction. For this reason, *in vitro* fertilization (IVF) has been extensively studied and applied, but the *in vitro* produced bovine embryos are not up to that of *in vivo* produced embryos in many respects (Boni *et al.,* 1999; Duby *et al.,* 1997; Viuff *et al.,* 1999). In many species, production of *in vitro* fertilized embryo is dependent on the intrinsic quality of oocyte and the culture environment of the fertilized oocyte (Rizos *et al.,* 2002), and thus the study of culture media is one of the most important parts.

Serum has not been perfectly analyzed and varies depending on individual animals and batches (Pinyopummintr *et al.,* 1994), an addition of serum to the *in vitro* fertilized egg culture media has been applied in almost all the research processes. Several studies have reported that the addition of serum to the culture media has the effect to inhibit earlier cleavage divisions than that of the non-added media and to accelerate development after the cleavage (Rosenkrans *et al.,* 1994; Carolan *et al.,* 1996; Van Langendonckt *et al.,* 1997; Thompson *et al.,* 1998; Lonergan *et al.,* 1999a; Lonergan *et al.,* 1999b; Gutierrez-Adan *et al.,* 2001), however the addition of serum to the culture media causes morphological, biochemical and physiological abnormality (Dorland *et al.,* 1994; Gardner *et al.,* 1994; Thomson *et al.,* 1995a; Abe *et al.,* 1999; Krisher *et al.,* 1999; Crosier *et al.,* 2000; Crosier *et al.,* 2001; Fair *et al.,* 2001; Rizos *et al.,* 2003). One example is that the number of fat globules and their size in the blastocyst are different from those of *in*

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† This reserch supported by Korea institute of planning and Evaluation for Technology in Food, Agriculture, Forestry and Fixheries (No. of the project; 109029-3).
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vivo fertilized egg (Thomson *et al.,* 1995b; Abe *et al.,* 2002) and, some reported abnormality such as the overweight calf syndrome among newborn calves caused by these kinds of differences. On the other hand, some reports on the culture media without serum showed that the abnormality found in the serum added culture media has been reduced (Rizos *et al.,* 2003; Mucci *et al.,* 2006).

In this study, we substituted BSA for serum which has been conventionally used for the bovine IVF culture solution and produced IVF Hanwoo (Korean native beef cattle) embryos within a simple culture solution excluding the nutrients such as hormones, growth factors and essential and nonessential amino acids. In this process, we compared the development ratio (to 4cells), blastocyst formation rate, and pregnancy rate after ET depending on the culture conditions as well as the abnormality ratio of the newborn calf and post-thaw survival ratio of the frozen blastocysts. This study was performed to find and confirm convenient and appropriate culture methods to avoid the complications such as the overweight calf syndrome which is known to be caused from serum addition, to enhance the quality and production efficiency of the blastocyst, and to increase the pregnancy rate.

MATERIALS AND METHODS

1. *In Vitro* Maturation

The ovaries of Hanwoo were transferred from the butchery to the laboratory, washed more than five times with 25℃ of physiological saline. COCs (Cumulus Ooperus Complexes) were collected from ovarian follicles (2～7 mm). After settling for 10 minutes. the precipitated matters excluding the supernatant were transfered to 60ϕ Petri dish with washing media contained. Excluding the COCs of which cytoplasm is declined extremely, all the COCs including denuded eggs were collected. The collected COCs were washed for five times and the unnecessary cumulus cells were removed. After that, those COCs were transfered to the maturation media and cultured at 38.5℃ in 5% CO_2, and maximum humidity in air. The maturation media was prepared in 4-well dishes in 900μl of culture solution and covered with 250μl of mineral oil. The 80～100 COCs per well were inserted and cultured for 22 hours in the maturation media. The media was prepared by adding 5% FBS (Gibco, 12483) and 0.1mg/ml Libostamycin in Medium-199 (Gibco, 12340) and used for collecting and washing COCs. The culture solution was used for maturation media by adding EGF 30 ng/ml (Sigma, E4127).

2. *In Vitro* Fertilization

Basic BO solution (Brackett *et al.,* 1975) was used as the fertilization media composed of 112.0 mM NaCl, 4.02 mM KCl, 2.25 mM $CaCl_2$, 0.83 mM Na_2HPO_4, 0.52 mM $MgCl_2 \cdot 6H_2O$, 25.0 mM $NaHCO_3$, 1.25 mM Na-Pyruvate, 13.9 mM glucose, 12.5 mM HEPEs, and 10 mg BSA(Sigma, A8806) /ml. Libostamycin was added as 0.1 mg/ml. The basic BO solution to which heparin 10 μg/ml and 5 mM Caffeine-Sodium Benzoate added was used for COCs washing and semen treatment. The COCs were put into the fertilization media after being washed three times with fertilization media. The thawed semen was washed two times at 1,000 rpm for 5min, and then added to the fertilization media, making the number of motil spermatozoa 1×10^6 /ml. The COCs were cultured for 8 hours in the fertilization media and then washed five times in the Medium199 solution to which 5%FBS and antibiotics was added. The presumptive zygotes were then cultured again in the maturation media which had been exchanged with the maturation solution by 50%.

3. Postfertilization Culture

48 hours after fertilization, the embroys over 4 cell stage were selectively put into the culture media which were exchanged for each experiment and cultured. Every 48 hour, 50% of the culture solutions were exchanged for each concentration, and the growth status in each kind of solution was recorded for each age in day. The discrimination of all the fertilized eggs was based on IETS (International Embryo Transfer Society) Guideline. The embryos which grew over the stage of blastocyst were immediately transfered to recipients or frozen and stored.

4. Freezing of Blastocyst and Post-Thaw Culture

The expanded blastocysts that normally developed were frozen between the 6～9th day for the post-thaw culture experiment. The freezing medium was prepared by adding 1.8 M ethylene glycol and 0.1 M sucrose to D-PBS. The freezing was performed first by settling blastocysts in the freezing media for 10 minutes at room temperature. Then, 5～10 blastocysts were put into 0.25 ml straw, then put into the alcohol freezer at −7℃ and then seeded. The temperature was lowered to −30℃ at a rate of −0.3℃ /min and the straw was immersed in liquid nitrogen. For post-thaw survival ratio, the frozen blastocysts

were thawed in warm water at 35℃ for 30 seconds, and then washed for two times with Medium199 solution to which 20% FBS was added. After settling for 1 hour in the same solution (20%FBS), each samples were cultured for 72 hours in the same culture solution in which each of them were cultured before freezing until each of them hatched.

5. Experimental Method

Experiment 1) Cleavage rate was observed in totally four kinds of solutions, which are Medium199 with FBS added by 5% and 10%, and Medium199 with fatty acid free BSA (Sigma, A8806) added by 0.5% and 1%.

Experiment 2) To compare the growth rate to the expanded blastocyst stage after culture, the fertilized eggs at 4 cell stage which were developed in Medium199+5% FBS solution were cultured in 0.3% BSA, 0.5% BSA, 0.7% BSA, 1% BSA, and 2% BSA in Medim199 solutions for 8 days as well as in Medium199+10% FBS solution in the same method, and the development rate and the environment in the culture medium were compared.

Experiment 3) The expanded blastocysts, at the age of 6～9 days, that were developed in each of the methods abovementioned were frozen and the post-thaw survival ratios were compared.

6. Statistics

The COCs were divided equally to each group for all the experiments. The data were derived by six times of repetition and the significance test in *t*-test was conducted to analyze the data.

RESULTS AND DISCUSSION

The initial development ratio for Experiment 1 was shown in Table 1.

According to this result, the development ratio was significantly higher in the case of FBS addition than that of BSA addition, to the extent that it can be concluded that FBS addition is even necessary. The development ratio was dependent on FBS concentration, lower as 33.8% for 3% FBS solution, but higher as 49.2% and 49.8% for 5% and 10% FBS solutions ($p < 0.05$) with no significant difference between the two groups ($p > 0.5$).

This result can be considered as almost equivalent with the results of Im *et al*. (2004) which showed that the development solution and the results of Park *et al*. (2007) showed which

Table 1. Comparison of differentiation rates to 4 cell stage in different medium

Sort of medium (in Medium199)	No. of oocytes	No. of fertilized Ova.	No. of 4 cell (%)
3% FBS	480	378	128 (33.8)[b]
5% FBS	480	390	192 (49.2)[a]
10% FBS	480	389	194 (49.8)[a]
0.5% BSA	480	386	29 (7.5)[c]
1% BSA	480	388	30 (7.7)[c]

[a～c] Values with different superscripts are differrent ($p < 0.05$).

development ratio was 86.4% since the ratio in this experiment is the results of full 4 cell stage fertilized eggs although the numerical value of this study is smaller than their results. The development of the cumulus cells matured for 22 h in 10% FBS solution was more active than that in 5% FBS, but it is regarded not good for the development of ova. This is assumed that because the nutrients such as amino acids, vitamin, growth factors and other unknown contents are necessary during maturation period of the immature ova and the development period after fertilization, while the culture solution which contains only BSA is not appropriate for maturation of the immature ova to be substituted for serum.

The blastocyst production ratio in Experiment 2 is shown in Table 2 and Table 3. As shown in Table 2 and Table 3, there was no difference in blastocyst production ratio depending on the age in day and the BSA concentration in the media of 5% FBS (Table 2) and 10% FBS (Table 3). 5% FBS seems to be the sufficient condition for initial development stage.

The blastocyst production ratio was lower in 0.3% BSA, but increased as the BSA concentration increased from 0.5%. However, there was no difference in the BSA concentration higher than 1%. This result is higher than that of Jaakma *et al*. (1997) which the development ratio in BSAITS until 9th day was 20.7% (19/92) and the result of Palasz *et al*. (2006) the development ratio of the cleaved eggs after fertilization in SOF + 5 mg/ml BSA until 9th day was 38.8%, and the result of Lim *et al*. (2007) which blastocyst production ratio in the defined media containing 8 mg/ml BSA was 35% (blastocysts/cleaved). The results of this study were also better compared to 34.7% of blastocyst production ratio reported by Im *et al*. (2004) and 34.2% by Park *et al*. (2007). In harmony with the result of Rizos *et al*. (2003) the blastocyst production ratio in creased

Table 2. Developmental rates of Hanwoo IVF embryos cultured in different media (Cultured in 5%FBS-Medium199 to 4 cell stages)

Concentrations of BSA (in Medium199)	No. of 4 cell	No. of expanded blastocysts at				Total (%)
		D6	D7	D8	D9	
0.3% BSA	392	9	62	52	28	149 (38.0)[a]
0.5% BSA	408	11	64	68	33	176 (43.1)[b]
0.7% BSA	397	10	65	67	37	179 (45.0)[b]
1% BSA	388	8	65	70	35	178 (45.8)[b]
2% BSA	399	8	59	66	40	173 (43.3)[b]

[a,b] Values with different superscripts are different ($p < 0.05$).

Table 3. Developmental rates of Hanwoo IVF embryos cultured in different media (Cultured in 10%FBS-Medium199 to 4 cell stages)

Concentrations of BSA (in Medium199)	No. of 4 Cell	No. of Expanded Blastocysts at				Total (%)
		D6	D7	D8	D9	
0.3% BSA	395	12	59	57	26	155 (39.2)[a]
0.5% BSA	389	13	64	66	33	169 (43.4)[b]
0.7% BSA	393	13	64	63	36	176 (44.8)[b]
1% BSA	396	12	64	66	41	183 (46.2)[b]
2% BSA	389	12	58	60	43	170 (43.7)[b]

[a,b] Values with different superscripts are different ($p < 0.05$).

as BSA concentration increased. Based on this tendency and the high blastocyst production ratio result, we could conclude that addition of hormones, growth factors and nutrients to the culture solution is not necessary. In addition, the fertilized eggs in 1% BSA culture media did not stick to the cumulus cells settled at the bottom of the culture media, and the fertilized eggs at expanded blastocyst stage in 2% BSA solution floated on the surface of the solution. This means that the osmorality of the culture medium is higher than that of inside of the expanded blastocyst, indicating that BSA concentration above 1% is not needed for the culture after development of the fertilized eggs.

Post-thaw survival ratio of the cryopreserved blastocyst in each experimental group in Experiment 3) is shown in Table 4.

In this experiment, we followed the method using 1.8M ethylene glycol and 0.1M sucrose that according to several reports (Yoon *et al.,* 1999) shows good post-thaw re-expanded rate. The re-expanding rates of the blastocysts cultured in the BSA-containing media were higher at 0.7, 1.0 and 2% BSA concentration than of cultured in serum-containing media. The

Table 4. Re-expansion and hatching rates of frozen-thawed Hanwoo IVF blastocysts cultured in different medium (Cultured in 5% FBS-Medium199 to 4 cell stages) from 4 cell stage to blastocyst

Culture medium (in medium 199)	No. of thawed embryos	No. of re-expanded embryos (%)	No. of hatched embryos (%)	*No. of restricted embryos (%)
0.3% BSA	95	51 (53.7)[a]	23 (24.2)[a]	8 (8.4)
0.5% BSA	102	67 (65.7)[b]	34 (33.3)[b]	5 (4.9)
0.7% BSA	97	69 (71.1)[c]	35 (36.1)[c]	3 (3.1)
1% BSA	98	77 (78.6)[d]	40 (40.8)[d]	1 (1.1)
2% BSA	95	75 (78.9)[d]	38 (40.0)[d]	1 (1.1)
5% FBS	89	56 (62.9)[e]	17 (19.1)[e]	6 (6.7)
10% FBS	99	66 (66.7)[b]	25 (25.3)[a]	3 (3.0)

[a~e] Values with different superscripts in same column are different ($p < 0.05$).

*Number of restricted embryos of hatched embryos.

re-expanding ratio was 71.1%, 78.6% and 78.9% for the solutions of 0.7%, 1% and 2% BSA concentrations, respectively. The development of the hatched blastocyst was better as the concentration of BSA increased. This re-expanding ratio was higher than 55.0% of Yoon *et al*. (1999) and 46.1% of Im *et al*. (2004). It seemed that the compaction of the cell mass inside the blastocyts affects cryotolerance, indicating that cryotolerance is better as the concentration of the culture solution is higher. In addition, the serum-added groups showed better re-expanding ratio when the concentration was higher, with better re-expanding ratio in 1% BSA than that in 10% FBS (66.7: 78.6%). This result also indicates that culture in BSA-containing culture medium is better than serum-containing one.

Experiment 4) As shown in Table 5, there was no significant dependency ($p > 0.5$) on the pregnancy ratio for the transplantation of the fertilized eggs that were cultured in 10% FBS solution and each BSA-containing culture medium while the overall pregnancy ratio was 63.8% with 79.2% of delivery ratio. But, as shown in many reports that there was morphological and physiological abnormality in the embryos cultured in the serum-containing solution, there was a considerably high ratio of abnormality (14%) among the calves. These abnormalities were not found in the cases in which the culture solution containing only BSA was used for the culture after development.

The purpose of ET is to produce healthy calves. Thus, in order to avoid making the efforts of the breeders and produces vain, abnormality in newborn calves should be removed in advance. The overweight calf syndrome among the calves born from the IVF embryos has been reported to be due to accumulation of fat globules inside the cytoplasm caused by the use of serum for the IVF culture medium which leads to decreased cryotolerance for further cryopreservation and abnormality after birth (Behboodi *et al.,* 1995; Walker *et al.,* 1996; Young *et al.,* 1998; Farin *et al.,* 2001). Consistent with these reports, we could not find any abnormality in the cases where serum was replaced with BSA. However, the reason for this needs to be investigated more, whether it is simply the effect of serum, or the effect of the growth factors and hormones or even other unknown effects.

Table 5. Pregnancy rates of transferred fresh embryos cultured in different media to Holstein heifers (Cultured in 5%FBS-Medium199 till 4 cell stages)

Concentrations of BSA	No. of ET	No. of pregnant cows (%)	No. of live calves	No. of great birth weight calves (%)
0.5% BSA	98	66 (67.3)[a]	61	0 (0)
0.7% BSA	108	74 (68.5)[a]	68	0 (0)
1% BSA	85	57 (67)[a]	53	0 (0)
10% FBS	80	52 (65)[a]	50	7 (14)

[a] Value of superscript is not differ ($p > 0.5$).

REFERENCES

Abe H, Yamashita S, Itoh T, Satoh T and Hoshi H. 1999. Ultrastructure of bovine embryos developed from *in vitro*-matured and *in vitro*-fertilized oocytes? comparative morphological evaluation of embryos cultured either in serum-free medirm or serum-supplrmented medium. Mol. Reprod. Dev. 53:325-335.

Abe H, Yamashita S, Satoh T and Hoshi H. 2002. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. Mol. Reprod. Dev. 61:57-66.

Behboodi E, Anderson GB, Bon Durant RH, Cargill SL, Krescher BR, Medrano JF and Murray JD. 1995. Birth of large calves that developed from *in vtro*-derived bovine embryos. Theriogenology 44:227-232.

Boni R, Tosti E, Roviello S and Dale B. 1999. Intercellular communication in *in vivo*-and *in vitro*-produced bovine embryos. Biol. Reprod. 61:1050-1055.

Brackett BB and Oliphant G. 1975. Capacitation of rabbit spematozoa *in vitro*. Biol. Repod. 13:260-274.

Carolan C, Lonergan P, Khatir H and Mermillod P. 1996. *In vitro* production of bovine embryos using individual oocytes. Mol. Reprod. Dev. 45:145-150.

Crosier AE, Farin PW, Dykstra MJ, Alexander JE and Farin CE. 2001. Ultrastructural Morphometry of bovine blastocysts produced *in vivo* or *in vitro*. Biol. Reprod. 64:1375-1385.

Crosier AE, Farin PW, Dykstra MJ, Alexander JE and Farin CE. 2000. Ultrastructural morphometry of bovine compact mourlae produced *in vivo* or *in vitro*. Biol. Reprod. 62: 1459-1465.

Dorland M, Gardner DK and Trounson AO. 1994. Serum in synthetic oviduct fluid causes mitochondrial degeneration

in ovine embryos. J. Reprod. Fertil. 13:70.

Duby RT, Hill JL, O'Callaghan D, Overstrom EW and Boland MP. 1997. Changes induced in the bovine zona pellucida by ovine and bovine oviducts. Theriogenology 47:332.

Fair P, Lomergan P, Dinnyes A, Cottell D, Hyttel P, Ward FA and Boland MP. 2001. Ultrastructure of bovine blastocysts followong cryopreservation: effect of method of embryo production on blastocyst quality. Mol. Reprod. Dev. 58: 186-195.

Farin PW, Crosier AE and Farin CE. 2001. Influnce of *in vitro* systems on embryo survival and fetal development in cattle. Theriogenology 55:151-170.

Gardner DK, Lane M, Spitzer A and Batt PA. 1994. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. Biol. Reprod. 50:390-400.

Gutierrez-Adan A, Lonergan P, Rizos D, Ward FA, Boland MP, Pintado B and De La Fuente J. 2001. Effect of the in vitro culture system on the kinetics of blastocyst development and sex ratio of bovine embryos. Theriogenology 55:1117-1126.

Im YJ, Kim JH, Song HB and Jung YG. 2004. Transfer, cryopreservation and production of bovine embryos cultured in serum-free system. J. Emb. Trans. 19:133-145.

Jaakma U, Zhang BR, Larsson B, Niwa K and Rodriguez-Martinez H. 1997. Effects of sperm treatments on the *in vitro* development of bovine oocytes in semidefined and defined media. Theriogenology 48:711-720.

Krisher RL, Lane M and Bavister BD. 1999. Developmental competendce and metabolism of bovine embryos cultured in semi-defined and defined culture media. Biol. Reprod. 60:1345-1352.

Lim KT, Jang G, Ko KH, Lee WW, Park HJ, Kim JJ, Lee SH, Hwang WS, Lee BC and Kang SK. 2007. Improved *in vitro* bovine embryo development and increased efficiency in producing viable calves using defined media. Theriogenology 67:293-302.

Lonergan P, Khatir H, Piumi F, Rieger D, Humblot P and Boland MP. 1999a. Effect of time interval from insemination to first cleavage on the developmental characteristics, sex and pregnancy rates following transfer of bovine preimplantation embryos. J. Reprod. Fertil. 117:159-167.

Lonergan P, O'Kearney-Flylnn M and Boland MP. 1999b. Effect of protein supplementation and presence of an antioxidant on the development of bovine zygotes in synthetic oviduct fluid medium under high or low oxygen tension. Theriogenology 51:1565-1576.

Mucci N, Aller J, Kaiser GG, Hobor F, Cabodvila J and Alberio RH. 2006. Effect of estrous cow serum during bovine embryo culture on blastocyst development and cryotolerance after slow freezing or vitrification. Theriogenology 65:1551-1562.

Palasz AT, Rodriguez-Martinez H, Beltran-Breña P, Perez-Garnelo S, Martinez MF, Gutierrez-Adan A and De la Fuente J. 2006. Effects of hyaluronan, BSA, and serum on bovine embryo *in vitro* development, ultrastructure, and gene expression patterns. Mol. Reprod. Dev. 73:1503-11.

Park SJ, Takahashi Y, Park SB, Baek KS,. Ahn BS, Jeon BS, Ryu IS and Kim HS. 2007. Maturation, embryonic development, and subsequent embryo survival after freezing and thawing following a single or group culture condition of japanese black cattle oocytes using modified synthetic oviduct medium. J. Emb. Trans. 22:21-26.

Pinyopummintr T and Bavister BD. 1994. Development of bovine embryos in a serum-free medium; effects of type of serum, timing of its inclusion and heat inactivatation. Theriogenology 41:1241-1249.

Rizos D, Gutiérrez-Adán A, Pérez-Garnelo S, De La Fuente J, Boland MP and Lonergan P. 2003. Bovine embryo culture in the presence or absence of serum; implication for blastocysts development, cryotolerance, and messenger RNA expression. Biol. Reprod. 68:236-243.

Rizos D, Ward F, Duffy P, Boland MP and Lonergan P. 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*; implications for blastocysts quality. Mol. Reprod. Dev. 61:234-248.

Rosenkrans CF Jr and First NL. 1994. Effects of free amino acids and vitamins on cleavage and development rate of bovine zygotes *in vitro*. J. Anim. Sci. 72:434-437.

Thompson JG, Allen NW, McGowan LT, Bell AC, Lambert MG and Tervit HR. 1998. Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryo development *in vitro* and following transfer. Theriogenology 49:1239-1249.

Thompson JG, Gardner DK, Pugh PA, McMillan WH and Tervit HR. 1995b. Lamb birth weight is affected by culture system utilised during *in vitro* pre-elongation development of ovine embryos, Biol. Reprod. 53:1385-1391.

Thomson JG. 1995a. Defining the requirements for bovine embryo culture. Theriogenology 45:27-40.

Van Langendonckt A, Donnay I, Schuurbiers N, Auquier P, Caroan C, Massip A and Dessy F. 1997. Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium. J. Reprod. Fetil. 109:87-93.

Viuff D, Rickords L, Offenberg H, Hyttel P, Avery B, Greve T, Olsaker I, Williams JL, Callesen H and Thomsen PD. 1999. A high proportion of bovine blastocysts produced *in vitro* are mixoploid. Biol. Reprod. 60:1273-1278.

Walker SK, Hartwich KM and Seamark RF. 1996. The production of unusually large offspring following embryo manipulation: concepts and challenges. Theriogenology 45: 111-120.

Yoon JT, Lee HJ, Roh S, Jung YG, Park YS, Choi EJ, Lee JW, Kim YY and Jung HY. 1999. Effect of culture conditions on survival of frozen-thawed blastocysts fertilized *in vitro*. J. Emb. Trans. 14:163-169.

Young LE, Sinclair KD and Wilmut I. 1998. Large offspring syndrome in cattle and sheep. Rev. Reprod. 3:155-163.

(접수일: 2009. 12. 4 / 채택일: 2009. 12. 25)