Korean J. Animal Reprod. 27(4) : 339~347 (2003)

Developmental Potential of Bovine Nuclear Transfer Embryos Cultured in Serum-free Medium

Lee, S. K.[1,2], D. H. Kim[1], G. S. Im[1], B. C. Yang[1], H. S. Park[1], W. K. Chang[1] and H. T. Lee[2†]

[1]Animal Biotechnology Division, National Livestock Research Institute. RDA., Suwon, Korea

ABSTRACT

The purpose of this study was to investigate the development of bovine nuclear transfer (NT) embryos cultured in serum-free conditions. Bovine NT embryos cultured in various culture conditions were compared blastocyst development, total cell number and apoptosis using TUNEL assay. In experiment 1, blastocyst rates of NT embryos were significantly higher ($P<0.01$) in FBS (22.0%) and BSA (26.6%) groups than in PVA (6.3%) group. Total cell number was significantly higher in FBS (78.4±19.4) and BSA (90.9±29.1) groups than in PVA group (46.0±0.0). Apoptotic cell number was significantly fewer in FBS (3.1±1.4) and BSA (1.7±1.4) groups than in PVA group (7.0±20.0) However, all of results were not different between the FBS and BSA group. In experiment 2, blastocyst rates of NT embryos were significantly higher ($P<0.05$) in fatty acid free-BSA (FAF-BSA) group (26.8%) than in fraction V-BSA group (11.2%). Total cell number were somewhat higher in FAF-BSA group (89.8±30.7) than in fraction V-BSA group (88.1±19.3). Apoptotic cell number were somewhat fewer in FAF-BSA (1.7±1.5) group than in fraction V-BSA group (4.2±2.9). These findings suggest that serum free condition were effective for the *in vitro* development of bovine NT embryos. Therefore, we concluded that fatty acid free-BSA has beneficial effect in development bovine NT embryos and can be use as a serum substitute.

(Key words : Nuclear transfer, FBS, BSA, PVA, Apoptosis)

Ⅰ. INTRODUCTION

The overall efficiency of somatic cell nuclear transfer (NT) in cloning animal is very low (Kato et al., 1998; Schnieke et al., 1997; Wells et al., 1999). Moreover, somatic cell nuclear transfer has rised severe developmental failures including high rate of abortion during early gestation and incredaed perinatal death (Hill et al., 1999; Hill et al., 2000; Schnieke et al., 1997; Wilmut et al., 1997). Even the surviving offsprings show large placenta (Hill et al., 1999; Wakayama et al., 1998) and increased birth weights (Eggan et al., 2001) referred to as "large offspring syndrome (LOS)". The LOS is a typical phenotype observed in cloned neonates

These authors contributed equally to this work.

† Correspinding author : Hoon-Taek Lee, Animal Resources Research Center, Konkuk University, Seoul, Korea, E-mail : htl3675@konkuk.ac.kr

[2] Animal Resources Research Center, Konkuk University, Seoul, Korea

of mammalian species such as cattle, mouse and sheep, but the factors responsible for LOS still remain elusive. It has been reported that the LOS were attributed to variety of *in vitro* culture procedures before embryo transfer (Eggan et al. 2001; Sinclair et al., 2000 Young et al., 2000).

In general, bovine embryos are cultured in media supplemented with serum. Several studies have shown that serum has a biphasic effect; the presence of serum can inhibit the early cleavage divisions, while it can have an accelerating effect later in development (Carolan et al., 1996; Langendonckt et al., 1997; Thomson et al., 1998; Gutierrez et al., 2001). This phenomenon has been exploited by several laboratories to maximize the yield of blastocysts following *in vitro* fertilization by adding serum to serum-free medium at approximately day 2 to 4 of development (Carolan et al., 1995; Massip et al., 1996; Langendonkt et al., 1996; Pinyopummintr et al., 1996). However, data on the development of sheep and cattle blastocysts in the presence of serum have raised serious issues regarding the use of serum for embryo culture. Serum can adversely affect the development of embryos at several levels: precocious blastocoel formation, sequestration of lipid, abnormal mitochondria ultrastructure, perturbations in metabolism, and association with abnormally large offspring (Gardner and Lane, 1999). Removing serum and replacing them with BSA as a protein source in a variety of culture media has resulted in good embryo development (Takahashi et al., 1992; Rosenkrans et al., 1994; Liu et al., 1995; Edwards et al., 1997). Study in early mouse embryos revealed a reduced embryotoxicity through addition of BSA to the culture medium (Flood et al., 1991).

On the basis of previous reports, it is postulates that serum free condition may be improve the viability of somatic cell nuclear transfered bovine embryos. Therefore, the objectives of the present study were to investigate first to compare the development of NT bovine embryos with various culture conditions such as protein source and BSA source, second to compare the cell number and apoptosis of these NT bovine embryos.

Ⅱ. MATERIALS AND METHODS

1. *In Vitro* Maturations (IVM) of Bovine Oocytes

Bovine ovaries obtained from slaughterhouse were transported in saline at 37℃ to the laboratory. Cumulus oocytes complexes (COCs) were aspirated from follicles 2~6 mm in diameter, washed in HEPES-buffered TCM 199 (H-TCM), and selected on the basis of their morphology for *in vitro* maturation according to the density of their cumulus cell layers. For *in vitro* maturation, COCs were incubated in TCM 199 (GIBCO, USA) supplemented with 10% fatal bovine serum (FBS; GIBCO BRL, USA), 1 ㎍/ml FSH, and 1 ㎍/ml E_2 for 20~22 h at 39℃ in a humidified atmosphere of 5% CO_2 in air.

2. Preparation of Bovine Ear Skin Fibroblast

A small ear skin biopsy was obtained from 2-year-old Korean native cattle (Hanwoo). Ear skin fibroblast cells were cultured on 50 ml tissue culture flask (Falcon, USA) in Dulbecco's Modified Eagles's medium (DMEM; Gibco BRL, USA) supplemented with 10% FBS. After 5 days of culture, the cells were trypsinized with 0.05% trypsin and washed three time with Ca^{2+}, Mg^{2+}-free PBS (Gibco BRL, USA) by centrifugation at 300×g for 5 min. The pellet was resuspended and cultured in 50 ml tissue culture flasks in DMEM containing 10% FBS. Ear skin fibroblast cells were cultured over several passage (2 to 12) and used as donor cells for nuclear transfer.

3. Nuclear Transfer (NT) of Bovine Oocytes

Enucleation and nuclear transfer procedure of bovine oocytes were carried out as described previously (Im et al., 2001). The oocytes were placed in a 30 ㎕ drop of H-TCM 199 supplemented with 20% FBS and 10 ㎕/ml phytohemaglutinin (PHA; Sigma, USA). An oocyte was held by holding pipette (approximately 90～120 ㎛). Enucleation of oocyte was carried out by cutting a part of zona pellucida near the first polar body. A small volume of cytoplasm surrounding the polar body was squeezed out through the slit which was made at enucleation with cutting needle. After manipulation, enucleated oocytes were incubated in H-TCM 199 containing 20% FBS until injection of donor cells. Enucleation was confirmed by staining enucleated polar body and cytoplasm with 2 ㎍/ml Hoecthst 33342 (Sigma, USA).

Enucleated oocytes were placed in a 30 ㎕ drop of the same medium as the enucleation, and donor cells were placed in a 20 ㎕ drop of Ca^{2+}, Mg^{2+}-free PBS supplemented with 0.5% FBS. Donor cells were introduced into the perivitelline space of the enucleated oocytes through slit made at enucleation by injection pipette (approximately 15～20 ㎛).

4. Fusion and Activation

Fusion between karyoplast and cytoplast was induced by electric pulse. The karyoplast and cytoplast complexes were placed in the Zimmerman cell fusion medium for equilibration and then placed between the electrofusion needles. Cell fusion was induced with a single DC pulse of 25 V/mm for 10 ㎲ec. After the fusion treatment, the karyoplast-cytoplast complexes were washed in H-TCM 199 supplemented with 20% FBS, and the fusion was evaluated by microscopic examination after 30 min. The fused embryos were activated in CR2 medium containing 1.5 mg/ml BSA, 5% FBS and 10㎛M calcium ionophore for 5 min, and followed by 2 mM 6-dimethylaminopurine (DMAP) for 3 h.

5. *In Vitro* Culture Systems

After the activation treatment, fused embryos were washed three times with CR2 medium and allocated into each experiment medium.

In experiment 1, Fused embryos were cultured in 10 ㎕ drops of CR2 medium supplemented with 10% FBS, 0.5% Fatty acid free BSA (FAF-BSA; Sigma, USA) and 0.1% polyvinyl alcohol (PVA; Sigma, USA). In experiment 2, Fused embryos were cultured in 10 ㎕ drops of CR2 medium supplemented with 0.5% fraction Ⅴ-BSA or 0.5% FAF-BSA at 39℃ in 5% O_2, 5% CO_2, and 90% N_2 humidified air.

6. Detection of Apoptotic Cells by Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End-Labeling (TUNEL) and Propidium Iodide Labeling

Zona pellucida intact Day 7 blastocyst were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature. The blastocysts were washed twice in PBS containing 0.3% polyvinyl pyrrolidone (P-PBS). Fixed blastocysts permeabilized with 0.5% Triton X-100 in PBS for 30 min at room temperature. The blastocyst were washed twice in P-PBS and were placed in to 20 ㎕ drops of FITC labelled TUNEL reagent (Roche Molecular Biochemicals, USA) covered with mineral oil and incubated in the dark at 37 ℃ for 1 h. The blastocysts were washed with 0.5% Trition X-100 in PBS (for 5 min) and followed by P-PBS. For counterstain, the blastocysts were incubated in PBS containing RNase A (50 ㎕/ml) for 1 h at room temperature and counterstained with propidium iodide (10 ㎍/ml) in dark for 1 h at room temperature. After washed twice in P-PBS, blastocysts were mounted in vectashield antifade medium (Vector Lab, USA). The evaluation was performed

by laser scanning confocal microscopy (Olympus, Japan). FITC-labelled nuclei were counted as apoptotic cells and PI-labelled nuclei were counted as total cell (Fig. 1).

7. Statistical Analysis

The statistical difference among treatment groups in each experiment was determined by using General Linear Models Procedure and *t*-test of SAS.

Ⅲ. RESULT

1. *In Vitro* Development of Bovine NT Embryos according to Protein Sources

In vitro development of bovine NT embryos according to protein sources were shown in Table 1. The cleavage rates of NT embryos cultured in CR2 medium supplemented with 10% FBS, 0.5% BSA and 0.1% PVA were 65.4, 81.3 and 66.9%, respectively. Developmental rates upto blastocysts were 22.0, 26.6 and 6.3%, respectively. The development of bovine NT embryos was significantly higher in the culture medium supplemented with FBS and BSA when compared to the culture medium supplemented with PVA ($P<0.01$).

2. Total and Apoptotic Cell Number according to Protein Sources

Bovine NT blastocysts produced by different protein sources were analysed total and apoptotic cell numbers, Total cell number in 10% FBS (78.4±19.4) and 0.5% BSA (90.9±29.1) were significantly ($P<0.05$) higher than in 0.1% PVA (46±0.0) (Fig. 2). Apoptotic cell number in 0.1% PVA (7.0±0.0) were significantly ($P<0.05$) higher compared to 10% FBS (3.1±1.4) and 0.5% BSA (1.7±1.4) (Fig. 2) And also apoptotic index in 0.1%

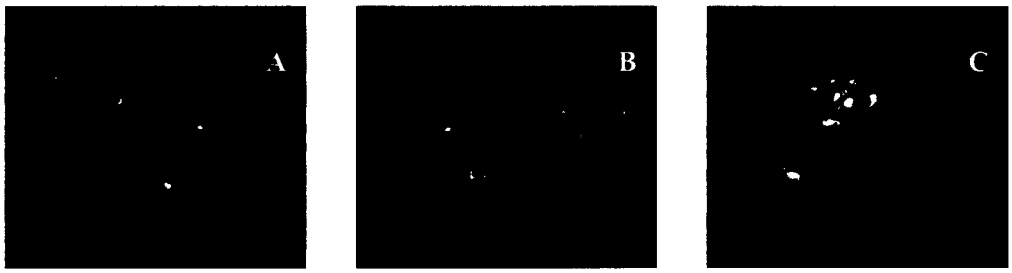

Fig. 1. Confocal microscopic images of TUNEL in bovine NT blastocysts. Blastocysts were developed in (A) 10% FBS, (B) 0.5% BSA, (C) 0.1% PVA and labeled with fluorescein isothiocyanat (FITC)-coniugated dUTP (green) and propidium iodide (red).

Table 1. The effect of protein sources on the development of bovine NT embryos

Protein source	Fused oocytes	Cleaved (%)	Blastocyst (%)
10% FBS	157	100 (65.4)	32 (22.0)[a]
0.5% BSA*	138	111 (81.3)	36 (26.4)[a]
0.1% PVA	115	76 (66.9)	8 (6.3)[b]

* Fatty acid free-BSA.

[a,b]: Multiple Range Test ($P<0.01$).

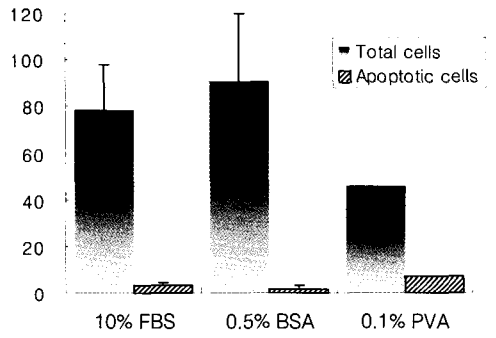

Fig. 2. Total and apoptotic cell numbers in bovine NT blastocyst cultured with various protein sources(P<0.05).

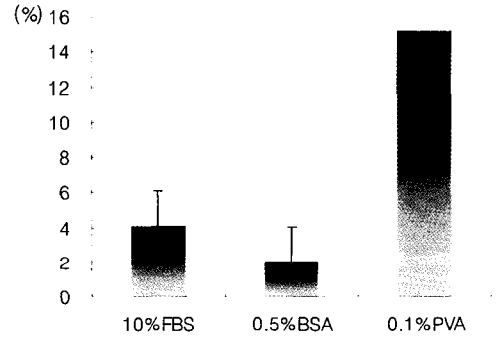

Fig. 3. Apoptotic index in bovine NT blastocyst cultured with various protein sources (P<0.05).

PVA (15.2%) was significantly (P<0.05) higher than those in 10% FBA (4.1%) and 0.5% BSA (2.0%) (Fig. 3).

3. *In Vitro* Development of Bovine NT Embryos Cultured with Different BSA Sources

In vitro development of NT embryos cultured with different were shown in Table 2. The cleavage rates of NT embryos cultured in CR2 medium supplemented with 0.5% FAF-BSA or fraction V-BSA were 81.3 and 67.5%, respectively. Developmental rates upto blastocysts were 26.8 and 11.2%, respectively. The development of bovine NT embryos was significantly higher in the culture medium supplemented with FAF-BSA when compared to the culture medium supplemented with fraction V-BSA (P<0.05).

4. Total and Apoptotic Cell Number according to BSA Sources

Total and apoptotic cell number in bovine NT blastocysts produced by different BSA sources were analysed.

In apoptotic cell number, there was no difference between the FAF-BSA (1.7±1.5) and fraxtion V-BSA (4.2±2.9) groups (Fig. 4). Apoptotic index was also not different between the FAF-BSA (2.1%) and fraction V-BSA (6.0%) groups (Fig. 5).

Ⅳ. DISCUSSION

The results of the present study demonstrate that fatty acid free-BSA can be use for *in vitro* production of NT bovine embryos, as a serum substitute. Changing culture supplement from serum to BSA had previously been shown to reduce the frequency of LOS (Thompson et al., 1995; Wagtendonk et al., 2000). Serum have been replaced by macromolecules such as BSA and PVA to generate

Table 2. The effect of BSA sources on the development of bovine NT embryos

BSA source	Fused oocytes	Cleaved (%)	Blastocyst (%)
0.5% FAF*	122	98 (81.3)	32 (26.8)
0.5% Fraction V	158	108 (67.5)	20 (11.2)

*FAF: Fatty acid free.
P<0.05.

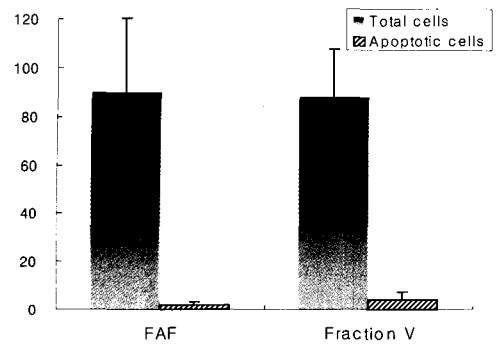

Fig. 4. Total and apoptotic cell number in bovine NT blastocyst cultured in FAF-BSA and Fraction V-BSA.

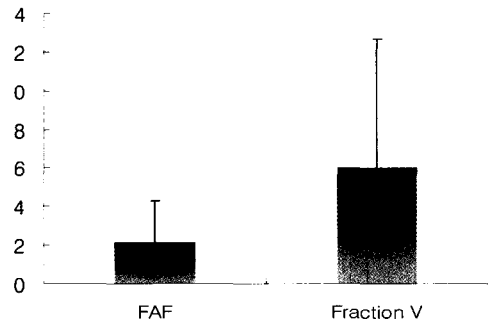

Fig. 5. Apoptotic index in bovine NT bovine blastocyst cultured in FAF-BSA and Fraction V-BSA.

chemically media without detrimental effects on early embryonic development. The complete removal of serum from culture protocol has been challenging, and was apply to embryo culture media. In this study, supplementation of BSA to the culture medium did not changed in development rates of bovine NT embryos.

The process of apoptosis cell death in preimplantation mammalian embryos has been well described. Apoptosis plays an important role in embryo development (Levy et al., 2001; Feugang et al., 2002; Gjorret et al., 2002). The incidence of apoptosis was higher in bovine blastocysts produced *in vitro* than in embryos produced *in vivo* (Feugang et al., 2002). Generally assessment of embryo apoptosis cell death has been carried out using TUNEL. This method facilitates the analysis of nuclear DNA fragmention *in situ*. This technique has proved useful in the quantitative measurement of cell death in mouse and human embryos in which only a few cells are present and therefore small amounts of apoptotic DNA (Jurisicova et al., 1996; Pampfer et al., 1997; Brison et al., 1998). Research has suggested that a major cause for the level of cell death can be reconciled with the high level of embryo arrest.

In this study, fragmented DNA detected by the TUNEL method was investigated in bovine NT embryos. IVP bovine embryos is associated with apoptotic cell death and that the extend of apoptosis is affected by *in vitro* culture conditions (Byrne et al., 1999). Apoptosis may also be related to 'embryo quality' Although it is not possible to measure the number of blastocyst cells before embryo transfer, it is thought that embryos with a large number of cells are more likely to implant and give rise to live offspring (Van Soon et al., 1997). In this study, NT blastocysts produced culture medium supplemented with PVA showed lower developmental rates, reduced total number and high incidence of apoptosis when compared to NT blastocysts produced from culture medium supplemented with BSA and FBS. These results suggest that PVA is not a good supplement for the culture of bovine NT embryos.

In vitro culture a part of failed development may be attributed to embryo culture containing high serum concentrations. (Thompson et al., 1995). Recently, it is reported that the presence of serum in the medium significantly reduced the cryotolerance of blastocyst (Rizos et al., 200) and increased large offspring syndrome (Lazzari et al., 2002), due to deviations of the relative abundance of developmentally important gene transcripts. Thus the

omission of serum during the culture period may be improve the developmental capacity of bovine NT blastocysts. Further studies are needed to investigate post-implantation development of bovine NT blastocyst produced from serum-free culture condition.

In this study, our results suggest that serum free condition is effective for the *in vitro* development of bovine NT embryos. Therefore, it is concluded that fatty acid free-BSA has beneficial effect in development of bovine NT embryos and can be use as a serum substitute.

V. REFERENCES

1. Brison, D. and Schultz, R. 1998. Increased incidence of apoptosis in transforming growth factorα-deficient mouse blastocyst. Biol. Reprod., 59:136-144.
2. Byrne, A., Southgate, J., Brison, D. and Leese, H. J. 1999. Analysis of apoptosis in the preimplantation bovine embryo using TUNEL. J. Reprod. Fetil., 117:97-105.
3. Carolan, C., Lonergan, P., Van Langendonckt, A. and Mermillod, P. 1995. Factors affecting bovine embryos development in Synthetic Oviduct Fluid following oocyte maturation and fertilization *in vitro*. Theriogenology, 43:1115-1128.
4. 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.
5. Edwards, L., Batt, P. A., Gandolfi, F. and Gardner, D. K. 1997. Modifications made to culture medium by bovine oviduct epithelial cells: changes to carbohydrates stimulate bovine embryos development. Mol. Reprod. Dev., 46: 146-154.
6. Eggan, K., Akutsu, H., Loring, J., Jackson-Grusby, Klemm, M. and Rideout, W. M. 2001. Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. Proc. Natl. Acad. Sci. USA., 98;6209-14.
7. Flood, L. P. and Shirley, B. 1991. Reduction of embryostoxicity by protein in embryos culture media. Mol. Reprod. Dev., 30:226-231.
8. Feugang, J. M., Roover, D. E., Leonard, S., Dessy, F. and Donnay, I. 2002. Kinetics of apoptosis in preimplantation bovine embryos produced *in vitro* and *in vivo*. Theriogenology, 57:494.
9. Gardner, D. and Lane, M. 1999. Development of Viable mammal embryos *in vitro*: Evolution of sequential media. In "Handbook of Principles of Cloning," 9nd Ed. (Gardner DK and Lane M, eds), pp. 188-208. AP press.
10. Gjorret, J., Wengle, J., King, W. A., Schllander, K. and Hyttel, P. 2002. Occurrence of apoptosis in bovine embryos reconstructed by nuclear transfer or derived *in vivo*. Theriogenology, 57:495.
11. Gutierrez-Adan, A., Lonergan, P., Rizos, D., Ward, F. A., Boland, M. P., Pintado, B. and 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.
12. Hill, J. R., Roussel, A. J., Cibelli, E. J., Hooper, N. L. and Miller, M. W. 1999. Clinical and pathologic features of cloned transgenic claves and fetuses. Theriogenology, 51:1451-65.
13. Hill, J. R., Winger, Q. A., Looney, C. R., Thompson, J. A. and Westhusin, M. E. 2000. Development rates of mail bovine nuclear transfer embryos derived from adult and fetal cells. Biol. Reprod., 62:1135-40.
14. Im, G. S., Yang, B. S., Yang, B. S., Chang, W. K., Yi, Y. J. and Park, C. S. 2001. Effect of cell cycle stage on the development of embryos produced by cumulus cell nuclear transfer in

Hanwoo (Korea cattle). Asian-Ausu. J. Anim. Sci., 14:759-764.

15. Jursicova, A., Varmuza, S. and Casper, R. F. 1996. Programmed cell death and human embryo fragmentation. Mol. Hum. Reprod., 2: 93-98.
16. Kato, Y., Sotomaru, Y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H. and Tsunoda, Y. 1998. Eight Calves Cloned from Somatic Cells of a single Adult. Science, 282: 2095- 2098.
17. Langendonckt, A. V., Donnay, I., Schuurbiers, N., Auquier, P., Carolan, C., Massp, A. and Dessy, F. 1997. Effect of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium. J. Reprod. Fertil., 109:87-93.
18. Langendonk, A., Auquier, P., Donnay, I. and Massip, A. 1996. Acceleration of *in vitro* bovine embryo development in the presence of fetal calf serum. Theriogenology, 45:194 abstr.
19. Lazzari, C., Wrenzycki, C., Herrmann, D., Duchi, R., Kruip, T., Niemann, H. and Galli, C. 2002. Cellular and molecular deviations in bovine *In vitro*-produced embryos are related to the large offspring syndrome. Biol. Reprod., 67:767-775.
20. Levy, R. R., Cordonier, H., Czyba, J. C. and Gocrin, J. E. 2001. Apoptosis in preimplantation mammalian embryo and genetics. Ital. J. Anat. Embryo., 106(suppl 2):101-108.
21. Liu, Z. and Foote, R. H. 1995. Development of bovine embryos in KSOM with added superoxide dismutase and trurine and five and twenty-five percent O_2. Biol. Reprod., 53 : 786-790.
22. Massip, A., Mermillod, P., Langendonkt, A., Reichenbach, H. D., Lonergan, P., Berg, U., Carolan, C. and Roover, R. 1996 Calving outcome following transfer of embryos produced *in vitro* in different condictions. Amim. Reprod. Sci., 44:1-10.
23. Pinyopummintr, T. and Bavister, B. D. 1996. Effect of amino acids on development *in vitro* of cleavage stage bovine embryos into blastocyst. Reprod. Fertil. Dev., 8:835-841.
24. Pampfer, S., Vanderheyden, I., McCracken, J., Vasela, J. and Hertogh, R. D. 1997. Increased cell death in rat blastocysts exposed to maternal diabetes in utero and to high glucose or tumour necrosis factor-α *in vitro*. Development, 124: 4827-4836.
25. Rizos, D., Gutierrez-Adan., Garnelo, P., Fuente, J., Boland, M. and Lonergan, P. 2003. Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. Biol. Reprod., 68:236-243.
26. Rosenkrans, C. F. and First, N. L. 1994. Effect of free amino acid and vitamins on cleavage and developmental rate of bovine zygotes *in vitro*. J. Anim. Sci., 71:434-437.
27. Schnieke, A. E., Kind, A. J., Ritchie, W. A., Mycock, Scott, A. R., Ritchie, M., Willmut, I., Colmon, A. and Campbell, K. H. 1997. Human Factor Ⅸ Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts. Science, 278:2130-2133.
28. Sinclair, K. D., Young, L. E., Wilmut, I. and McEvoy, T. G. 2000. In-utero overgrowth in ruminants following embryo culture: lessons from mice and warming to men. Hum. Reprod., 15 (suppl) 5:68-86.
29. Takahashi, Y. and First, N. L. 1992. *In vitro* development of bovine one-cell embryos: influence of glucose, lactate, pyruvate amino acids and vitamin. Theriogenology, 37:963-978.
30. Thompson, J. G., Gardner, D. K., Pugh, P. A., McMillan, W. H. and Tervit, H. R. 1995. Lamb birth weight is affected by culture system utilized during *in vitro* pre-elongation develop-

ment of ovine embryos. Blol. Reprod., 53 : 1385-1391.

31. Thompson, J. G., Allen, N. W., McGowan, L. T., Bell, A. C. S., Lambert, M. G. and Tervit, H. R. 1998. Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryos development *in vitro* and following transfer. Theriogenology, 49:1239-1249.
32. Van Soon, A., Ysebaert, M. and Kruif, A. D. 1997. Relationship between timing of development, morula morphology and cell allocation to inner cell mass and trophectoderm in *in vitro* produced embryos. Mol. Reprod. Dev., 47:47-56.
33. Wakayama, T., Perry, A. C., Zuccotti, M., Johson, K. R. and Yanagimachi, R. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature, 394: 369-74.
34. Wagtendonk-de., Leeu, A. M., Mullaart, E., Roos, A. P. W., Merton, J. S., Den Dass, J. H. G., Kemp, B. and Ruigh, L. 2000. Effect of different reproduction techniques: AI, MOET or IVP, health and welfare of bovine offspring. Theriogenology, 53:575-597.
35. Wells, D. N., Misica, P. M. and Tervit, H. R. 1999. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. Biol. Reprod., 60:996-1005.
36. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. and Cambell K. H. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature, 385:810-3.
37. Young, L. E. and Fairburn, H. R. 2000. Improving the safety of embryos technologies: possible role of genomic imprinting. Theriogenology, 53:627-48.

(Received October 21, 2003;
Accepted November 15, 2003)