# *IN VITRO* DEVELOPMENT OF BOVINE ONE-CELL EMBRYOS FERTILIZED *IN VITRO* IN SERUM- AND FEEDER CELL-FREE CULTURE SYSTEMS

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## Summary

The purpose of this study was to evaluate some factors in the bovine embryonic development from one-cell to blastocyst using modified synthetic oviduct fluid medium (mSOFM), after maturation and *in vitro* fertilization of the oocytes. The embryonic development to the blastocyst stage was assessed at 7-10 days after *in vitro* fertilization, and the total cells in the blastocysts were counted by staining nuclei with fluorochrome. Some commercial calf sera (CS) and a superovulated cow serum had different effects on the embryonic development to the blastocyst stage (8.6-21.4%), dependent upon their product lots, although the development might not be affected at least by serum progesterone levels. $\beta$-Mercaptoethanol ($\beta$-ME) supplemented into mSOFM was effective to the embryonic development (27.8%), as well as the co-culture system with cumulus cells (19.5%). In a serum- and feeder cell-free culture using mSOFM containing several growth factors and $\beta$-ME instead of CS plus co-cultured cumulus cells, bovine serum albumin (BSA, fraction V), but not polyvinyl alcohol (PVA), was highly effective in embryonic development to the blastocyst stage, almost comparable to CS in the serum-contained culture (CS, BSA and PVA; 27.8, 19.5 and 5.7%, respectively). However, fatty acid free BSA rather reduced the number of developed blastocysts, compared with fraction V BSA (7.3 vs 29.4%). In the serum- and feeder cell-free culture, supplement of glucose to the medium (final 2.0 mM) stimulated the cell proliferation of developing embryos 120 hr after *in vitro* fertilization. These results indicated that a serum-free medium supplemented with $\beta$-ME could successfully support the development of bovine one-cell embryos to the blastocyst stage. Moreover, supplement of glucose and fatty acids to the medium might support preferably the development and cell proliferation of embryos.

(**Key Words** : Bovine Embryonic Development, Serum-free Medium, $\beta$-Mercaptoethanol, Bovine Serum Albumin)

## Introduction

Bovine morulae and blastocysts have been routinely obtained from *in vitro* matured and fertilized oocytes in many laboratories. In many cases, culture media for embryo development supplemented with sera and various somatic cells were used as co-culture systems to overcome the developmental block that occurs *in vitro* at the 8-16 cell stage (Goto et al., 1988; Eyestone and First, 1989). On the other hand, several workers have cultured one-cell bovine embryos *in vitro* up to the blastocyst stage without using sera and/or co-culture systems (Pinyopummintr and Bavister, 1991; Kim et al., 1993; Takagi et al., 1993; Keskintepe et al., 1995). Comparison of these culture conditions would enable the isolation and identification of any embryotrophic components.

A study on sheep embryos revealed that the blastocysts developing in medium containing serum have apparent differences in embryo metabolism and morphology, compared with those cultured in serum-free medium and the suitability of serum as a nitrogen source is questionable for embryo development (Gardner et al., 1994). Energy substrates and protein sources have

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Received March 4, 1996
Accepted June 13, 1996

important roles in the *in vivo* and *in vitro* development of mammalian embryos with glucose being one of the most significant factors (Leese, 1992; Rieger, 1992).

Takahashi et al. (1993) and Hamano et al. (1994) reported that the addition of β-mercaptoethanol (β-ME) to culture media facilitates culture of bovine embryo through the *in vitro* developmental block in the absence of any feeder cells, and calves were born after the developed embryos were transfered to recipients. However, these authors did not estimate the quality of the cultured embryos.

In the present study, the effects of protein sources, β-ME or glucose on the development of one-cell *in vitro* fertilized embryos was evaluated. The aim was to develop blastocysts *in vitro* from bovine one-cell embryos without serum and feeder cells, using a reliable and simple medium.

## Materials and Methods

### *In vitro* maturation and fertilization of oocytes

Bovine ovaries obtained at a local abattoir were transported to the laboratory in physiological saline containing antibiotics at 30-35℃ within 2 hr. Follicular oocytes were aspirated from follicles of 1-8 mm diameter. Oocytes with more than two-thirds of their surface surrounded by cumulus cells were used for maturation and culture. The maturation medium consisted of SFRE 199-2 (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% calf serum (CS; Gibco, Grand Island, NY, USA), 5 μg/ml insulin (Sigma) and 50 μg/ml gentamycin sulfate (Sigma). Approximately 50 to 120 cumulus-oocyte-complexes (COCs) were transferred to 2.5 ml of maturation medium in a 35 mm culture dish (Costar, Cambridge, MA, USA) and cultured for 24 hr at 39℃ under a humidified gas phase of 5% $CO_2$ in air. The medium was also supplemented with $0.5 \times 10^6$/ml granulosa cells.

Frozen semen of one Holstein bull in straw was thawed in a water bath at 37℃ for 1 min, and the spermatozoa were washed with modified HEPES-TALP (Brackett and Oliphant, 1975). The sperm suspension was diluted with modified HEPES-TALP containing 10 μg/ml heparin (Katayama Chemical, Osaka, Japan), and 5 mM theophylline (Sigma) and 5 mg/ml BSA. The final sperm concentration was adjusted to $10 \times 10^6$/ml.

After the maturation of the oocytes, COCs were introduced into 100 μl droplets of the sperm suspension (11-20 COCs/drop) and maintained at 39℃ for 6 hr under mineral oil (Katayama Chemical) in a gas phase of 5% $CO_2$ in air. The insemination day was defined as Day 0.

### *In vitro* culture of embryos

In this study, modified synthetic oviduct fluid medium supplemented with 0.5 mM glucose (mSOFM) was used as the basal medium for embryo development (Takahashi and First, 1992). After insemination, the COCs were washed with the culture medium intended for embryo development according to the different experiments. The oocytes were transferred to 500 μl of culture medium covered with a layer of mineral oil in a humidified 95% air, 5% $CO_2$ incubator at 39℃. At 42 hr after insemination (on Day 2), the cumulus cells surrounding embryos were separated from embryos by pipetting and the culture medium was replaced with fresh medium. At the time, the cleavage rate (2-8 cell stage) from one-cell embryos was evaluated morphologically under a microscope.

### Experiment 1 (Effect of lots of calf sera)

Three lots (A-C) of CS and superovulated cow serum (SCS) were compared for their ability to support the *in vitro* development of embryos. For the initial 42 hr after insemination, the embryos under co-culture with cumulus cells attached to the bottom of the dish were incubated in mSOFM supplemented with 1% CS or SCS. The media were then exchanged every 48 hr with fresh mSOFM supplemented with 5% CS or SCS. The SCS was collected from one superovulated cow treated with a total of 20 mg FSH (Antrin; Denka Pharmaceuticals, Kanagawa, Japan). Luteolysis and oestrus were induced by treatment with prostaglandin $F_2\alpha$-analogue (Syncrocept; Dainihon Pharmaceuticals, Osaka, Japan) approximately 50 hr after the initial injection of gonadotropin. Whole blood was collected on the seventh day after standing heat and immediately centrifuged twice (750 g, 10 min, 4℃) and the serum obtained was then heat-inactivated at 56℃ for 30 min. Progesterone levels in each CS and SCS were directly measured using an enzyme-linked immunosorbent assay (ELISA) kit (Denka Pharmaceuticals).

### Experiment 2 (Effect of co-cultured cumulus cells and β-ME)

Effect of β-ME on development of embryos was examined. Lot C of CS on the basis of the results in the Experiment 1 was used in the following experiments. The culture was carried out using the same procedure as described for Experiment 1. The treatments are as follows: 1) co-culture with cumulus cells after Day 2 in 5% CS/mSOFM, 2) culture without cumulus cells after Day 2, using 5% CS/mSOFM or 3) culture without cumulus cells after Day 2, using 5% CS/mSOFM supplemented with 50

$\mu$M $\beta$-ME.

### Experiment 3 (Effect of BSA and PVA)

This experiment investigated the effects of substituting BSA and polyvinyl alcohol (PVA) for CS on development of embryos in mSOFM supplemented with 50 $\mu$M $\beta$-ME under non co-culture after Day 2. The additional reagents in the medium in each treatment were as follows: 1) mSOFM supplemented with 1% CS between Day 0 and Day 2, and the medium was replaced with mSOFM supplemented with 5% CS on Day 2 for subsequent development, 2) mSOFM supplemented with 5 $\mu$g/ml insulin, 5 $\mu$g/ml transferrin, 5 ng/ml sodium selenite (premixed ITS; Sigma), 10 ng/ml epidermal growth factor (EGF; Sigma) and 3 mg/ml BSA (fraction V; Sigma), and the medium was replaced fresh one on Day 2 or 3) mSOFM supplemented with 5 $\mu$g/ml insulin, 5 $\mu$g/ml transferrin, 5 $\mu$g/ml sodium selenite, 10 ng/ml EGF and 1 mg/ml PVA (Sigma) and the medium was replaced fresh one on Day 2.

### Experiment 4 (Effect of high glucose)

Effect of glucose on embryo development was examined. The culture medium used was mSOFM supplemented with $\beta$-ME, ITS, EGF and BSA. The medium was replaced on Day 2 in two groups and Day 5 in one of two groups. On Day 5, the medium in another group was changed to the medium containing 2.0 mM glucose.

### Experiment 5 (Effect of fatty acid free BSA)

In this experiment, the effect of fatty acid free BSA (3 mg/ml; Sigma) on development of embryos was determined. The culture medium, mSOFM with $\beta$-ME, ITS, EGF and BSA of either fraction V or fatty acid free, was replaced on Day 2 and Day 5. On Day 5, furthermore, the concentration of glucose in the medium was changed to 2.0 mM.

### Experiment 6 (Cell number of blastocysts developed *in vivo*)

The cell numbers of blastocysts developed *in vivo* was compared with the cell number of blastocysts developed *in vitro*. Blastocysts developed *in vivo* were collected on the eighth day after standing heat from two cows superovulated by treatment with gonadotropin as described above. The uterus was removed at a local abattoir within 30 minutes after slaughter and transported to the laboratory in physiological saline containing antibiotics at 30-35℃ within 2 hr after slaughter. The uterus was flushed with modified Dulbecco's phosphate-buffered saline (mDPBS; Gibco) supplemented with 4 mg/ml BSA and antibiotics, then the embryos were collected and washed twice with mDPBS. Immediately the embryos were stained for counting the total cells of the blastocysts as described below.

### Assessment of embryonic development and cell count of blastocyst

The cleavage rate of the oocytes inseminated was evaluated on Day 2. Development to blastocyst was assessed between Day 7 and Day 10. Blastocysts were stained with Hoechst 33258 (10 $\mu$g/ml in ethanol) or 33342 (10 $\mu$g/ml in 25% ethanol solution) according to the method of Pursel et al. (1985) with slight modification, and the number of nuclei were counted by fluorescence microscopy (Nikon, Tokyo, Japan).

### Data analysis

The proportions of embryos cleaving (2-8 cell stage) and blastocysts were compared by chi-square test. Total cell numbers in blastocysts are expressed as the mean ± S.E. Analysis of variance followed by Duncan's new multiple range test was used to assess the statistical difference at $p < 0.05$ among more than two group means. Comparisons between two groups were analysed using Student's *t*-test (Steel and Torrie, 1980).

## Results

### Experiment 1 (Effect of lots of calf sera)

The cleavage rate of ova was significantly higher in lot C of CS than in lot A and in SCS ($p < 0.05$, table 1). The proportion of cleaved oocytes developing to blastocysts was significantly higher in SCS than in lot B ($p < 0.05$). The cell number of blastocysts in lot C and in SCS showed higher values than those in lots A and B, although this was not statistically significant. Progesterone levels were 3.8 to 5.5 ng/ml in the three lots of CS and 17.0 ng/ml in SCS.

### Experiment 2 (Effect of co-cultured cumulus cells and $\beta$-ME)

After Day 2, the embryos were co-cultured with or without cumulus cells in the presence of 5% CS (lot C) or 50 $\mu$M $\beta$-ME. As shown in table 2, the embryos co-cultured with cumulus cells showed a high proportion of developed blastocysts, as compared with those cultured without cumulus cells. However, supplement with $\beta$-ME was effective in the development of blastocysts. There were no significant differences in the cell number of blastocysts among the treatments.

TABLE 1. DEVELOPMENT OF BOVINE ONE-CELL EMBRYOS FERTILIZED *IN VITRO* AND CULTURED IN MEDIA CONTAINING THREE DIFFERENT LOTS OF CALF SERUM (CS) AND SUPEROVULATED COW SERUM (SCS)[1]

| Serum | No. of oocytes | No. of cleaved oocytes (%) | No. of blastocysts (%)[2] | Mean cell numbers of blastocysts (ranges)[3] |
|---|---|---|---|---|
| A | 95 | 63 (66.3)[b] | 8 (12.7)[ab] | 65.4 ± 7.5 (42- 92) |
| B | 96 | 70 (72.9)[ab] | 6 ( 8.6)[a] | 58.2 ± 15.7 (25-100) |
| C | 100 | 83 (83.0)[a] | 14 (16.9)[ab] | 75.2 ± 7.1 (37-121) |
| SCS | 101 | 70 (69.3)[b] | 15 (21.4)[b] | 72.1 ± 7.6 (27-144) |

[1] Data from two replicates.
[2] Percentages of cleaved oocytes.
[3] Values are means ± standard error.
[ab] Different superscripts in the same column denote significant difference ($p < 0.05$, $\chi^2$-test).

TABLE 2. EFFECT OF CUMULUS CELLS AND β-MERCAPTOETHANOL (β-ME) ON *IN VITRO* DEVELOPMENT OF BOVINE IVF EMBRYOS[1]

| Culture system | No. of oocytes | No. of cleaved oocytes (%) | No. of blastocysts (%)[2] | Mean cell numbers of blastocysts (ranges)[3] |
|---|---|---|---|---|
| with cumulus cells | 152 | 123 (80.9)[a] | 24 (19.5)[ab] | 81.9 ± 7.4 (24-176) |
| without cumulus cells | 114 | 84 (73.7)[ab] | 8 ( 9.5)[a] | 86.9 ± 16.4 (29-176) |
| without cumulus cells, (+) β-ME | 118 | 79 (66.9)[b] | 22 (27.8)[b] | 90.4 ± 8.9 (33-163) |

[1] Data from two or three replicates.
[2] Percentages of cleaved oocytes.
[3] Values are means ± standard error.
[ab] Different superscripts in the same column denote significant difference ($p < 0.05$, $\chi^2$-test).

### Experiment 3 (Effect of BSA and PVA)

Development of blastocysts in cultures with BSA as a supplement was comparable to that of CS ($p > 0.05$, table 3). However, PVA was less effective compared to CS or BSA ($p < 0.05$). There were no significant differences in the cell number of blastocysts among the treatments.

TABLE 3. *IN VITRO* DEVELOPMENT OF BOVINE IVF EMBRYOS IN SERUM-FREE MEDIUM[1]

| Supplement | No. of oocytes | No. of cleaved oocytes (%) | No. of blastocysts (%)[2] | Mean cell numbers of blastocysts (ranges)[3] |
|---|---|---|---|---|
| CS | 118 | 79 (66.9) | 22 (27.8)[a] | 90.4 ± 8.9 (33-163) |
| BSA | 104 | 82 (78.8) | 16 (19.5)[a] | 94.4 ± 11.4 (33-175) |
| PVA | 114 | 87 (76.3) | 5 ( 5.7)[b] | 76.6 ± 13.7 (43-113) |

[1] Data from two replicates.
[2] Percentages of cleaved oocytes.
[3] Values are means ± standard error.
[ab] Different superscripts denote significant difference ($p < 0.05$, $\chi^2$-test).

### Experiment 4 (Effect of high glucose)

After Day 2, the embryos were cultured in the presence of low glucose (0.5 mM), and on Day 5 the glucose level was shifted to 2.0 mM in half the cultures.

High glucose had no effect on the formation of blastocysts compared with low glucose. However, the cell number of blastocysts in high glucose increased significantly than that in low glucose ($p < 0.01$, table 4).

TABLE 4. EFFECT OF GLUCOSE CONCENTRATION ON *IN VITRO* DEVELOPMENT OF BOVINE IVF EMBRYOS[1]

| Glucose concentration | No. of oocytes | No. of cleaved oocytes (%) | No. of blastocysts (%)[2] | Mean cell numbers of blastocysts (ranges)[3] |
|---|---|---|---|---|
| 0.5 mM | 182 | 139 (76.4) | 31 (22.3) | 88.4 ± 6.7[a] (33-163) |
| 2.0 mM | 177 | 124 (70.1) | 26 (21.0) | 127.0 ± 8.7[b] (42-190) |

[1] Data from two replicates.
[2] Percentages of cleaved oocytes.
[3] Values are means ± standard error.
[ab] Different superscripts denote significant difference ($p < 0.05$, Student's *t*-test).

### Experiment 5 (Effect of fatty acid free BSA)

The development of blastocysts was significantly lower in the presence of fatty acid free BSA, as compared with that in fraction V BSA ($p < 0.05$, table 5). The cell number of blastocysts was also reduced by adding fatty acid free BSA, although this did not reach statistical significance.

TABLE 5. EFFECT OF BSA ON *IN VITRO* DEVELOPMENT OF BOVINE IVF EMBRYOS[1]

| Source of BSA | No. of oocytes | No. of cleaved oocytes (%) | No. of blastocysts (%)[2] | Mean cell numbers of blastocysts (ranges)[3] |
|---|---|---|---|---|
| Fraction V | 144 | 109 (75.7) | 32 (29.4)[a] | 103.2 ± 10.1 (63-148) |
| Fatty acid free | 112 | 82 (73.2) | 6 ( 7.3)[b] | 81.7 ± 15.8 (48-147) |

[1] Data from three replicates.
[2] Percentages of cleaved oocytes.
[3] Values are means ± standard error.
[ab] Different superscripts denote significant difference ($p < 0.05$, $\chi^2$-test).

### Experiment 6 (Cell number of blastocysts developed *in vivo*)

To compare the quality of blastocysts developed *in vitro* and *in vivo*, the total number of cells of blastocysts obtained from superovulated cows was determined. Of 15 embryos recovered from the cows, 3 unfertilized and degenerated embryos were discarded. The average cell number per blastocyst was 111.1 ± 8.9 (mean ± S.E., n = 12) with a range from 79 to 168. This value was not significantly different from blastocysts developed *in vitro* in the basal medium supplemented with β-ME, ITS, EGF and fraction V BSA ($p > 0.05$).

## Discussion

This study demonstrates that one-cell bovine embryos derived from *in vitro* maturation and fertilization of oocytes can develop to the blastocyst stage in serum-free medium. A different efficacy among product lots of calf sera was observed in bovine embryonic development *in vitro* (table 1), indicating that the biological activities of sera differ considerably from lot to lot. Geshi et al. (1993) reported that various sera had different effects on the *in vitro* development of bovine embryos, and that superovulated donor serum improved embryonic development. The serum levels of progesterone do not seem to correlate with the rate of embryonic development under our experimental conditions. Our result agrees with the previous report of Boediono et al. (1994) in which the serum levels of progesterone and LH had little effect on embryonic development.

The involvement of somatic cells in the *in vitro* development of bovine embryos has been reported by many researchers. However, the precise mechanisms of

their beneficial roles remain unknown. Goto et al. (1994) demonstrated that the developmental rate and quality of blastocysts cultured only in medium of TCM-199 were poor, compared with those co-cultured with granulosa cell monolayers. The present results on blastocyst development using co-culture with cumulus cells agree with the previous results (table 2; Goto et al., 1994). However, there was no significant difference in the cell number per blastocyst between co-culture and cell-free culture systems in our experiments. This study also showed that β-ME stimulated the development of cleaved oocytes to the blastocyst stage in the cell-free culture system. In a preliminary experiment, a higher concentration of β-ME (100 μM) inhibited embryonic development to the blastocyst stage (13/124, 10.5%), but not the formation of cleaved embryos (124/154, 80.5%). Recent reports (Takahashi et al., 1993; Hamano et al., 1994) indicate that thiol compounds in culture media facilitate embryonic development in the absence of feeder cells, and the optimal concentration of β-ME is dependent on the embryonic stage and culture conditions.

The proportion of blastocysts developed in serum-free medium containing BSA and growth factors (ITS and EGF) was as high as that seen in CS-containing medium. However, the replacement of CS or BSA with PVA in the medium resulted in poor development to the blastocyst stage from one-cell embryos (table 3). Takahashi and First (1992) also reported poor embryonic development after substitution of PVA for BSA in mSOFM, probably owing to the presence of an embryotoxic component or deficiency of nutrients and/or growth factor(s). Shamsuddin et al. (1994) suggested that the frequency of formation of blastocysts in media containing BSA and ITS was stimulated mainly by ITS. Although EGF was also contained in our medium, it seemed to have little effect on development to the blastocyst stage. Flood et al. (1993) revealed that EGF slightly improved bovine embryo development to the blastocyst stage.

The cell numbers of blastocysts markedly increased in the presence of glucose in the medium, although the developmental rate was similar between low (0.5 mM) and high (2.0 mM) glucose concentrations (table 4). The presence of glucose is important for mammalian preimplantation embryo, since an increase in glucose metabolism from bovine two-cell to blastocyst stage occurs in steps, especially at the morula stage (Riegar, 1992). The increased requirement of glucose at the molura stage promotes cell proliferation to allow the development to the blastocyst stage. Takahashi and First (1992) reported that comparatively high concentrations of glucose (5.56 mM) on Day 5 had neither beneficial nor detrimental effects on *in vitro* embryonic development, and suggested that glucose concentrations in the medium was one of the main factors causing the developmental block in early bovine embryos maintained in a cell-free culture system. Kim et al. (1993) demonstrated that the addition of glucose at 2.78-5.56 mM to the chemically defined medium promoted the development of the 120 hr post-inseminated embryos.

Two types of commercially available BSA were compared for their effects on embryonic development. Fatty acid free BSA decreased the developmental rate to the blastocyst stage compared with fraction V BSA (table 5). The cell number of blastocysts developed in this medium also decreased, as compared with fraction V BSA, although no statistical difference was seen. It is well known that commercial preparations of serum albumin are contaminated with peptides other than albumin, as well as fatty acids, energy substrates and many other small molecules (Kane and Headon, 1980; Batt and Miller, 1988). Kane and Headon (1980) showed that commercial BSA has at least two effects on growth of rabbit blastocyst: (1) energy provision via albumin-bound fatty acids and (2) promotion of blastocyst hatching by a non-albumin component. Our results suggest that the decreased development to the blastocyst stage seen in the medium containing fatty acid free BSA may result from a deficiency of albumin-bound substances. Gray et al. (1992) confirmed that commercial BSA was heavily contaminated with citrate which stimulated cell proliferation and expansion of rabbit blastocysts. However, citrate alone has little effect on *in vitro* development of bovine embryos to the blastocyst stage (Rorie et al., 1994), while citrate is beneficial in combination with non essential amino acids, and addition of glutamine to this combination depresses morula and blastocyst development (Keskintepe et al., 1995). The utilization of fatty acids as an energy source requires further investigation for bovine embryonic development.

Greve et al. (1993) reported that 7 to 8 day-old embryos obtained from superovulated cattle consisted of about 80-100 cells in early blastocysts and about 120-160 cells in expanded blastocysts. In the present study, the blastocysts developed *in vitro* compared favorably with those obtained from the superovulated cows with regard to cell number, suggesting that the embryonic development to the blastocyst stage in the serum- and feeder cell-free culture systems was normal.

In conclusion, the results of this study indicate that the substitution of β-ME and BSA (fraction V) with co-cultured cumulus cells and CS, respectively, can support the *in vitro* development of bovine embryos, and glucose

and fatty acids have possible roles in cell proliferation and embryonic development, respectively.

## Acknowledgements

The authors gratefully acknowledge T. Etoh and A. Minami, Kuju Agriculture Research Center, for their assistance with the recovery of serum and embryos from superovulated donors, and also thank Dr. J. L. Voogt, the University of Kansas Medical Center, for reading this manuscript.

## Literature Cited

Batt, P. A. and B. G. Miller. 1988. Development of sheep embryos *in vitro* in a medium supplemented with different batches of serum albumin. Aust. J. Biol. Sci. 41:371-376.

Boediono, A., M. Takagi, S. Saha and T. Suzuki. 1994. Influence of Day-0 and Day-7 superovulated cow serum during development of bovine oocytes *in vitro*. Reprod. Fertil. Dev. 6:261-264.

Brackett, B. G. and G. Oliphant. 1975. Capacitation of rabbit spermatozoa *in vitro*. Biol. Reprod. 12:260-274.

Eyestone, W. H. and N. L. First. 1989. Co-culture of early cattle embryos to the blastocyst stage with oviductal tissue or in conditioned medium. J. Reprod. Fertil. 85:715-720.

Flood, M. R., T. L. Gage and T. D. Bunch. 1993. Effect of various growth-promoting factors on preimplantation bovine embryo development *in vitro*. Theriogenology 39:823-833.

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

Geshi, M., M. Yonai, H. Hanada, T. Komiyama and M. Takahashi. 1993. Effect of serum on the *in vitro* development of bovine embryos derived from *in vitro* maturation and fertilization. Anim. Sci. Technol. (Jpn.) 64:480-483. (In Japanese)

Goto, K., Y. Kajihara, S. Kosaka, M. Koba, Y. Nakanishi and K. Ogawa. 1988. Pregnancies after co-culture of cumulus cells with bovine embryos derived from *in vitro* fertilization of *in vitro* matured follicular oocytes. J. Reprod. Fertil. 83:753-758.

Goto, K., N. Iwai, K. Ide, Y. Takuma and Y. Nakanishi. 1994. Viability of one-cell bovine embryos cultured *in vitro* : comparison of cell-free culture with co-culture. J. Reprod. Fertil. 100:239-243.

Gray, C. W., P. M. Morgan and M. T. Kane. 1992. Purification of an embryotrophic factor from commercial bovine serum albumin and its identification as citrate. J. Reprod. Fert. 94:471-480.

Greve, T., B. Avery and H. Callesen. 1993. Viability of *in vivo* and *in vitro* produced bovine embryos. Repord. Domest. Anim. 28:164-169.

Hamano, S., M. Kuwayama, M. Takahashi, N. Okamura, A. Okano and T. Nagai. 1994. Effect of $\beta$-merchaptoethanol on the preimplantation development of bovine embryos fertilized *in vitro*. J. Reprod. Dev. 40:355-359.

Kane, M. T. and D. R. Headon. 1980. The role of commercial bovine serum albumin preparations in the culture of one-cell rabbit embryos to blastocysts. J. Reprod. Fert. 60:469-475.

Keskintepe, L., C. A. Burnley and B. G. Brackett. 1995. Production of viable bovine blastocysts in defined *in vitro* condition. Bio. Reprod. 52:1410-1417.

Kim, J. H., K. Niwa, J. M. Lim and K. Okuda. 1993. Effects of phosphate, energy substrates, and amino acids on development of *in vitro*-matured, *in vitro*-fertilized bovine oocytes in a chemically defined, protein-free culture medium. Biol. Reprod. 48:1320-1325.

Leese, H. J. 1992. Metabolism of the preimplantation mammalian embryo. In: S. R. Milligan (Ed.), Oxford Reviews of Reproductive Biology. Oxford University Press. Oxford. Vol. 13. pp. 35-72.

Pinyopummintr, T. and B. D. Bavister. 1991. *In vitro*-matured/*in vitro*-fertilized bovine oocytes can develop into molurae/blastocysts in chemically defined, protein-free culture media. Biol. Reprod. 45:736-742.

Pursel, V. G., R. J. Wall, C. E. Rexroad Jr., R. E. Hammer and R. L. Brinster. 1985. A rapid whole-mount staining procedure for nuclei of mammalian embryos. Theriogenology 24:687-691.

Riegar, D. 1992. Relationships between energy metabolism and development of early mammalian embryos. Theriogenology 37:75-93.

Rorie, R. W., G. F. Miller, K. B. Nasti and R. W. McNew. 1994. *In vitro* development of bovine embryos as affected by different lots of bovine albumin and citrate. Theriogenology 42:397-403.

Takagi, Y., K. Mori, M. Tomizawa, T. Takahashi, S. Sugawara and J. Masaki. 1991. Development of bovine oocytes matured, fertilized and cultured in a serum-free, chemically defined medium. Theriogenology 35:1197-1207.

Takahashi, Y. and N. L. First. 1992. *In vitro* development

of bovine one-cell embryos : influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology 37:963-978.

Takahashi, M., T. Nagai, S. Hamano, M. Kuwayama, N. Okamura and A. Okano. 1993. Effect of thiol compounds on *in vitro* development and intracellular glutathione content of bovine embryos. Biol. Reprod. 49:228-232.

Shamsuddin, M., B. Larsson, H. Gustafsson and H. Rodriguez-Martinez. 1994. A serum-free, cell-free culture system for development of bovine one-cell embryos up to blastocyst stage with improved viability. Theriogenology 41:1033-1043.

Steel, R. G. D. and J. H. Torrie. 1980. Principles and procedures of statics. 2nd ed. McGraw-Hill, New York. pp. 469-520.